

# PHYLOGENETIC ANALYSIS OF TWELVE BULGARIAN SEQUENCES BASED ON PARTIAL OPEN READING FRAME 2 GENOME FRAGMENT OF HEPATITIS E VIRUS

**Ch. Ismailova, A. Stoyanova, V. Yoncheva, T. Tenev, L. Nikolaeva-Glomb, E. Golkocheva-Markova**

Department of Virology, National Center for Infectious and Parasitic Diseases, Sofia, Bulgaria

**KEYWORDS:** hepatitis E virus, genotyping, Sanger sequencing

## ABSTRACT:

**Background:** Hepatitis E virus (HEV) causes both acute and chronic liver inflammation. HEV is transmitted through the fecal-oral mechanism and infects both animals and humans. The virus belongs to the *Hepeviridae* family and its genome is a single stranded RNA molecule. Thanks to molecular sequencing methods different genotypes and subgenotypes have been established. The aim of the present study was to identify and characterize Bulgarian HEV sequences by applying Sanger sequencing technique for a genome fragment in Open Reading Frame 2 (ORF2 region).

**Material and methods:** Twelve retrospective samples from patients with serologically confirmed HEV infection (anti-HEV IgM and IgG positive) were sequenced by Sanger sequencing. Sequencing data were analysed by BioEdit, MEGA11 and NCBI Genbank software tools.

**Results:** The results revealed that all isolates assign to species *Paslahepevirus balayani*. Phylogenetic analysis showed that HEV isolates were characterized with considerable genetic diversity. The sequences were sub-clustered into the following subgenotypes: HEV-3e, 3m, 3f and 3c.

**Conclusion:** We successfully applied the Sanger

method for hepatitis E virus RNA sequencing. The established heterogeneity of subgenotypes requires further study in order to determine the circulation of all possible subgenotypes of HEV in the country.

## INTRODUCTION

Hepatitis E virus is an important human and animal pathogen that can cause acute and chronic liver infections in humans. According to the World Health Organization (WHO), there are an estimated 20 million HEV infections worldwide each year, resulting in an estimated 3.3 million symptomatic cases of hepatitis E (1).

## HEV classification

HEV belongs to *Hepeviridae* family, which comprises two subfamilies - *Parahepevirinae* and *Orthohepevirinae*. Members of *Parahepevirinae* infect trout and salmon, and *Orthohepevirinae* - mammals and birds. *Orthohepevirinae* subfamily includes four genera: *Avihepevirus*, *Chirohepevirus*, *Paslahepevirus* and *Rocahepevirus*. The genus *Paslahepevirus* includes the species *Paslahepevirus balayani*, which is isolated from humans and mammals (2). *Paslahepevirus balayani* is subdivided into eight genotypes, of which the most important for humans are genotypes 1-4, 7, and 8. There are significant differences between each of the genotypes in terms of transmission patterns, epidemiology and course of infection. Genotypes 1 and 2 have been found only in humans, they circulate in Asia and Africa and are transmitted mainly by the fecal-oral route. Unlike them, genotypes 3 and 4 circulate in both human and animal species (domestic pigs, wild boar, deer and rabbits), and are transmitted to humans by consumption of under-cooked infected meat products (1). Genotypes 7 and 8 were identified in dromedary and Bactrian camels (3). Within these 8 genotypes, 31 subgenotypes were identified (4).

## HEV-3 subgenotypes distribution

HEV-3 genotype is transmitted worldwide and is the most frequently detected genotype in Europe (5). Initially, based on their nucleotide differences, HEV-3 isolates were sub-grouped into 10 subgenotypes, named with the small letters of the alphabet from "a" to "j" (6). Subsequently, new potential subgenotypes

## ADDRESS FOR CORRESPONDENCE:

Chiydem Ismailova  
National Centre of Infectious and Parasitic Diseases  
44A Gen. Stoletov Blvd., Sofia, Bulgaria  
e-mail: chiydem.ismailova@abv.bg

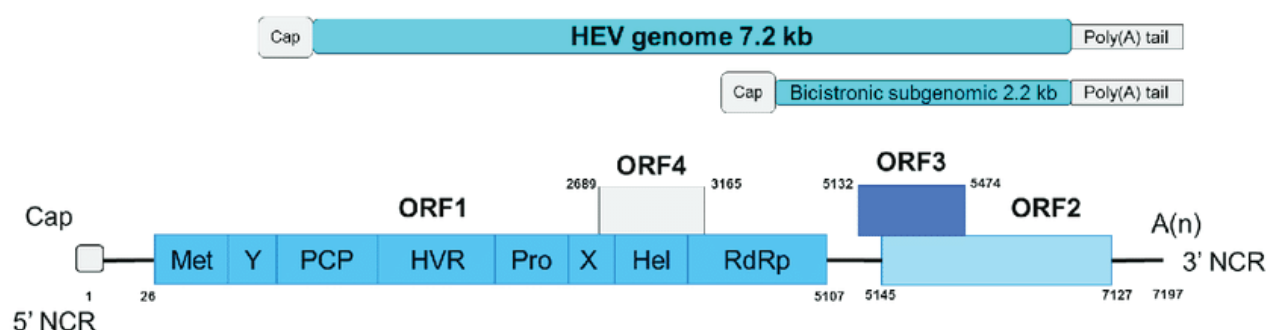
have been proposed identifying 3k, 3l, and 3m (previously named 3chi-new) as new subgenotypes. Later, with the accumulation of complete genome sequencing data from a large number of viral isolates and based on up to 26% nucleotide divergence, HEV-3 genotype was sub-divided into three main groups (clades) - 3abchij (referred to as Gt3-2), 3efg (Gt3-1) and sub-type 3ra (7, 8). Each subtype is associated with a specific geographic area, and some of the subgenotypes can be isolated simultaneously from different continental locations. Subgenotype HEV-3a is widespread and has been isolated in Europe, Asia, and the Americas (9, 10, 11, 12). HEV-3b and HEV-3d are reported mainly in Asian and American countries (13). However, according to a publication from 2022, HEV-3b and HEV-3m were isolated in a wild boar population in Italy (14). HEV-3j were described in Canada, Australia and Mexico (15, 16). HEV-3e, 3c, 3f, 3h and 3i are prevalent in Europe and subgenotypes 3e, 3c and 3h, in particular, are considered as autochthonous for Europe and they were detected in Bulgaria, as well (17, 18, 19, 20, 21, 22, 23). Subgenotype HEV-3i was described not only in Europe but also in Latin America (24, 25). In addition, there are several unassigned subgenotypes and new ones are being discovered all the time, due to the extreme heterogeneity of HEV-3 genotype (4).

### HEV structure and viral genome

There are two different forms of the HEV virions – non-enveloped (neHEV) and quasi-enveloped (eHEV) particles (26). Non-enveloped virions are icosahedral, spherical, 27–34 nm in diameter and are present in the faces and the bile of the infected individual.

Virions that circulate in the blood are coated with a host-derived membrane (27). Spikes are observed on the surface of the viral capsid (28). The viral capsid formed by capsomeres consisting of homodimers of the single capsid protein, where neutralizing epitopes are located. The capsid protein also has a receptor binding function (29). The viral genome is a linear, single stranded RNA molecule with a positive polarity. The size of the viral genome is approximately 7200 nucleotides in length and is structured as follows: 7-methylguanosine cap structure in the 5'-end followed by 5'-untranslated region (UTR), three open reading frames (ORFs) ORF1, ORF2 and ORF3, and 3'-UTR followed by a poly A tail (30). An additional ORF4 (nt 2835–3308), overlapping with ORF1 has been reported only for HEV genotype 1 (31). ORF1 is the largest ORF in the HEV genome with an approximate length of 5082 nucleotides (32). It starts from the 26<sup>th</sup> nucleotide and ends at the 5107<sup>th</sup>. The functional domains within ORF1 include: methyltransferase (Met), Y domain, papain-like cysteine protease (PCP), hypervariable region (HVR) or proline-rich region (PRR), X domain, Hel, and the RdRp (33). ORF2 is 1983 nucleotides long and is situated between the 5145<sup>th</sup> and 7127<sup>th</sup> nucleotides. ORF2 overlaps with ORF3 and codes the viral capsid protein. ORF3 begins 24 nucleotides downstream from the ORF1 stop codon and overlaps with ORF2 by 300 nucleotides. It is situated between nucleotides 5132 and 5474 (34).

HEV typing is based on successful amplification followed by targeted Sanger sequencing of the specific gene region and, finally - implementation of bioinformatics tools for genotype and sub-genotype assignment. Such approach is a powerful tool in



**Figure 1.** Schematic presentation of the genome structure of HEV (adapted from: Hepatitis E in High-Income Countries: What Do We Know? And What Are the Knowledge Gaps? - Scientific Figure on ResearchGate. Available from: [https://www.researchgate.net/figure/Organization-of-the-Hepatitis-E-virus-HEV-genome-open-reading-frames-ORFs-and\\_fig1\\_325400981](https://www.researchgate.net/figure/Organization-of-the-Hepatitis-E-virus-HEV-genome-open-reading-frames-ORFs-and_fig1_325400981) [accessed 14 Dec, 2022])

detecting and tracking similarities between HEV sequences obtained from different sources and samples. The aim of the present study was to sub-genotype twelve Bulgarian sequences of HEV using targeted Sanger sequencing of the specific HEV genome region in ORF2.

## MATERIALS AND METHODS

Twelve retrospective samples from patients, with serologically confirmed HEV infection (anti-HEV IgM and IgG positive), were recruited from archived serum samples tested between 2018 and 2021 in the National Reference Laboratory (NRL) for Hepatitis Viruses at the National Center of Infectious and Parasitic Diseases. Ethical approval was not required due to the retrospective character of the study.

### *Virological assays*

Detection and quantification of HEV RNA was performed using the RealStar HEV RT-PCR kit2.0 (Altona diagnostics, Germany) according to the manufacturer's instructions. Briefly, 25 µl of extracted RNA was mixed with an equal volume of Master Mix, after which the reverse transcription PCR reaction was set up. Four quantification standards (QS) were used to generate a standard curve for quantitative analysis. The results are considered valid if the generated standard curve reaches the control parameter value  $\geq 0.98$ . The linear range of the RT-PCR was from 10 to  $1 \times 10^7$  IU/µl. (as determined by the manufacturer).

### *Genotyping/sub-genotyping*

HEV genotyping was performed by sequencing the ORF2 region according to the method of Boxman et al. (35). Viral RNA was automatically extracted on ExiPrep 16Dx (Bioneer, RK). The extracted RNA was reverse transcribed and amplified using a first round and nested PCR. Reverse transcription was performed with a specific HEV gene primer using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA), in accordance with the manufacturer's instructions. The reaction was carried out at 42°C for 60 minutes, followed by 95°C for 5 minutes. The first and nested PCRs were carried out using the Platinum II Hot-start PCR master mix (Invitrogen, Lithuania), following the manufacturer's instructions. The cycling conditions for the first PCR were 35 cycles of 95°C for 30 seconds, 42°C for 30

seconds, and 60°C for 45 seconds. Respectively, the cycling conditions for the nested PCR were 40 cycles of 95°C for 30 seconds, 60°C for 20 seconds, and 72°C for 15 seconds. The amplification products were analyzed by agarose gel electrophoresis. After purification, PCR products were sequenced using the GenomeLab Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit on an automated DNA sequencer (Beckman Coulter, Inc., Fullerton, CA).

### *Statistical and bioinformatic analysis*

Numbers and percentages (n, %) were used to present qualitative variables. Data obtained after Sanger sequencing were processed and analyzed by two types of software - BioEdit and MEGA software version 11 (MEGA11). The raw data obtained from the sequencer represent paired-end nucleotide reads in FASTA format were analyzed by BioEdit, the biological sequence alignment editor. With the BioEdit the reads were filtered and low-quality reads were removed, the forward and reverse paired reads were normalized and a consensus nucleotide sequence was generated. The set of HEV reference sequences was obtained from the GenBank - genetic sequence database of the National Center for Biotechnology Information (NCBI) (36). In total 34 sequences were downloaded from the GenBank: 24 reference HEV sequences (AB248521; AB369687; AF455784; FJ906895; L08816; FJ457024; MH809516; KX387865; KJ496143; AB197673; AB856243; AB573435; AP003430; AF082843; AY115488; AB369689; MF959765; LC260517; KP294371; FJ998008; FJ705359; JQ013794; JQ953664; KU513561) (7); 8 sequences from Bulgarian patients with acute viral hepatitis E (MH203185; MH203169; MH203164; MH203227; MH203186; MH203187; MH203191; MH203166) (17); and 2 - from Bulgarian slaughterhouse pigs (MZ555942, MZ555941) (37). Using the Molecular Evolutionary Genetics Analysis (MEGA) software version 11 (MEGA11) [<https://www.megasoftware.net>], both the consensus sequences, created by BioEdit, and the reference sequences, were aligned together using ClustalW option. After that a maximum-likelihood analysis of the resulting alignment was performed to generate a phylogenetic tree. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed.

## RESULTS

### *Characteristics of the case-samples*

From a total of 12 sequenced samples, 10 were from patients with laboratory-confirmed acute viral hepatitis type E (AVH-E). Two of the serum samples were from patients with cirrhosis (CI) (Table 1).

Real-Time PCR showed variable levels of viremia in the case samples. The median viral load was 167 759 IU/ml, ranging from 360 IU/ml to 4 927 500 IU/ml (Table 1). The lowest detected viral load (360 IU/ml) was measured in a sample from a patient with AVH-E. The highest (4 927 500 IU/ml) concentration of HEV RNA was measured in a sample from a patient with CI. Both patients with clinically proven CI were characterized with a high viral load with a median value of 2 624 688 IU/ml. The median viral load in AVH-E patients was 11 235 IU/ml.

### *Phylogenetic analysis – genotyping and sub-genotyping*

A phylogenetic tree was built using the 12 case-sequences and 14 complete HEV genome reference sequences (Figure 1/case-seq-Probl\_g-t\_1-8). The studied case-sequences were stably defined into monophyletic group with HEV genotype 3 reference sequences. At the same time the case-sequences were separated in well-characterized sub-groups: A (n=8) and B (n=3). One of the sequences (696AVH20) forms a separate branch from the common ancestors with European and Japanese reference genotype 3

complete genome sequences. The sequences from subgroup A shared the most recent common ancestor with a reference sequence from Mongolia. Sub-group B clustered next to reference sequences from Italy and Germany.

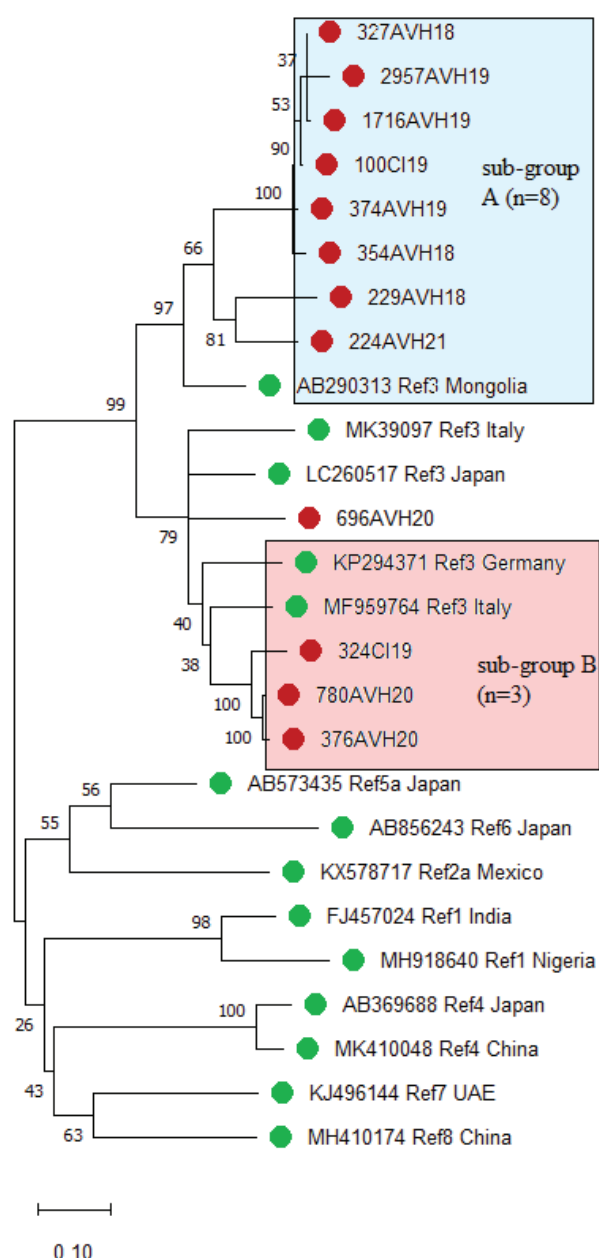
Further, the sub-genotyping phylogenetic tree was computed including the 12 case-sequences, previously published 8 human and 2 swine Bulgarian sequences, 13 HEV sub-genotype 3 reference complete genome sequences and the complete genome rabbit sequence (Ref3ra) (7), which was well defined in a separate branch within HEV-3 genotype (Figure 2/Aligh\_case-seq&ISS&pig\_sub\_g-t\_cut\_13-01-2024) (17, 37). The sub-genotyping tree revealed three well-defined clusters from the Bulgarian sequences. The majority (n=13) were grouped into a separate clade (C) with a common ancestor with reference HEV-3e sub-genotype. The second small but well-defined clade (D, n=3) was composed by three separate branches of the Bulgarian sequences, and formed separate monophyletic sub-group with the reference HEV-3f subgenotype. Taking into account that both clades C and D, were characterized by significant bootstrap values (91 and from 60 to 95, respectively) the sequences could be assigned as HEV sugenobtypes 3e and 3f, respectively. The third cluster (E, n=3) was statistically well supported (bootstrap value of 100) and represents a group of Bulgarian sequences that could not be assigned to any HEV-3 subgenotype. After sub-genotyping, the case-

**Table 1.** Main characteristics of the analyzed case- samples.

Sample ID	Diagnosis	Viral Load IU/ml	Genotype /Sub-cluster-ing/	Genome re-gion
224AVH21	AVH-E	367 375	HEV-3f	5923-6488
229AVH18	AVH-E	1 560	HEV-3f	5923-6486
327AVH18	AVH-E	2 783 750	HEV-3e	5960-6444
354AVH18	AVH-E	13 643	HEV-3e	5923 -6491
374AVH19	AVH-E	2 931 250	HEV-3e	5920 -6489
376AVH20	AVH-E	360	HEV-3	5923-6489
1716AVH19	AVH-E	8 826	HEV-3e	5958-6490
324CI19	CI	4 927 500	HEV-3	5923-6487
100CI19	CI	321 875	HEV-3e	5956-6487
2957AVH19	AVH-E	432 375	HEV-3e	5930-6492
780AVH20	AVH-E	2 872	HEV-3	5924-6491
696AVH20	AVH-E	6 756	HEV-3m	5921-6442

Legend: AVH-Acute viral hepatitis; CI = cirrhosis





**Figure 1.** Genotyping of the 12 case-sequences.

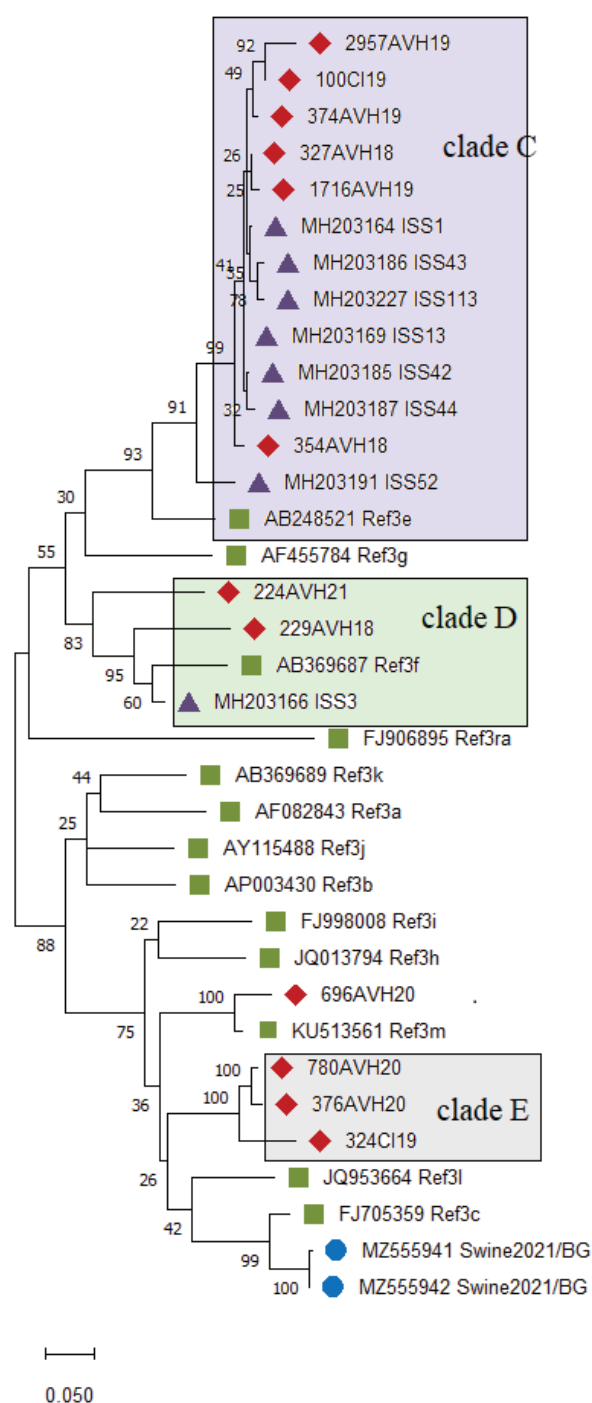
Evolutionary analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model (47)[1]. The tree with the highest log likelihood (-4926.66) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.1542)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 51.17% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 26 nucleotide sequences. There were a total of 598 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (48, 49). Bootstrap 500. Reference sequences are marked with green circles; study sequences are marked with red circles.

sequence 696AVH20 that formed a separate branch during the genotyping, shared a common ancestor with the reference HEV subgenotype 3m sequence. Due to maximal bootstrap support (of 100) the case-sequence was assigned as HEV subgenotype 3m. Bulgarian swine HEV sequences were grouped as well/maximum supported bootstrap branch with a referent HEV subgenotype 3c whole genome sequence.

## DISCUSSION

In recent years, HEV infection has emerged from the group of neglected diseases. In Bulgaria, HEV was isolated from patients with different clinical manifestation and slaughterhouse pigs Bulgaria (17, 38, 37). In the present study, phylogenetic analysis of

the Bulgarian sequences (case- and NCBI published ones, of human and animal origin, was conducted. After partial ORF2 sequencing of the case-sequences phylogenetic reconstruction was performed for HEV genotype subgenotype identification. All evaluated sequences belonged to HEV-3 genotype. Further case-sequences were assigned as sub-genotypes 3e, 3f and 3m. Our results are consistent with those of other recent studies on HEV. The majority of HEV strains sequenced in other European countries belong to subgenotypes 3c, 3f, and 3e. For instance, in a 2020 study, two main HEV-3 subgenotypes, 3c and 3f, were found in France, isolated from patients with AVH. The study also demonstrated correlations between the clinical course and particular subgenotypes, highlighting the importance of understanding the



**Figure 2.** Sub-genotyping analysis

Evolutionary analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (50)[1]. The tree with the highest log likelihood (-4446.30) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4411)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 30.35% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 35 nucleotide sequences. There were a total of 598 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (48). Reference sequences are marked with green squares, study sequences are marked with red rhombuses, sequences from Bulgarian patients with acute viral hepatitis E are marked with purple triangles and the sequences from Bulgarian slaughterhouse pigs with blue circles.

genetic diversity of HEV (39). A genotyping study conducted in Hungary, found that all sequences isolated from patients with AVH belonged to subgenotypes 3e and 3f. Similarly, a study conducted in Croatia between December 2021 and March 2022 found HEV-3c subgenotype sequences in two infected blood donors (41). A German study aimed at establishing the phylogenetic and epidemiological situation regarding HEV between 2010 and 2019, identified HEV-3c as the predominant subgenotype, with a continuing increase in the proportion of HEV-3 3abchijklm strains (accepted as a new taxonomic HEV clade). HEV-3c (67.3%) was the most prevalent

subgenotype, followed by HEV-3f, HEV-3e, and HEV-3i(-like) with 14.3%, 9.7%, and 4.0%, respectively (42). Although the virus exhibits significant genetic diversity, subgenotypes 3f, 3c, and 3e are the most commonly isolated among HEV-infected individuals in Europe (43). Bulgarian sequences show homology with referent HEV-3 complete genome sequences. The sequences in the present study were grouped into two main clades, homologous to both European (originating from Italy and Germany) and Asian (originating from Mongolia) whole genome HEV-3 sequences. These data are in agreement with the spatio-temporal reconstruction conducted by

Zehender et.al. of the currently circulating in Europe HEV-3 genotype. It was assumed that HEV-3 diverged into two main clades and one of them further diverged into the less common European sub-genotypes and the Asian sub-genotypes common ancestor (44).

Based on sub-genotyping the analyzed human sequences were further assigned as HEV-3e, 3f and 3m and porc sequences were close to sub-genotype HEV-3c. Previous phylogenetic analysis of Bulgarian human sequences for the period 2013-2015 established a similar distribution of HEV sub-genotypes with the prevalence of sequences assigned as HEV-3e and 3f (17). In their original study Palombieri and co-authors (37) established high nucleotide identity (92.0-93.9%) between the analyzed Bulgarian porc sequences and HEV-3c human sequences. HEV-3c was detected in pig porc fecal samples from Germany, Italy, Netherlands and Poland (45). It is worth mentioning that a switch from sub-genotypes HEV-3f and 3e to sub-genotype 3c has been observed in France, the United Kingdom, and Belgium. This is due to the circulation of HEV sub-genotypes in pig reservoirs (46).

A major drawback of our study is the small number of the analyzed sequences – case-sequences and selected ones from the NCBI. Although a two-step alignment process was used, involving genotyping followed by sub-genotyping, one of the sequences could not be assigned to a specific sub-genotype.

**Conclusion:** A larger study is required to determine the circulation of all possible subgenotypes of HEV in the country because of the significant genetic diversity of the virus and the constant emergence of new subgenotypes.

## ACKNOWLEDGMENTS

The study was supported by the National Science Fund of Bulgaria [Grant No KP-06-N33/2].

## REFERENCES

1. World health organization. [Online] <https://www.who.int/news-room/fact-sheets/detail/hepatitis-e>.
2. Purdy MA, Drexler JF, Meng XJ, et al. ICTV Virus Taxonomy Profile: Hepeviridae 2022. *J Gen Virol.* 2022;103(9):10.1099/jgv.0.001778. <https://doi.org/10.1099/jgv.0.001778>
3. Woo P, Lau S, Teng J, et al. New Hepatitis E Virus Genotype in Camels, the Middle East. *Emerging Infectious Diseases.* 2014;20(6):1044-1048. <https://doi.org/10.3201/eid2212.160979>
4. Smith DB, Simmonds P, Izopet J, et al. Proposed reference

- sequences for hepatitis E virus subtypes. *J Gen Virol.* 2016;97(3):537-542. <https://doi.org/10.1099/jgv.0.000393>
5. Kamar N, Bendall R, Legrand-Abravanel F, et al. Hepatitis E [published correction appears in *Lancet.* 2012 Aug 25;380(9843):730]. *Lancet.* 2012;379(9835):2477-2488. [https://doi.org/10.1016/S0140-6736\(11\)61849-7](https://doi.org/10.1016/S0140-6736(11)61849-7)
6. Lu L, Li C, Hagedorn CH. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. *Rev Med Virol.* 2006;16(1):5-36. <https://doi.org/10.1002/rmv.482>
7. Smith DB, Izopet J, Nicot F, Simmonds P, Jameel S, Meng XJ et al. Update: proposed reference sequences for subtypes of hepatitis E virus (species Orthohepevirus A). *J. gen. virol.* 2020 Jul;101(7):692-698. doi: 10.1099/jgv.0.0014358.
8. Subissi L, Peeters M, Lamoral S, Klamer S, Suin V, Van Gucht S. Subtype-specific differences in the risk of hospitalisation among patients infected with hepatitis E virus genotype 3 in Belgium, 2010-2018. *Epidemiol Infect.* 2019;147:e224. <https://doi.org/10.1017/S0950268819001122>
9. Huang FF, Haqshenas G, Guenette DK, et al. Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *J Clin Microbiol.* 2002;40(4):1326-1332. <https://doi.org/10.1128/JCM.40.4.1326-1332.2002>
10. Takahashi M, Nishizawa T, Yoshikawa A, et al. Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. *J Gen Virol.* 2002;83(Pt 8):1931-1940. <https://doi.org/10.1099/0022-1317-83-8-1931>
11. Ahn JM, Kang SG, Lee DY, Shin SJ, Yoo HS. Identification of novel human hepatitis E virus (HEV) isolates and determination of the seroprevalence of HEV in Korea. *J Clin Microbiol.* 2005;43(7):3042-3048. <https://doi.org/10.1128/JCM.43.7.3042-3048.2005>
12. van der Poel WH, Verschoor F, van der Heide R, et al. Hepatitis E virus sequences in swine related to sequences in humans, The Netherlands. *Emerg Infect Dis.* 2001;7(6):970-976. <https://doi.org/10.3201/eid0706.010608>.
13. Wu JC, Chen CM, Chiang TY, et al. Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. *J Med Virol.* 2002;66(4):488-492. <https://doi.org/10.1002/jmv.2170>
14. Battistini R, Serracca L, Razzuoli E, et al. Hepatitis E Virus (HEV): Identification of Subtypes 3b and 3m in Wild Boar Population in Liguria Region, Italy. *Pathogens.* 2022;11(11):1236. Published 2022 Oct 26. <https://doi.org/10.3390/pathogens11111236>
15. Pei Y, Yoo D. Genetic characterization and sequence heterogeneity of a canadian isolate of Swine hepatitis E virus. *J Clin Microbiol.* 2002;40(11):4021-4029. <https://doi.org/10.1128/JCM.40.11.4021-4029.2002>
16. Ward P, Müller P, Letellier A, et al. Molecular characterization of hepatitis E virus detected in swine farms in the province of Quebec. *Can J Vet Res.* 2008;72(1):27-31.
17. Bruni R, Villano U, Equestre M, et al. Hepatitis E virus genotypes and subgenotypes causing acute hepatitis, Bulgaria, 2013-2015. *PLoS One.* 2018;13(6):e0198045. Published 2018 Jun 7. <https://doi.org/10.1371/journal.pone.0198045>
18. Banks M, Bendall R, Grierson S, Heath G, Mitchell J, Dalton H. Human and porcine hepatitis E virus strains, United Kingdom. *Emerg Infect Dis.* 2004;10(5):953-955. <https://doi.org/10.3201/eid1005.030908>.
19. Zanetti AR, Schlauder GG, Romanò L, et al. Identification of a novel variant of hepatitis E virus in Italy. *J Med Virol.* 1999;57(4):356-360. [https://doi.org/10.1002/\(sici\)1096-9071\(199904\)57:4<356::aid-jmv5>3.0.co;2-d](https://doi.org/10.1002/(sici)1096-9071(199904)57:4<356::aid-jmv5>3.0.co;2-d)
20. Festa S, Garbuglia AR, Baccini F, et al. Acute fulminant

- hepatitis E virus genotype 3e infection: description of the first case in Europe. *Scand J Infect Dis*. 2014;46(10):727-731. <https://doi.org/10.3109/00365548.2014.928417>
21. Schlauder GG, Desai SM, Zanetti AR, Tassopoulos NC, Mushahwar IK. Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV. *J Med Virol*. 1999;57(3):243-251. [https://doi.org/10.1002/\(sici\)1096-9071\(199903\)57:3<243::aid-jmv6>3.0.co;2-r](https://doi.org/10.1002/(sici)1096-9071(199903)57:3<243::aid-jmv6>3.0.co;2-r)
22. Renou C, Pariente A, Cadranet JF, Nicand E, Pavo N. Clinically silent forms may partly explain the rarity of acute cases of autochthonous genotype 3c hepatitis E infection in France. *J Clin Virol*. 2011;51(2):139-141. <https://doi.org/10.1016/j.jcv.2011.02.013>
23. Martelli F, Toma S, Di Bartolo I, et al. Detection of Hepatitis E Virus (HEV) in Italian pigs displaying different pathological lesions. *Res Vet Sci*. 2010;88(3):492-496. <https://doi.org/10.1016/j.rvsc.2009.12.011>
24. Dell'Amico MC, Cavallo A, Gonzales JL, et al. Hepatitis E virus genotype 3 in humans and Swine, Bolivia. *Emerg Infect Dis*. 2011;17(8):1488-1490. <https://doi.org/10.3201/eid1708.100769>
25. Worm HC, Schlauder GG, Wurzer H, Mushahwar IK. Identification of a novel variant of hepatitis E virus in Austria: sequence, phylogenetic and serological analysis. *J Gen Virol*. 2000;81(Pt 12):2885-2890. <https://doi.org/10.1099/0022-1317-81-12-2885>
26. Wang B, Meng XJ. Structural and molecular biology of hepatitis E virus. *Comput Struct Biotechnol J*. 2021;19:1907-1916. Published 2021 Apr 7. <https://doi.org/10.1016/j.csbj.2021.03.038>
27. Nagashima S, Takahashi M, Kobayashi T, et al. Characterization of the Quasi-Enveloped Hepatitis E Virus Particles Released by the Cellular Exosomal Pathway. *J Virol*. 2017;91(22):e00822-17. Published 2017 Oct 27. <https://doi.org/10.1128/JVI.00822-17>
28. Bradley DW. Enterically-transmitted non-A, non-B hepatitis. *Br Med Bull*. 1990;46(2):442-461. <https://doi.org/10.1093/oxfordjournals.bmb.a072409>
29. Mori Y, Matsuura Y. Structure of hepatitis E viral particle. *Virus Res*. 2011;161(1):59-64. <https://doi.org/10.1016/j.virusres.2011.03.015>
30. 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(12):5919-5926. <https://doi.org/10.1128/JVI.00046-06>
31. Emerson SU, Purcell RH. Hepatitis E virus. *Rev Med Virol*. 2003;13(3):145-154. <https://doi.org/10.1002/rmv.384>
32. Tsarev SA, Emerson SU, Reyes GR, et al. Characterization of a prototype strain of hepatitis E virus. *Proc Natl Acad Sci U S A*. 1992;89(2):559-563. <https://doi.org/10.1073/pnas.89.2.559>
33. Ropp SL, Tam AW, Beames B, Purdy M, Frey TK. Expression of the hepatitis E virus ORF1. *Arch Virol*. 2000;145(7):1321-1337. <https://doi.org/10.1007/s007050070093>
34. Graff J, Nguyen H, Yu C, et al. The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. *J Virol*. 2005;79(11):6680-6689. <https://doi.org/10.1128/JVI.79.11.6680-6689.200535>
35. Boxman ILA, Jansen CCC, Hägele G, et al. Porcine blood used as ingredient in meat productions may serve as a vehicle for hepatitis E virus transmission. *Int J Food Microbiol*. 2017;257:225-231. <https://doi.org/10.1016/j.ijfoodmicro.2017.06.029>
36. Benson DA, Cavanaugh M, Clark K, et al. GenBank. *Nucleic Acids Res*. 2013;41(Database issue):D36-D42. <https://doi.org/10.1093/nar/gks1195>
37. Palombieri A, Tsachev I, Sarchese V, et al. A Molecular Study on Hepatitis E Virus (HEV) in Pigs in Bulgaria. *Vet Sci*. 2021;8(11):267. Published 2021 Nov 6. <https://doi.org/10.3390/vetsci8110267>
38. Golkocheva-Markova E, Ismailova C, Kevorkyan A, et al. Age and Gender Trends in the Prevalence of Markers for Hepatitis E Virus Exposure in the Heterogeneous Bulgarian Population. *Life (Basel)*. 2023;13(6):1345. Published 2023 Jun 8. <https://doi.org/10.3390/life13061345>
39. Abravanel F, Dimeglio C, Castanier M, et al. Does HEV-3 subtype play a role in the severity of acute hepatitis E? *Liver Int*. 2020;40(2):333-337. <https://doi.org/10.1111/liv.14329>
40. Ulbert ÁB, Bukva M, Magyar A, et al. Characteristics of hepatitis E viral infections in Hungary. *J Clin Virol*. 2022;155:105250. <https://doi.org/10.1016/j.jcv.2022.105250>
41. Gorski I, Babić I, Bingulac-Popović J, et al. Prevalence of HEV RNA in Croatian blood donors. *Transfus Clin Biol*. 2023;30(2):244-248. <https://doi.org/10.1016/j.traccli.2023.01.005>
42. Schemmerer M, Wenzel JJ, Stark K, Faber M. Molecular epidemiology and genotype-specific disease severity of hepatitis E virus infections in Germany, 2010-2019. *Emerg Microbes Infect*. 2022;11(1):1754-1763. <https://doi.org/10.1080/22221751.2022.2091479>
43. Zahmanova G, Takova K, Tonova V, et al. The Re-Emergence of Hepatitis E Virus in Europe and Vaccine Development. *Viruses*. 2023;15(7):1558. Published 2023 Jul 16. <https://doi.org/10.3390/v15071558>
44. Zehender G, Ebranati E, Lai A, et al. Phylogeography and phylodynamics of European genotype 3 hepatitis E virus. *Infect Genet Evol*. 2014;25:138-143. <https://doi.org/10.1016/j.meegid.2014.04.016>
45. De Sabato L, Ianiro G, Alborali GL, Kroneman A, Grierson SS, Krumova-Valcheva GL et al. Molecular Characterization and Phylogenetic Analysis of Hepatitis E Virus (HEV) Strains from Pigs Farmed in Eight European Countries between 2020 and 2022. *Transbound. Emerg. Dis.*. 2023 Dec 7;2023:2806835. <https://doi.org/10.1155/2023/2806835>
46. Nicot F, Dimeglio C, Miguieres M, et al. Classification of the Zoonotic Hepatitis E Virus Genotype 3 Into Distinct Subgenotypes. *Front Microbiol*. 2021;11:634430. Published 2021 Jan 28. <https://doi.org/10.3389/fmicb.2020.634430>
47. Nei M. and Kumar S. . Molecular Evolution and Phylogenetics. Oxford University Press, New York. 2000; doi:10.1017/S0016672301219405
48. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol Biol Evol*. 2021;38(7):3022-3027. <https://doi.org/10.1093/molbev/msab120>
49. Felsenstein J. CONFIDENCE LIMITS ON PHYLOGENIES: AN APPROACH USING THE BOOTSTRAP. *Evolution*. 1985;39(4):783-791. <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>
50. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*. 1993;10(3):512-526. <https://doi.org/10.1093/oxfordjournals.molbev.a040023>