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Hepatitis E Virus Genotype 3 Genomes from RNA-Positive but Serologically Negative Plasma Donors Have CUG as the Start Codon for ORF3

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Keywords
HEV3 · Blood donors · Mutation · Nucleotide sequence

Abstract
Hepatitis E virus (HEV) is a pathogen that causes hepatitis worldwide. Molecular studies have identified HEV RNA in blood products although its significance is not understood. This study was undertaken to characterize HEV genomes in asymptomatic plasma donors from Sweden and Germany lacking anti-HEV. Complete open reading frames (ORFs) were obtained from HEV strains in 5 out of 18 plasma donors who tested positive for HEV RNA. All strains had CUG as the start codon of ORF3, while 147 GenBank strains all had AUG as the start codon ($p < 0.0001$). This substitution was found in both interrelated and unrelated strains belonging to different phylogenetic clades. The HEV strains from the seronegative plasma donors had no other substitution in common, which may be why the CUG substitution seems to explain the seronegativity.

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Hepatitis E virus (HEV) is a positive strand RNA virus of approximately 7.2 kb belonging to the family Hepeviridae. Strains infecting humans belong to five genotypes (HEV1-HEV4 and HEV7) within *Orthohepevirus A* [1]. HEV is mainly transmitted by the faecal-oral route, but blood transmission may occur [2]. HEV1 and HEV2 cause sporadic cases or large waterborne outbreaks in developing countries, while HEV3 and HEV4 have zoonotic reservoirs, mainly pigs and wild boar, and probably cause most endemic human HEV3 infections by faecal-oral spread [2, 3]. There are nine HEV3 subtypes in two major phylogenetic clades, 3I by subtypes abchij, and 3II by efg [4, 5].

HEV3 is the common genotype in Europe [2], where the number of notified cases increases yearly. Serological studies have shown relatively high rates of HEV exposure in blood donors [6, 7] and molecular analysis has demonstrated HEV-RNA in blood and blood products [7–12], such as in Sweden where about 1/8,000 blood donations has HEV RNA [10]. It has been shown that blood donors

All sequences reported from this study have been deposited in GenBank; accession No. MH377721–27.

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with HEV RNA may be anti-HEV negative for up to 48 days, before seroconversion [8, 11]. There are studies showing a low risk of transmission of HEV from HEV RNA-positive blood donations [13], but also that it is cost effective to screen blood products for HEV to minimize hepatitis E posttransfusion [14]. Five European countries have introduced HEV RNA screening of blood products based on this [15].

Seventy per cent of HEV3 infections are subclinical [16]. However, the infection may even be fulminant [2]. It may also cause chronic hepatitis in immunosuppressed individuals [17, 18], in whom viral RNA can be detected for 6 months before seroconversion [18]. During a resolving infection, HEV RNA can be detected in serum prior to and 1–2 weeks after the onset of illness and for another 2 weeks in faeces [19]. Immune response against HEV can be detected shortly after viremia and may persist for at least 5–6 years [19].

The HEV genome has three open reading frames, ORF1–ORF3, and two short non-coding regions, a 5′-methylguanine-cap and a 3′-poly A-tail [20]. ORF1 is the longest ORF and is only present in the genomic RNA. ORF2 and ORF3 are expressed by the same bicistronic subgenomic RNA.

ORF1 encodes a non-structural polyprotein of 1,693 amino acids (aa) with several putative domains implicated in viral replication, pathogenesis, and host tropism [20]. Among these are a methyltransferase [20], a Y-domain with unknown function, a papain-like protease [20], a hypervariable region with a proline-rich domain [21], an X-domain or ADP-Ribose-binding domain designated as a macro domain [20], a helicase with nucleotide and $MgCl_2$-binding sites [20], and the C-terminal longest domain encoding the RNA-dependent RNA polymerase (RdRP).

ORF2 encodes a 660-aa-long protein which is processed by cleavage of a signal sequence of 22 aa at the N-terminus [22], and thereafter self-associates into viral capsids that can be glycosylated [23]. The N-terminal 111 residues are responsible for packaging of the viral RNA genome and residues 112–660 for forming virus-like particles. This latter region has the S-domain forming the capsid shell, the P1-domain, which together with the S-domain forms the icosahedral capsid symmetry, and the P2-domain, which protrudes out from the viral capsid [24] and is important for receptor binding and has immune-dominant epitopes.

ORF3 is the smallest ORF and encodes a 113-aa-long multifunctional phosphoprotein. In patient sera it is bound to viral particles associated with cellular membranes [25]. It has been proposed to affect the host inflammatory response [26, 27]. At the N-terminal part of the protein two hydrophobic domains are located, D1 and D2, where D1 is important for cytoskeleton association [28]. Part of D1 and D2 act as an ion channel important for viral egress [29]. Two other regions, P1 and P2, are proline-rich domains. P1 has motifs homologous to kinase substrates, and Ser79 in this region has been shown to be phosphorylated in vitro [28], and when phosphorylated shown to be involved in the binding of ORF3 to non-glycosylated viral capsids. The P2 region contains a ProSerAlaPro motif (residues 104–107) important for the formation of membrane-associated HEV particles and together with another $Pro^{95}XXPro^{98}$ motif critical for viral egress [30].

A junction region (JR) located between ORF1 and ORF3 contains a *cis*-reactive element (CRE) that may serve as a promoter for subgenomic mRNA [31]. This CRE is 15 nucleotides long and conserved in all HEV genotypes infecting humans. Part of the 3′-end of ORF2 and the 3′UTR-region of the genome form a secondary structure predicted to be another CRE essential for HEV replication [32].

Complete HEV3 genomes have mainly been sequenced from patients with acute, fulminant, and chronic HEV, or from pigs and wild boar. Even if clinical HEV infections in immunocompetent hosts are well characterised, less is known regarding strains causing asymptomatic often undiagnosed infections in immunocompetent individuals. In this study, we have therefore characterized near complete HEV RNA genomes from 4 Swedish and 1 German HEV RNA-positive but seronegative plasma donors. They were selected from 18 plasma donors who were positive for HEV RNA during the screening process for plasma donations at Octapharma AB (Stockholm, Sweden) between 2010 and 2013.

The 18 samples were tested for IgM antibodies to hepatitis E (anti-HEV IgM) and IgG (anti-HEV IgG) by commercial enzyme-linked assays (HEV IgM Dia.pro and HEV IgG Dia.pro, Diagnostic BioProbes, Milan, Italy). Only 3 had both anti-HEV IgG and IgM, 2 had borderline values for anti-HEV IgG, and the remaining 13 were negative (Table 1). All 5 donors with a sequenced HEV strain were anti-HEV negative (Table 1).

Nucleic acids were extracted from 250 μL of plasma from the 18 donors as described [3]. Viral RNA was detected in 17 by an in-house TaqMan qPCR assay [3]. A plasmid with the target region was used to set the threshold to measure the C_t value for product quantification. Five microlitres of extracted RNA were reversely transcribed to

cDNA and detected by PCR [3]. Sets of primers were used to amplify the complete genome of five selected HEV strains (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000491926). The first PCR mixtures consisted of 5 µL of cDNA added to a 45-µL master mix containing 200 µM of dNTPs (Roche Diagnostics), 400 nM of each primer, 1× Advantage GC-2-PCR-Buffer, 500 µM of GC-Melt and 1× Advantage-GC-2-Polymerase Mix (Clontech Laboratories, Mountain View, CA, USA). A second nested PCR was performed with reaction mixtures containing the same reagents at the same concentrations as the first PCR, but with different primers and 1 µL of the first amplified product as a template. Human sera with HEV RNA were used as positive controls, and nuclease-free water as a negative control. The amplification products were purified and sequenced as described [3].

Part of the 5′-half of the genomes was difficult to amplify by PCR with primers with sequences present in the strains. Therefore, the amplification was performed with a mixture of primers with known HEV sequences mixed with random primers and sequenced by next generation sequencing (NGS). A total of 5 µL of cDNA was used in triplicate as the template in a touch-up gradient PCR using specific primers in a first round amplification with 50 µL of reaction mix containing 6.5 µL of cDNA, 1× Taq buffer (Applied Biosystems), 2 mM of $MgCl_2$ (Applied Biosystems), 0.5 mM dNTP (Sigma-Aldrich), 1 U Taq polymerase (Roche Diagnostics), and 0.8 µM of each primer. The PCR reaction was performed for 1 cycle at 94 °C for 3 min, followed by 12 cycles of touch-up PCR with 94 °C for 30 s, 20 °C for 190 s (2 °C increase per cycle), followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s, and 68 °C for 2 min, and with 5 min of final extension at 68 °C.

Libraries were built on the PCR products using an Ion-Plus-Fragment-Library Kit on Library Builder™ System (ThermoFisher, Waltham, MA, USA) according to the

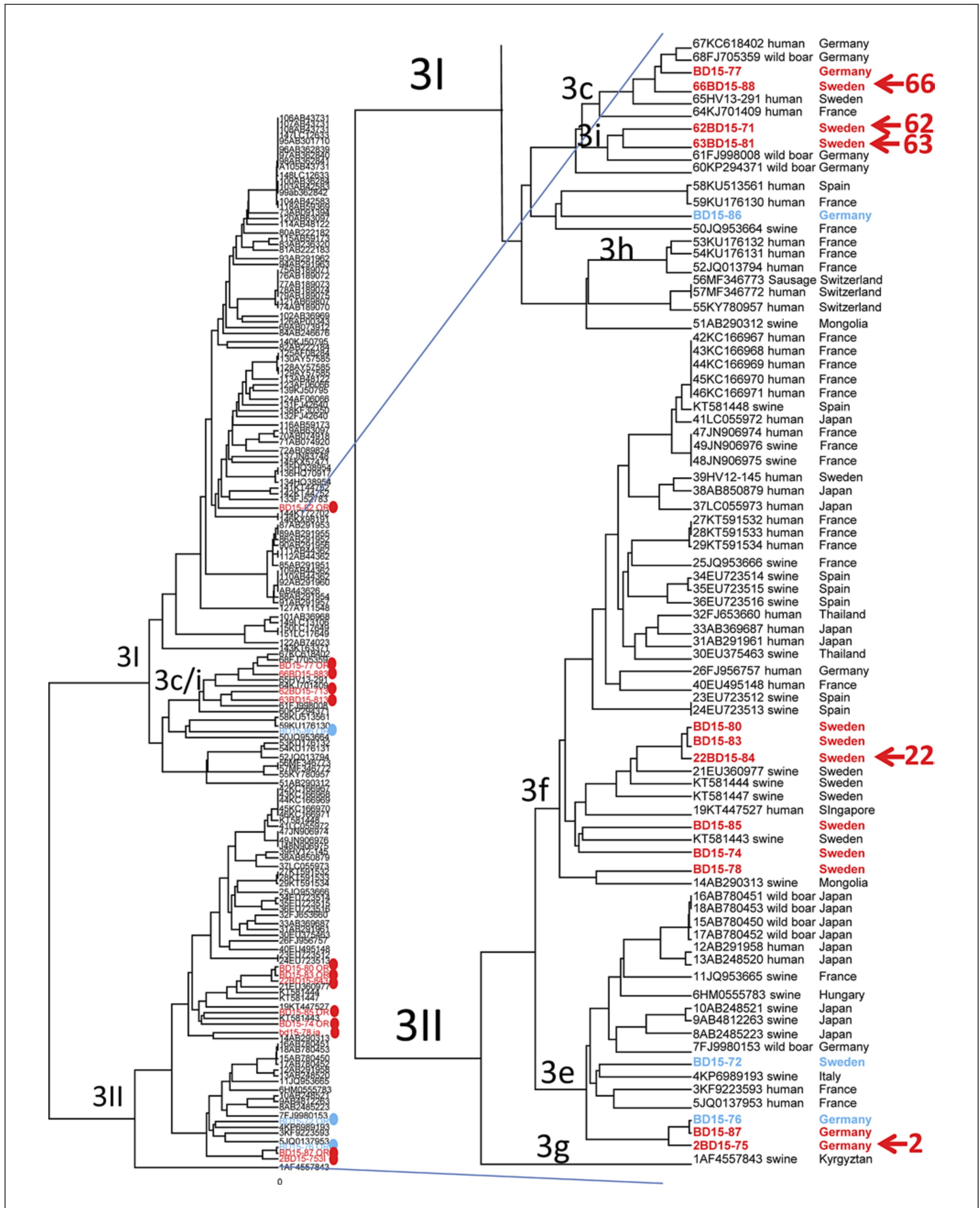
Table 1. Designation and characterization of samples from 18 plasma donors sequenced in partial ORF1

Strain No.	Designation	Country of origin	Belonging to clade	Anti-HEV IgG sample/cut-off	Anti-HEV IgM sample/cut-off	HEV RNA, copies/mL
2	BD15-75	Germany	3II	0.83	0.75	23,100
22	BD15-84	Sweden	3II	0.19	0.05	1,500
62	BD15-71	Sweden	3c/i	1.64	0.25	10,700
63	BD15-81	Sweden	3c/i	0.17	0.06	4,650
66	BD15-88	Sweden	3c/i	0.18	0.05	13,400
	BD15-72	Sweden	3II	1.56	**9.2**	900
	BD15-73	Sweden	3II	0.29	0.11	<100
	BD15-74	Sweden	3II	0.24	0.16	350
	BD15-76	Germany	3II	**6.47**	**2.88**	700
	BD15-77	Germany	3c/i	1.16	0.70	3,700
	BD15-78	Sweden	3II	0.43	0.99	<100
	BD15-79	Germany	3c/i	0.35	0.51	18,700
	BD15-80	Sweden	3II	0.73	0.13	93,000
	BD15-82	Sweden	3I	0.20	0.08	57,000
	BD15-83	Sweden	3II	0.29	0.17	<100
	BD15-85	Sweden	3II	0.19	0.03	6,000
	BD15-86	Germany	3c/i	**1.73**	**3.80**	1,000
	BD15-87	Germany	3II	0.17	0.04	5,700

Strains with numbers were subjected to complete genomic sequencing. Values considered reactive for anti-HEV-IgG and IgM are shown in bold. Cut-off was calculated according to the manufacturer. Sample/cut-off values >1 and <1.7 are considered as borderline values and shown as regarded negative [40].

Fig. 1. UPGMA dendrogram based on 356 nucleotides of partial ORF1 in 165 HEV genotype 3 sequences. The branches with 3II and 3i/c strains are enlarged. The accession numbers, host, and origin of the strains are given at the nodes. Strains from 13 plasma donors without anti-HEV are marked in red, and three strains from plasma donors with anti-HEV are marked in blue. The five strains of which near complete genomes were sequenced are marked with arrows. One strain from a seronegative plasma donor belonged to clade 3I and is not shown in the enlarged tree. The two major HEV3 clades, 3I and 3II, and subtypes are indicated on the branches. Colors refer to the online version.

(For figure see next page.)


Color version available online
3I
3II
3c/i
3c
3i
3h
3f
3e
3g
67KC618402 human Germany
68FJ705359 wild boar Germany
BD15-77 Germany
66BD15-88 Sweden 66
65HV13-291 human Sweden
64KJ701409 human France
62BD15-71 Sweden 62
63BD15-81 Sweden 63
61FJ998008 wild boar Germany
60KP294371 wild boar Germany
58KU513561 human Spain
59KU176130 human France
BD15-86 Germany
50JQ953664 swine France
53KU176132 human France
54KU176131 human France
52JQ013794 human France
56MF346773 Sausage Switzerland
57MF346772 human Switzerland
55KY780957 human Switzerland
51AB290312 swine Mongolia
42KC166967 human France
43KC166968 human France
44KC166969 human France
45KC166970 human France
46KC166971 human France
KT581448 swine Spain
41LC055972 human Japan
47JN906974 human France
49JN906976 swine France
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39HV12-145 human Sweden
38AB850879 human Japan
37LC055973 human Japan
27KT591532 human France
28KT591533 human France
29KT591534 human France
25JQ953666 swine France
34EU723514 swine Spain
35EU723515 swine Spain
36EU723516 swine Spain
32FJ653660 human Thailand
33AB369687 human Japan
31AB291961 human Japan
30EU375463 swine Thailand
26FJ956757 human Germany
40EU495148 human France
23EU723512 swine Spain
24EU723513 swine Spain
BD15-80 Sweden
BD15-83 Sweden
22BD15-84 Sweden 22
21EU360977 swine Sweden
KT581444 swine Sweden
KT581447 swine Sweden
19KT447527 human SIngapore
BD15-85 Sweden
KT581443 swine Sweden
BD15-74 Sweden
BD15-78 Sweden
14AB290313 swine Mongolia
16AB780451 wild boar Japan
18AB780453 wild boar Japan
15AB780450 wild boar Japan
17AB780452 wild boar Japan
12AB291958 human Japan
13AB248520 human Japan
11JQ953665 swine France
6HM0555783 swine Hungary
10AB248521 swine Japan
9AB4812263 swine Japan
8AB2485223 swine Japan
7FJ9980153 wild boar Germany
BD15-72 Sweden
4KP6989193 swine Italy
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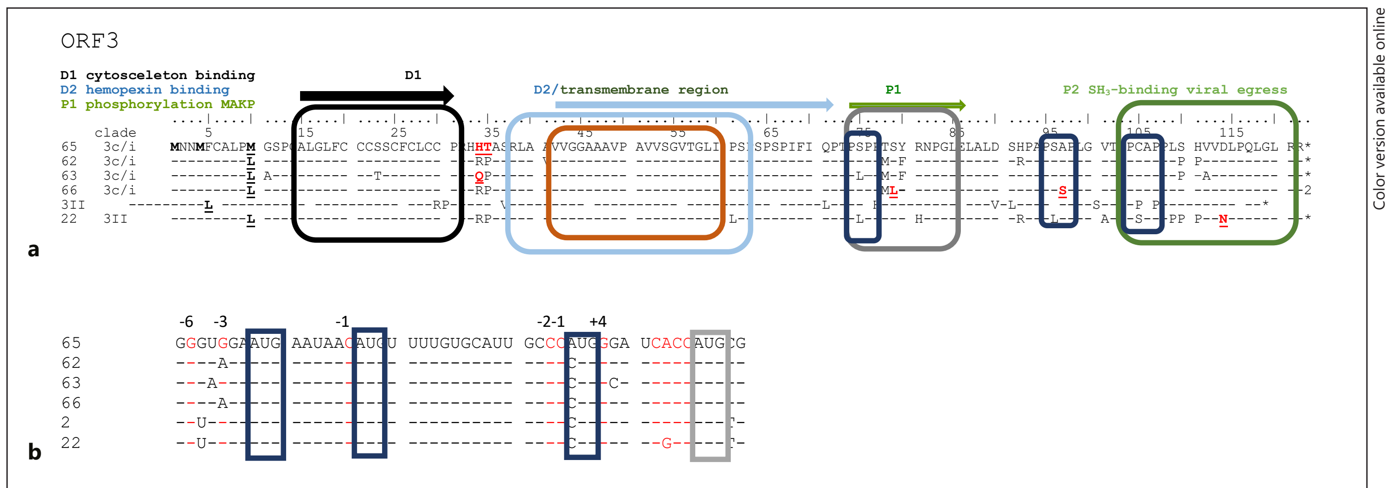

Fig. 2. a Deduced aa sequence of ORF3 protein for five strains from the plasma donors aligned with strain 65 from a patient with chronic hepatitis E. The different domains, D1, D2, P1, P2, and a transmembrane region are indicated. The three methionine residues are marked in bold, with the third also underlined, and assumed to be used as the start of ORF3. This residue is leucine in strains from the plasma donors. Unique substitutions are marked with bold red letters. The three PXXP regions, of which the third "PSAP" is assumed to be important for the phosphorylation of ORF3, are boxed in blue. All GenBank strains belonging to the 3II clade expressed the 104PSAP107 motif, while strains belonging to 3c/i expressed PCAP in this region. Three of these strains expressed PSAP at residues 95–98. **b** Nucleotide sequence. The three start codons in frame for ORF3 are boxed in blue. In the strains from the plasma donors, the third start codon is CUG, the most common noncanonical start codon. The AUG start codon for ORF2 is boxed in grey. The Kozak recognition nucleotides are marked in red. The optimal sequence is GCC**R**CC<u>AUG</u>**G**, with the most important nucleotides in bold. Colors refer to the online version.

Color version available online

manufacturer. Newly built libraries were amplified for 8 cycles and purified with AMPureXP-beads (Beckman Coulter, Brea, CA, USA) and thereafter analysed and loaded on an ion 318 chip and sequenced on an IonTorrent PGM with an Ion-PGM-Hi-Q-View-Sequencing Kit (ThermoFisher) as previously described [33]. The sequences obtained were imported into a CLC Genomic Workbench 9.5.1 (Qiagen, Hilden, Germany) and analysed as previously described [33]. The contigs and singleton reads were blasted against known HEV genomes using BLASTn, and a cut-off for E-value of 10^{-3} for significant hits. Between 7,194 and 19,175 contigs >100 bp could be matched to HEV. About 80% of the complete genomes for all five strains were obtained by combining Sanger sequencing and NGS. The remaining regions and sequences obtained by NGS were confirmed by PCR with primer sequences obtained by NGS and Sanger sequencing. By using these techniques complete ORF1-ORF3 regions and between 69 and 88% of the non-coding regions were obtained for all five strains.

Analysis of partial ORF1 of seventeen strains from the donors revealed that the strains belonged to both major clades, thirteen to clade 3II, one to clade 3I, and three were on the branch formed by subtypes 3c/i (Fig. 1). This was confirmed when complete genomes were compared.

Near-complete genomes, 7,202–7,227 nucleotides, were obtained for five strains (No. 2, 22, 62, 63, and 66). These were aligned with 147 complete HEV3 genomes from GenBank (online suppl. Table S2). Genetic distances were calculated using the Hasegawa-Kishino-Yano algorithm in the DNADIST program in the PHYLIP package version 3.65 [34] with a transition/transversion ratio of 9.14, and gamma correction with alpha 0.27. Phylogenetic trees were constructed as previously described [3].

All five sequenced genomes had the three predicted ORFs. ORF1 was 5,112–5,115 nucleotides long. Strain 2 had a 3-nucleotide insertion encoding a proline[739] in the hypervariable region.

The predicted structure of the reverse complement sequence of 58 nucleotides between nucleotides 5102 and 5159 (FJ998008) forming the JR of the genome differed between the strains (online suppl. Fig. S1). Within this region 8 nucleotides, GAATAACA, were conserved as in all other *Orthohepevirus A* strains.

ORF1 encoded 1,704–1,705 aa residues. Strain 66 had a unique substitution, Leu138Phe, in the methyl transfer-

ase domain. The Y domain was highly conserved and had the predicted N-linked glycosylation site at positions 363–365. The Gly-triplet proposed to be the substrate for proteolysis of the ORF1 protein in the X domain, at residues 825—827, was conserved in all sequenced strains [35]. All six predicted motifs in the 233-aa-long helicase domain between residues 971 and 1205 were also conserved. In this domain, two strains, 63 and 66, expressed one unique substitution each, Ile1165Arg and Ala-1071Cys, respectively.

In the region coding for the RdRP encompassing 487 aa of the C-terminal part of ORF1, six of the eight predicted motifs were conserved for the sequenced strains, including the GlyAspAsp (GDD) site at residues 1561–1563. Two unique substitutions were expressed, Asn1233Asp in strain 2 and Ala1269Ser in strain 22.

The deduced ORF2 proteins had the three predicted N-linked glycosylation sites at residues 137–139, 310–312, and 562–564. Three strains, 22, 62, and 66, had five unique substitutions in ORF2, two of which were adjacent to the C-terminal region of the assumed neutralisation epitope between residues 578 and 607, Ala608Val in strain 22 and Ile581Val in strain 62.

The gene product of ORF3 is assumed to be 113 residues long for HEV3. The only substitution in common for the strains of the 5 seronegative plasma donors was a substitution to CUG of the third of three AUG codons in the frame located in the JR (Fig. 2a). AUG is present in all known HEV3 strains and is considered the start codon for ORF3 [36]. The second AUG codon in the frame for ORF3 is located downstream of ORF1, the first within ORF1.

The second hydrophobic region of ORF3, D2, between residues 37 and 62, overlapped with a proposed transmembrane region at residues 41–60 identified in the sequenced strains. Strains 22, 63, and 66 expressed one to two unique substitutions each in ORF3. A Ser79Leu substitution in strain 66 may eliminate the phosphorylation site of its deduced ORF3 protein. This strain had a unique Ala97Ser substitution, which may serve the function of being phosphorylated in place of Ser79. Three of the 5 plasma donors shared the relatively uncommon mutation of Ser105Cys. Both the Ser79 and the Cys105 in the pORF3 may potentially affect the formation of viral capsids or egress (Fig. 2b).

As mentioned, all five sequenced genomes had CUG instead of AUG as the start codon for pORF3 [36, 37], while all 147 HEV3 genomes retrieved from GenBank had an AUG at this position ($p < 0.0001$, Fisher exact test). This AUG is the only one of the three AUG codons in the frame for ORF3 in HEV1–3 genomes with a strong Kozak consensus sequence. In addition, the other AUGs have been shown to be dispensable for the expression of ORF3 [36]. CUG is quite frequently used as a start codon for RNA viruses; these are mainly plant viruses [38], but also a few animal viruses, such as Sendai virus [38], as well as for some eukaryotic genes, although it is less efficient than AUG [39].

A lower expression of ORF3 may alter functions shown for this protein on the innate immune response [26] and type I interferon production [27]. The substitution of the third AUG to CUG may not only downregulate the expression of the ORF3 protein, but also cause structural changes of HEV RNA in regions with regulatory functions, since the CRE within the JR region is assumed to act as a promoter for the subgenomic mRNA [31, 37]. Therefore, the CUG substitution may also affect the expression of ORF2. This may render the viral strains less immunogenic or less virulent with a relatively lower level of replication and protein expression. It may also affect the virus ability to transmit and cause infection, since the pORF3 has been shown to be important for viral egress. This might mean that there is lower risk for hepatitis E transmission during the viraemic, although a seronegative state supports a study from Denmark [13]. Seroconversion to anti-HEV may occur after reversion of the ORF3 start codon to AUG. This would also result in a structural reversal of the promoter of ORF2 and ORF3 subgenomic mRNA, which in turn can lead to an increased expression of these proteins. This is in support of a study from the Netherlands showing cost effectiveness of blood screening for HEV RNA [14].

Previously assumed functions of the ORF3 protein have been identified through high expression of its product in cell culture. Less has been demonstrated on the functions of this protein in its natural host. This study shows the need for genomic sequencing of strains directly isolated from patients to understand the biology of HEV, since cell propagated virus may not always mirror the infection in the natural host. Further studies should investigate if strains from seronegative blood donors with HEV RNA are infectious and if the change in the start codon for ORF3 and the promotor for the subgenomic mRNA may also influence the ability of the virus to spread and cause infection, although it seems apparent that the non-canonical start codon for ORF3 and its accompanying effect on the subgenomic promotor will explain the absence of immune response in the seronegative plasma donors.

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Statement of Ethics

The study was approved by the Regional Ethical Review Board in Gothenburg (No. Dr534-16 and Dr150-08). All donors are anonymous and only the date and country of donation is known.

Disclosure Statement

The authors declare that they have no conflicts of interest.

References

1 Purdy MA, Harrison TJ, Jameel S, Meng XJ, Okamoto H, Van der Poel WHM, Smith DB; ICTV Report Consortium: ICTV virus taxonomy profile: *Hepeviridae*. J Gen Virol 2017;98:2645–2646.
2 Kamar N, Dalton HR, Abravanel F, Izopet J: Hepatitis E virus infection. Clin Microbiol Rev 2014;27:116–138.
3 Roth A, Lin J, Magnius L, Karlsson M, Belák S, Widén F, Norder H: Markers for ongoing or previous hepatitis E virus infection are as common in wild ungulates as in humans in Sweden. Viruses 2016;8:E259.
4 Widen F, Sundqvist L, Matyi-Toth A, Metreveli G, Belak S, Hallgren G, Norder H: Molecular epidemiology of hepatitis E virus in humans, pigs and wild boars in Sweden. Epidemiol Infect 2011;139:361–371.
5 Smith DB, Simmonds P, Izopet J, Oliveira-Filho EF, Ulrich RG, Johne R, Koenig M, Jameel S, Harrison TJ, Meng XJ, Okamoto H, Van der Poel WH, Purdy MA: Proposed reference sequences for hepatitis E virus subtypes. J Gen Virol 2016;97:537–542.
6 Galli C, Fomiatti L, Tagliacarne C, Velati C, Zanetti AR, Castaldi S, Romanò L: Seroprevalence of hepatitis E virus among blood donors in northern Italy (Sondrio, Lombardy) determined by three different assays. Blood Transfus 2017;15:502–505.
7 Mansuy JM, Gallian P, Dimeglio C, Saune K, Arnaud C, Pelletier B, Morel P, Legrand D, Tiberghien P, Izopet J: A nationwide survey of hepatitis E viral infection in French blood donors. Hepatology 2016;63:1145–1154.
8 Tedder RS, Tettmar KI, Brailsford SR, Said B, Ushiro-Lumb I, Kitchen A, Morgan D, Lattimore S, Tossell J, Ijaz S, Hewitt PE: Virology, serology, and demography of hepatitis E viremic blood donors in South East England. Transfusion 2016;56:1529–1536.
9 Hogema BM, Molier M, Sjerps M, de Waal M, van Swieten P, van de Laar T, Molenaar-de Backer M, Zaaijer HL: Incidence and duration of hepatitis E virus infection in Dutch blood donors. Transfusion 2016;56:722–728.
10 Baylis SA, Gärtner T, Nick S, Ovemyr J, Blümel J: Occurrence of hepatitis E virus RNA in plasma donations from Sweden, Germany and the United States. Vox Sang 2012;103:89–90.
11 Vollmer T, Diekmann J, Eberhardt M, Knabbe C, Dreier J: Hepatitis E in blood donors: investigation of the natural course of asymptomatic infection, Germany, 2011. Euro Surveill 2016;21:30332.
12 Tedder RS, Tettmar KI, Brailsford SR, Said B, Ushiro-Lumb I, Kitchen A, Morgan D, Lattimore S, Tossell J, Ijaz S, Hewitt PE: Virology, serology, and demography of hepatitis E viremic blood donors in South East England. Transfusion 2016;56:1529–1536.
13 Harritshoj LH, Holm DK, Saekmose SG, Jensen BA, Hogema BM, Fischer TK, Midgley SE, Krog JS, Erikstrup C, Ullum H: Low transfusion transmission of hepatitis E among 25,637 single-donation, nucleic acid-tested blood donors. Transfusion 2016;56:2225–2232.
14 de Vos AS, Janssen MP, Zaaijer HL, Hogema BM: Cost-effectiveness of the screening of blood donations for hepatitis E virus in the Netherlands. Transfusion 2017;57:258–266.
15 Domanovic D, Tedder R, Blumel J, Zaaijer H, Gallian P, Niederhauser C, Sauleda Oliveras S, O'Riordan J, Boland F, Harritshoj L, Nascimento MSJ, Ciccaglione AR, Politis C, Adlhoch C, Flan B, Oualikene-Gonin W, Rautmann G, Strengers P, Hewitt P: Hepatitis E and blood donation safety in selected European countries: a shift to screening? Euro Surveill 2017;22:30514.
16 Guillois Y, Abravanel F, Miura T, Pavio N, Vaillant V, Lhomme S, Le Guyader FS, Rose N, Le Saux JC, King LA, Izopet J, Couturier E: High proportion of asymptomatic infections in an outbreak of hepatitis E associated with a spit-roasted piglet, France, 2013. Clin Infect Dis 2016;62:351–357.
17 Fang SY, Han H: Hepatitis E viral infection in solid organ transplant patients. Curr Opin Organ Transplant 2017;22:351–355.
18 Dalton HR, Bendall RP, Keane FE, Tedder RS, Ijaz S: Persistent carriage of hepatitis E virus in patients with HIV infection. N Engl J Med 2009;361:1025–1027.
19 Clayson ET, Myint KS, Snitbhan R, Vaughn DW, Innis BL, Chan L, Cheung P, Shrestha MP: Viremia, fecal shedding, and IgM and IgG responses in patients with hepatitis E. J Infect Dis 1995;172:927–933.
20 Holla RP, Ahmad I, Ahmad Z, Jameel S: Molecular virology of hepatitis E virus. Semin Liver Dis 2013;33:3–14.
21 Purdy MA, Lara J, Khudyakov YE: The hepatitis E virus polyproline region is involved in viral adaptation. PLoS One 2012;7:e35974.
22 Tyagi S, Jameel S, Lal SK: The full-length and N-terminal deletion of ORF2 protein of hepatitis E virus can dimerize. Biochem Biophys Res Commun 2001;286:214–221.
23 Tyagi S, Jameel S, Lal SK: A yeast two-hybrid study on self-association of the ORF2 protein of hepatitis E virus. Biochem Biophys Res Commun 2001;284:614–621.
24 Guu TS, Liu Z, Ye Q, Mata DA, Li K, Yin C, Zhang J, Tao YJ: Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. Proc Natl Acad Sci USA 2009;106:12992–12997.
25 Nagashima S, Jirintai S, Takahashi M, Kobayashi T, Tanggis, Nishizawa T, Kouki T, Yashiro T, Okamoto H: Hepatitis E virus egress depends on the exosomal pathway, with secretory exosomes derived from multivesicular bodies. J Gen Virol 2014;95:2166–2175.
26 He M, Wang M, Huang Y, Peng W, Zheng Z, Xia N, Xu J, Tian D: The ORF3 protein of genotype 1 hepatitis E virus suppresses TLR3-induced NF-κB signaling via TRADD and RIP1. Sci Rep 2016;6:27597.
27 Nan Y, Ma Z, Wang R, Yu Y, Kannan H, Fredericksen B, Zhang YJ: Enhancement of interferon induction by ORF3 product of hepatitis E virus. J Virol 2014;88:8696–8705.
28 Zafrullah M, Ozdener MH, Panda SK, Jameel S: The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. J Virol 1997;71:9045–9053.

29 Ding Q, Heller B, Capuccino JM, Song B, Nimgaonkar I, Hrebikova G, Contreras JE, Ploss A: Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles. Proc Natl Acad Sci USA 2017; 114:1147–1152.
30 Nagashima S, Takahashi M, Jirintai, Tanaka T, Yamada K, Nishizawa T, Okamoto H: A PSAP motif in the ORF3 protein of hepatitis E virus is necessary for virion release from infected cells. J Gen Virol 2011;92:269–278.
31 Cao D, Huang YW, Meng XJ: The nucleotides on the stem-loop RNA structure in the junction region of the hepatitis E virus genome are critical for virus replication. J Virol 2010;84: 13040–13044.
32 Emerson SU, Zhang M, Meng XJ, Nguyen H, St Claire M, Govindarajan S, Huang YK, Purcell RH: Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. Proc Natl Acad Sci USA 2001;98: 15270–15275.
33 Wang H, Sikora P, Rutgersson C, Lindh M, Brodin T, Bjorlenius B, Larsson DGJ, Norder H: Differential removal of human pathogenic viruses from sewage by conventional and ozone treatments. Int J Hyg Environ Health 2018;221:479–488.
34 Felsenstein J: Inferring phylogeneies from protein sequences by parsimony, distance and likelyhood methods. Methods Enzymol 1996; 266:418–427.
35 Parvez MK: Molecular characterization of hepatitis E virus ORF1 gene supports a papain-like cysteine protease (PCP)-domain activity. Virus Res 2013;178:553–556.
36 Huang YW, Opriessnig T, Halbur PG, Meng XJ: Initiation at the third in-frame AUG codon of open reading frame 3 of the hepatitis E virus is essential for viral infectivity in vivo. J Virol 2007;81:3018–3026.
37 Graff J, Torian U, Nguyen H, Emerson SU: A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. J Virol 2006;80:5919–5926.
38 Firth AE, Brierley I: Non-canonical translation in RNA viruses. J Gen Virol 2012;93: 1385–1409.
39 Starck SR, Jiang V, Pavon-Eternod M, Prasad S, McCarthy B, Pan T, Shastri N: Leucine-tRNA initiates at CUG start codons for protein synthesis and presentation by MHC class I. Science 2012;336:1719–1723.