

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.

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 MgCl₂-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 mem-

branes [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 Pro⁹⁵XXPro⁹⁸ 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 MgCl₂ (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

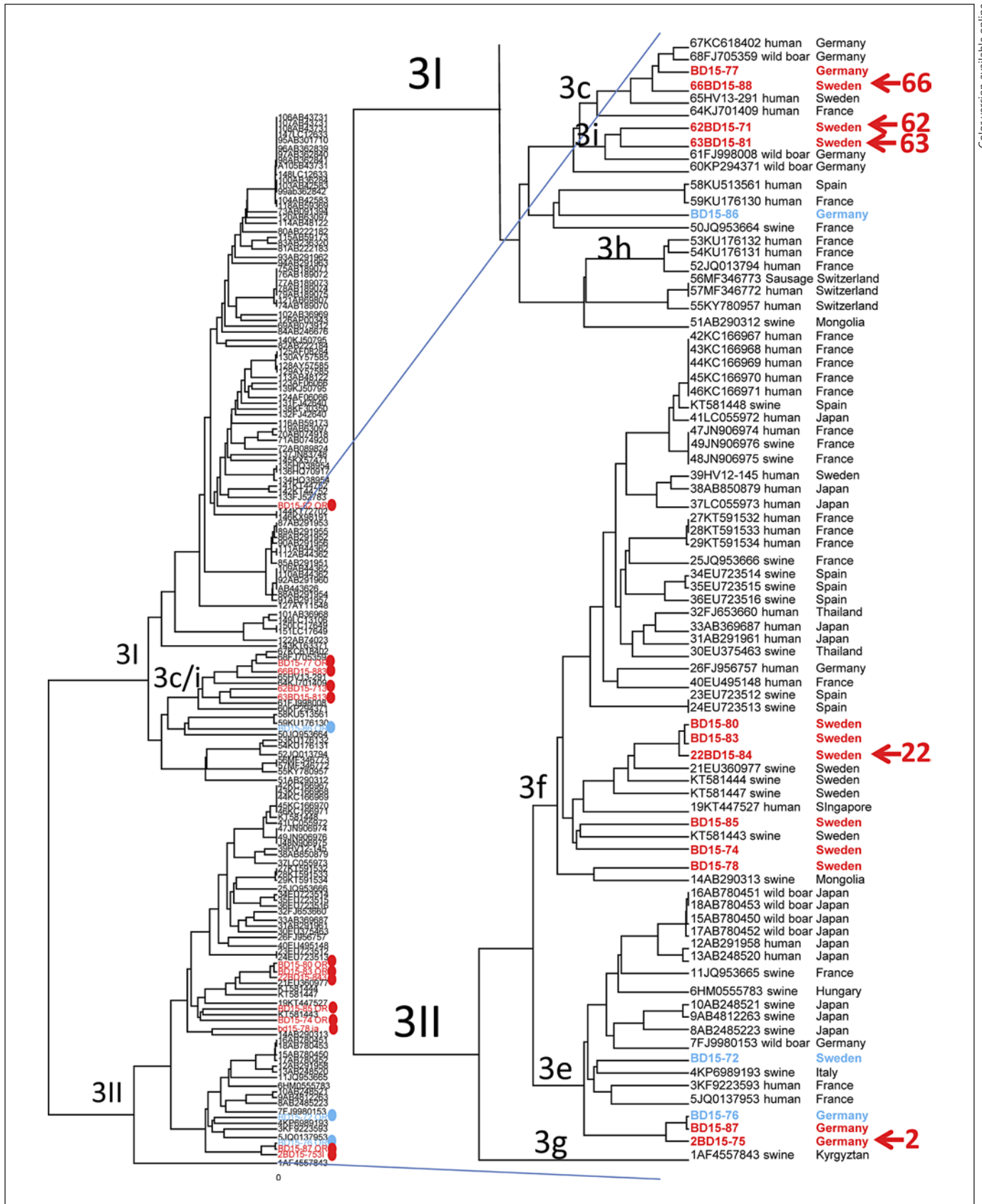
Strain No.	Designation	Country of origin	Belonging to clade	Anti-HEV IgG sample/cut-off	Anti-HEV IgM sample/cut-off	HEV RNA, copies/mL
2	BD15-75	Germany	3II	0.83	0.75	23,100
22	BD15-84	Sweden	3II	0.19	0.05	1,500
62	BD15-71	Sweden	3c/i	1.64	0.25	10,700
63	BD15-81	Sweden	3c/i	0.17	0.06	4,650
66	BD15-88	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.)



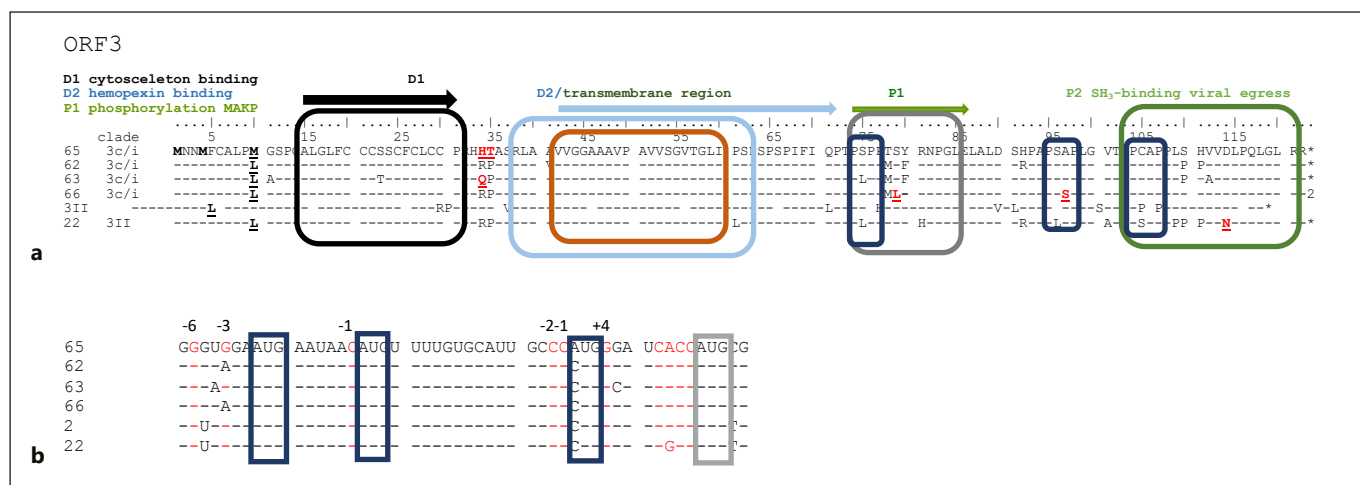


Fig. 2. a Deduced aa sequence of ORF3 protein for five strains from the plasma donors aligned with strain 65 from a patient with chronic hepatitis E. The different domains, D1, D2, P1, P2, and a transmembrane region are indicated. The three methionine residues are marked in bold, with the third also 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 GCCRCCAUGG, with the most important nucleotides in bold. Colors refer to the online version.

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 proline⁷³⁹ 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 Ala1071Cys, 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 promoter 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 promoter 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.

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