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RESEARCH ARTICLE

Genetic Diversity of Hepatitis E Virus in the Republic of Belarus

S. V. Zhavoronok[1], V. V. Davydov[1]*, E. L. Gasich[2], L.A. Anisko[1], S. I. Marchuk[1], I. V. Potemkin[3,4], A. A. Karlsen[3,4], K. K. Kyuregyan[3,4], M. I. Mikhailov[3,4], G.I. Alatorceva[3], P.A. Krasochko[5], D.S. Barysavets[6], Yu.V. Lamaka[6]

[1]Belarusian State Medical University, Minsk, Belarus
[2]The Republican Research and Practical Center for Epidemiology and Microbiology, Minsk, Belarus
[3]Mechnikov Research Institute for Vaccines and Sera, 105064, Moscow, Russia
[4]Russian Medical Academy of Continuous Professional Education, 125993, Moscow, Russia
[5]Vitebsk State Academy of Veterinary Medicine, Vitebsk, Belarus
[6]Institute of Experimental Veterinary Medicine named of S. N. Vyshelessky, Minsk, Belarus.

Abstract

The objective of this research was to study the genetic diversity of hepatitis E virus (HEV) strains obtained from humans and animals in Belarus. Samples of biological material from 97 patients, 79 pigs, 28 wild boars, 40 deer, 359 rabbits were tested for HEV RNA in RT-PCR. The obtained nucleotide sequences (n=9) were subjected to sequencing and phylogenetic analysis. A model of evolutionary relationships for the sequences encoding a fragment of the viral capsid protein was constructed. The sequences from this study split up between the two main clades of subgenotypes of the viral genotype 3. Within the "3abchij" clade, 5 of the 9 studied sequences were clustered, and within the "3efg" clade 2 studied isolates were the subject to clustering. The sequences from rabbits formed a separate clade on dendrogram within the genotype 3 with a 94% probability. The studied HEV nucleotide sequences obtained from humans and animals were clustered with subgenotype reference sequences 3c, 3f, 3i, and 3ra. The possibility of HEV import to the Republic of Belarus from Western Europe and the Russian Federation, as well as existence of autochthonous zoonotic cases of HEV infection have been proved.

Keywords: Viral hepatitis E, phylogenetic analysis, genetic polymorphism.

Abbreviations: HEV = Hepatitis E virus, ORF = Open reading frame

Introduction:

Hepatitis E virus (HEV) is a single-chain quasi-enveloped RNA virus with positive polarity, belongs to the *Orthohepevirus A* species, *Orthohepevirus* genus, *Hepeviridae* family. Typically, hepatitis E (HE) is an acute self-limiting disease with a mortality rate of 0.5 to 3% among young and immunocompetent individuals. In case of HEV infection during the third trimester of pregnancy mortality rate can reach 30% [1]. According to WHO estimates, 20 million infections occur annually, resulting in 3 million symptomatic cases of HE and about 70,000 deaths [2]. The true global burden of this disease is probably even greater [3]. Data from seroprevalence studies conducted in different countries among healthy people suggests that up to one third of the population have anamnestic anti-HEV [4].

HEV is characterized by fecal-oral route of transmission. Previously, it was believed that HEV infection is a health issue only in developing countries. Indeed, as reported by WHO, HEV is hyperendemic in Asia and Africa. However, as per the new paradigm, HEV also commonly causes acute viral hepatitis in the countries with developed infrastructure [5].

Based on phylogenetic analysis, all HEV strains refer to 8 genotypes [6]. Strains of genotypes 1 and 2 are obligate human pathogens. HEV strains of genotypes 3 and 4 are zoonotic, and have a wide range of hosts, among which are wild and domestic pigs, deers, rabbits, mongooses [7]. Animal reservoirs cause human infection and mainly trigger autochthonous sporadic HE cases in industrial countries.

It was previously thought that HEV causes only acute infection, whereas as is known today, the virus is also detected among immunocompromised individuals with chronic hepatitis [8]. HEV is a hepatotropic pathogen, meanwhile, certain extrahepatic manifestations, such as neurological disorders and kidney damage, have also been recorded [9]. Thus, given the wide prevalence and a variety of clinical manifestations, hepatitis E poses an underestimated public health threat in developed countries.

HEV genetic structure is quite simple. The virus RNA consists of 7.2 kb and contains only 3 open reading frames (ORF), where ORF2 and ORF3 partially overlap. ORF1 provides synthesis of non-structural polyprotein, required for virus

***Correspondence to:** Vladimir V Davydov, Belarusian State Medical University, Minsk, Belarus. E-mail: davidovvv@bsmu.by

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replication. OPF2 forms a 2 kb subgenomic bicystronic RNA and encodes capsid protein. ORF3 is the smallest of the three frames, which overlaps with ORF2 by about 300 nucleotides in the alternate reading frame [10]. ORF2 and ORF3 overlap region is the most conservative sequence, almost devoids of polymorphism.

Our research shows that all HEV strains, obtained from human and animal samples in the Republic of Belarus, refer to the viral genotype 3 (HEV-3) [11]. HEV subgenotype taxonomy, based on the study of the differences in the evolutionary distance (p-distance) of the virus genome sequences, shows a complex pattern with the formation of different phylogenetic groups. Thus, 3a, 3b, 3c, 3h, 3i and 3j subgenotypes (3abchij) and 3e, 3f and 3g subgenotypes (3efg) form two main phylogenetic clades. Virus diversity taxonomy process comes with a number of difficulties. Firstly, currently there is no generally accepted and approved list of reference sequences for these subgenotypes, although there have been attempts to standardize the classification of HEV-3[12]. Secondly, there are no standard criteria, that determine distances within and between subgenotypes. For example, p-distances between subgenotypes within HEV-1 is less than 0.12, between HEV-3 subgenotypes - from 0.12 to 0.26, and between HEV-4 subgenotypes - from 0.13 to 0.18. Besides p-distance ranges within and between subgenotypes overlap. Therefore, some complete genome sequences have acquired conflicting subtype assignments [13]. Thus, discrete artificially introduced categories used for classification often become arbitrary, as their genetic identity blurs into a variability continuum with description of additional new mutant strains or recombinants. Moreover, it is important to have a common set of reference sequences to compare results of different studies. The objective of this research was to study the genetic diversity of HEV strains obtained from humans and animals in the Republic of Belarus.

Materials and methods:

From 2017 to 2020, blood serum and stool samples were collected from 97 patients, 79 pigs, 28 wild boars, 40 deers, 359 rabbits. The obtained samples were used to detect HEV RNA by means of PCR analysis. The nucleic acid extraction kit (Jena Bioscience, Germany) was used to extract total RNA, following the manufacturer's protocol. To detect HEV RNA, we used the adapted reverse transcription nested PCR analysis (RT-PCR with degenerate primers, focused on the ORF2 section of the HEV genome from 5905 to 6635 nucleotides). Test sensitivity is not less than 10 MU/µl and 100% specificity. RT-PCR conditions were as described elsewhere [14]. Positive results' validation was carried out with the help of commercial HEV RT-PCR Kit 2.0 (RealStar®, Altona, Germany).

The QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) was used to extract amplification products from agarose gel. The nucleotide sequence of the HEV genome fragment was determined during direct amplicon sequencing on the 3500 GeneticAnalyzer automatic sequencer (ABI, Foster City, USA), using the BigDye Terminator v 3.1 Cycle Sequencing Kit. Analysis of HEV nucleotide sequences and their genotyping were performed with the help of MEGA X software [15]. 37 nucleotide sequences were analyzed, all of them are ORF2 HEV fragments, consisting of 273 nucleotides (nucleotide positions 6193 – 6466 against the Burma strain, GenBank number M73218). 9 sequences were obtained from humans and animals in the Republic of Belarus, 23 reference sequences for HEV genotypes 1-7 and HEV-3 subgenotypes, suggested by Smith D. B. and the co-authors [13], 3 sequences closest to the ones obtained in Belarus, that were found as a result of the search using BLAST tool. Avian HEV sequence was included into phylogenetic analysis as an outgroup. The list of HEV reference sequences included in analysis is shown in table 1. The phylogenetic analysis was carried out using maximum likelihood method based on the Hasegawa-Kishino-Yano model [16].

Results and discussion:

Based on the phylogenetic analysis of sequences corresponding to fragment of the viral capsid protein and, a phylogenetic tree was constructed, which allowed us to estimate the degree of genetic variability of HEV sequences isolated from humans and animals in Belarus in comparison to reference sequences for HEV genotypes and subgenotypes, as well as homologous sequences from the GenBank database (Figure 1). All HEV sequences from Belarus were clustered within the viral genotype 3. In most cases, the subgenotype was established for most sequences (table 2). The sequences from this study split up between two main clades of subgenotypes within genotype 3. Within the "3abchij" clade, 5 of the 9 studied sequences were clustered, and within the "3efg" clade 2 studied isolates

Table 1: HEV reference sequences, used for the phylogenetic analysis

No	Genotype	Subgenotype	No GenBank	Host
1.	1	1a	M73218	Homo sapiens
2.	1	1b	D11092	Homo sapiens
3.	1	1c	X98292	Homo sapiens
4.	1	1d	AY230202	Homo sapiens
5.	1	1e	AY204877	Homo sapiens
6.	1	1f	JF443721	Homo sapiens
7.	2	2a	M74506	Homo sapiens
8.	3	3a	AF082843	Domestic pig
9.	3	3b	AP003430	Homo sapiens
10.	3	3c	FJ705359	Wild boar
11.	3	3d	AF296165	Domestic pig
12.	3	3e	AB248521	Domestic pig
13.	3	3f	AB369687	Homo sapiens
14.	3	3g	AF455784	Domestic pig
15.	3	3h	JQ013794	Homo sapiens
16.	3	3i	FJ998008	Wild boar
17.	3	3j	AY115488	Domestic pig
18.	3	3ra	FJ906895	Oryctolagus cunicul
19.	4	4a	AB197673	Homo sapiens
20.	4	4b	DQ279091	Domestic pig
21.	5	5a	AB573435	Wild boar
22.	6	6a	AB602441	Wild boar
23.	7	7a	KJ496143	Camelus dromedarius

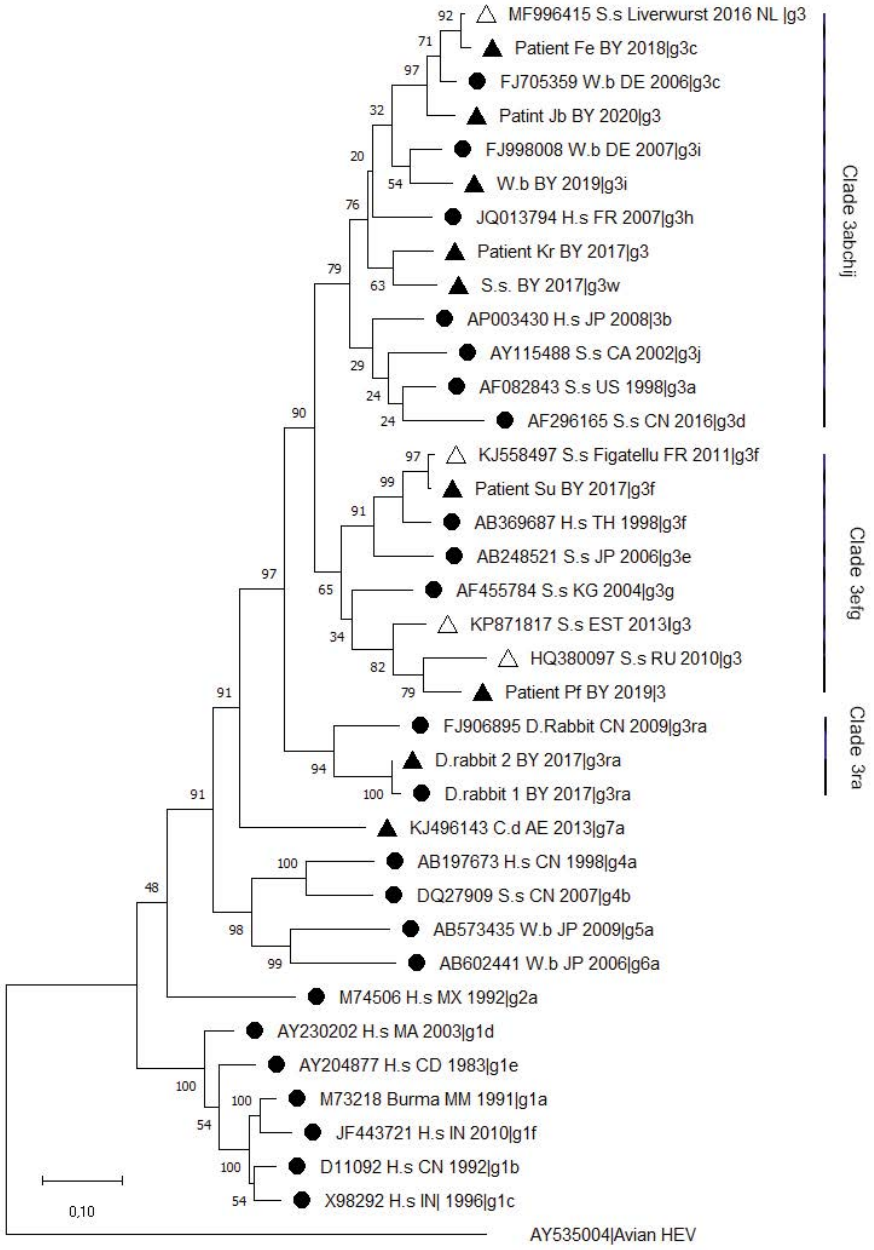

Figure 1: Phylogenetic tree for the partial HEV ORF2 sequences.

The tree with the highest logarithmic likelihood (-94549.25) is shown. The percentage of trees with the clustered linked taxons is given next to the branches. The tree is built in full scale; the length of branches is measured by the number of substitutions per site. This analysis included 36 nucleotide sequences. Symbols: ▲ – sequences obtained in Belarus, • – reference sequences, △ – closest to the sequences from Belarus.

Table 2: Genotyping of HEV nucleotide sequences, obtained from humans and animals in the Republic of Belarus

No	Genotype	Subgenotype	Code	Host
1	3	3c	Patient_Fe_BY_2018|g3c	Homo sapiens
2	3	3	Patient_Kr_BY_2017|g3	Homo sapiens
3	3	3g	Patient_Pf_BY_2019|3	Homo sapiens
4	3	3f	Patient_Su_BY_2017|g3f	Homo sapiens
5	3	3c	Patint_Jb_BY_2020|g3	Homo sapiens
6	3	3	S.s._BY_2017|g3	Domestic pig
7	3	3i	W.b_BY_2019|g3i	Wild boar
8	3	3ra	D.rabbit_2_BY_2017|g3ra	Oryctolagus cunicul
9	3	3ra	D.rabbit_1_BY_2017|g3ra	Oryctolagus cunicul

were clustered. Two nucleotide sequences, obtained from rabbits, formed a separate clade within genotype 3, creating a single branch with a reference sequence of rabbit HEV, with a 94% probability.

The viral genome sequence with the "Patient_Su_BY_2017|g3f" code on the dendrogram, obtained in 2017 from a patient, who had been to France one month before the onset of the disease and regularly consumed home-made smoked sausages, formed a single phylogenetic branch with a 97% probability with a sequence obtained from the traditional Corsican dry-cured pork sausage "Figatelli" ("KJ558497_S. s_figatellu_fr_2011|G3F"). There is practically no evolutionary distance between these two sequences (p-distance = 0.004±0.0041), which allows us to assert the importation of HEV in this medical case (table 3). A 3f subgenotype was determined for this isolate, since this sequence forms a single phylogenetic branch with the reference sequence "AB369687_H.s_TH_1998|g3f" with a 99% probability (p-d=0.09±0.021).

The possibility of HEV importation to Belarus from the European countries was further confirmed by a molecular genetic analysis of the nucleotide sequence, obtained in 2018 from patient “Fe” from Belarus who had a history of travel to the Western European countries during the incubation period of the disease. Isolate "Patient_Fe_BY_2018|g3c", obtained from this patient, refers to HEV 3c subgenotype and have a small evolutionary distance (0.05±0.015) from the reference strain "FJ705359_W.b_DE_2006|g3c" obtained from a wild boar in Germany. This sequence has 92% identity to the one obtained from the liver sausage in the Netherlands in 2016 with evolutionary distance between them being only 0.01±0.007. Isolate "Patient_jb_by_2020|g3" also belongs to the same group of sequences with a 97% probability. The fact that this isolate was obtained from a patient who arrived from Western Europe, together with results of genetic analysis, suggests the importation of HEV infection in this case.

The geographical location of the Republic of Belarus in the center of the European continent makes it possible the importation of HEV both from the West and the East. The phylogenetic tree (Figure 1) shows the result of a molecular genetic study of HEV nucleotide sequence obtained in 2019 from patient “Pf” (code "Patient_Pf_BY_2019|3"). This sequence demonstrated clustering within subgenotype 3g, but this result was confirmed only in 82% of replications, which is not statistically reliable. This sequence has the smallest evolutionary distance of 0.13±0.026 with sequence "HQ380097_S. s_RF_2010|g3" obtained from domestic pig in Russia in 2010, but also not statistically reliable.

Our study also demonstrated the autochthonous cases of HE in Belarus. The analysis of nucleotide sequence "Patient_Kr_BY_2017|g3" obtained in 2017 from a patient who had consumed untreated pork liver revealed a high degree of homology with the sequence isolated from the feces of domestic swine in the Minsk region, and with a sequence, isolated from a wild boar in Smolevichi district of the Minsk region. All three sequences were clustered in one phylogenetic branch of the dendrogram in 76% of replications. The value of the evolutionary distances between these sequences is 0.13±0.025

Table 3: The values of pairwise evolutionary distances between HEV nucleotide sequences obtained from humans and animals in the Republic of Belarus, as well as HEV sequences selected for comparison from the GenBank database.

No	Code	p-distance	Code
1.	Patient_Fe_BY_2018|g3c	0,05±0,015	FJ705359_W.b_DE_2006|g3c
2.	Patient_Fe_BY_2018|g3c	0,01±0,007	MF996415_S.s_Liverwurst_2016_NL_|g3
3.	Patient_Fe_BY_2018|g3c	0,12±0,023	S.s._BY_2017|g3
4.	Patient_Fe_BY_2018|g3c	0,12±0,023	W.b_BY_2019|g3i
5.	Patient_Kr_BY_2017|g3	0,14±0,025	Patient_Fe_BY_2018|g3c
6.	Patient_Kr_BY_2017|g3	0,14±0,024	Patint_Jb_BY_2020|g3
7.	Patient_Kr_BY_2017|g3	0,13±0,025	S.s._BY_2017|g3w
8.	Patient_Kr_BY_2017|g3	0,14±0,025	W.b_BY_2019|g3i
9.	Patient_Pf_BY_2019|3	0,14±0,025	W.b_BY_2019|g3i
10.	Patient_Su_BY_2017|g3f	0,004±0,0041	KJ558497_S.s_Figatellu_FR_2011|g3f
11.	Patient_Su_BY_2017|g3f	0,09±0,021	AB369687_H.s_TH_1998|g3f
12.	Patint_Jb_BY_2020|g3	0,08±0,019	Patient_Fe_BY_2018|g3c
13.	Patint_Jb_BY_2020|g3	0,13±0,022	S.s._BY_2017|g3w
14.	Patint_Jb_BY_2020|g3	0,08±0,018	MF996415_S.s_Liverwurst_2016_NL_|g3
15.	Patint_Jb_BY_2020|g3	0,07±0,016	FJ705359_W.b_DE_2006|g3c
16.	Patint_Jb_BY_2020|g3	0,14±0,025	W.b_BY_2019|g3i
17.	S.s._BY_2017|g3	0,13±0,024	MF996415_S.s_Liverwurst_2016_NL_|g3
18.	W.b_BY_2019|g3i	0,14±0,024	S.s._BY_2017|g3
19.	W.b_BY_2019|g3i	0,13±0,024	MF996415_S.s_Liverwurst_2016_NL_|g3
20.	W.b_BY_2019|g3i	0,10±0,021	FJ998008_W.b_DE_2007|g3i
21.	D.rabbit_2_BY_2017|g3ra	0,01±0,007	D.rabbit_1_BY_2017|g3ra

and 0.14±0.025, respectively. Isolate "W. b_BY_2019|g3i" was referred to the 3i subgenotype due to the high degree of the sequence identity with this genotype's reference strain "FJ998008_W.b_DE_2007|g3i" (pd=0.1±0.021). Isolate "Patient_Kr_BY_2017|g3" has statistically unreliable differences in evolutionary distances with reference sequences of 3h, 3i, 3j subgenotypes, thus, it is not possible to refer it to any of them.

Conclusions:

1. HEV sequences obtained in Belarus show a wide genetic diversity within genotype 3.
2. The studied HEV nucleotide sequences isolated from humans and animals are clustered in subgenotype reference sequences 3c, 3f, 3i, and 3ra.
3. The possibility of HEV importation to the Republic of Belarus from Western Europe and the Russian Federation, as well as existence of autochthonous zoonotic cases of HE have been proved.

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