NATIONAL CENTRE OF INFECTIOUS AND PARASITIC DISEASES

Department of Virology

National Reference Laboratory "Hepatitis Viruses"

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STUDIES ON THE SEROLOGICAL AND MOLECULAR GENETIC CHARACTERISTICS OF HEPATITIS E VIRUS

ABSTRACT OF A PhD THESIS

for the award of the educational and scientific degree of 'Doctor' in the scientific field of Virology. Field of Education: 4. Natural Sciences, Mathematics and Informatics; Professional field: 4.3. Biological Sciences.

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The doctoral thesis was developed in the Department of Virology, at the National Reference Laboratory (NRL) "Hepatitis Viruses" of the National Center of Infectious and Parasitic Diseases (NCIPD), Sofia.

The studies were funded by the National Centre for Infectious and Parasitic Diseases, Sofia, Bulgaria, Project „Studies on the genotypic diversity and the involvement of the polyproline region in the in vitro adaptation of Hepatitis E virus", funded by the National Science Fund of Bulgaria" (Grant No KP-06-N33/2) and Project BG05M2OP001-1.002-0001 'Fundamental, translational and clinical research in the field of infections and infectious immunology', funded by OP 'Science and Education for Smart Growth 2014-2020' and the European Regional Development Fund.

The internal defence took place before an extended panel of the Virology Department on 2nd June 2026 at 10:00.

The doctoral thesis consists of a list of abbreviations used, an introduction, a literature review, objectives and tasks, materials and methods, results and discussion, conclusions, contributions, a list of references used and appendices. The thesis contains 116 pages, 30 figures, 24 tables. The bibliography lists 198 literature sources

The materials related to the defense are published on the NCIPD website and are available at the NCIPD Library, Sofia.

The doctoral thesis is scheduled for public defence on 14.09.2026 at 13:00 in the auditorium of the NCIPD, 26 Yanko Sakazov Blvd., Sofia, at an open meeting of the scientific jury appointed by order No. 182/30.06.2026 of the Director of the NCIPD, in accordance with the Implementing rules of the Law on the Development of Academic Staff of the NCIPD and the Law on the Development of Academic Staff.

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List of abbreviations used:

cDNA – complementary DNA

Clade – monophyletic group, including a common ancestor and descendants

AVH – acute viral hepatitis

RNA – ribonucleic acid

WHO – World Health Organization

LD – liver disease

LC – liver cirrhosis

ACLF – *acute-on-chronic liver failure*

ALT – *alanine aminotransferase*

AST – *aspartate aminotransferase*

CRE – *cis reactive elements*

ddNTPs – *Dideoxynucleotides*

ELISA – *Enzyme Linked Immunosorbent Assay*

ET-NANB – *enterically-transmitted non-A, non-B hepatitis*

HAV – *hepatitis A virus*

HBV – *hepatitis B virus*

HBsAg – *hepatitis B surface antigen*

HEV – *hepatitis E virus*

HCV – *hepatitis C virus*

HDV – *hepatitis D virus*

Hel – *helicase-like domain*

HVR - *hypervariable region*

ICTV – *International Committee on Taxonomy of Viruses*

NTR –*Non-Translating Region*

ORF – *Open Reading Frame*

PCR – *Polymerase Chain Reaction*

PT-NANB – *parenterally transmitted non-A, non-B hepatitis*

PCP – *Papain-like cysteine protease*

MT – *Methyltransferase*

RdRp – *RNA-dependent RNA polymerase*

Introduction

Hepatitis E was first described in 1979 during the investigation of an outbreak in India. The hepatitis spread via the fecal-oral route through the consumption of contaminated water.

The hepatitis E virus (HEV) was discovered using electron microscopy in 1981, and in 1990, a portion of the viral genome was cloned and sequenced. It was subsequently recognized as the fifth hepatotropic virus, alongside hepatitis viruses A, B, C, and D.

HEV holds significant clinical importance in human pathology. Infection with HEV can manifest as acute and/or chronic, fulminant, or asymptomatic forms, and extrahepatic manifestations have also been described, which can sometimes be the sole presentation of the disease. The genetic characteristics of the virus influence the distribution, clinical course, and severity of the infection. Different HEV genotypes circulate in distinct geographic regions and target separate risk groups; in this regard, HEV remains without analogue among hepatotropic viruses.

In Bulgaria, HEV infection has been studied since the late 20th century, though systematic data on its prevalence during that period are lacking. Over the last two decades, interest in the disease has grown, accompanied by an increasing number of publications by Bulgarian authors. In 2019, "acute HEPATITIS E" was officially included in Ordinance No. 21 on the procedure for the registration, notification, and reporting of infectious diseases. Since then, new data regarding its clinical manifestations, affected demographics, and prevalence rates have been accumulating annually.

The present doctoral thesis aims to expand research on HEV infection in Bulgaria by investigating its seroprevalence across various populations and by analyzing the genetic characteristics of the virus. The obtained results will contribute to enriching the currently established data on the infection within the country, as well as to the development of targeted prevention programs.

I. Aim and Objectives

Aim

- To investigate the seroprevalence of hepatitis E and the molecular-genetic characteristics of HEV isolates within a heterogeneous population over the period 2015–2023.

Objectives

1. To establish the circulation of HEV by detecting infection markers in serum samples across various population groups.
2. To evaluate the significance of gender, age, occupation, and risk factors in the acquisition and development of HEV infection.
3. To screen for HEV RNA in serum samples with confirmed anti-HEV antibodies.
4. To assess the correlation between serological and molecular markers of HEV infection.
5. To perform genotyping of the isolated HEV strains.

II. Materials and methods

1. Materials

A total of 2,467 samples (whole blood and serum from patients and healthy individuals from various populations and different healthcare facilities in the country) received at the NRL "Hepatitis Viruses" of the NCIPD (Appendix 2).

Study Populations (Table. 1)

*1823 prospective serum samples from hospitalized individuals with various diagnoses:

-*with predominant hepatic manifestations*: unspecified acute viral hepatitis; acute viral hepatitis E; other liver disease – fatty degeneration, toxic hepatitis; individuals with incidentally detected elevated liver transaminase levels.

with other diseases- renal disease; oncological disease; neurological disease.

individuals with other clinical manifestations: diarrhea, colitis, viral infections, etc.

individuals with no diagnosis specified on the accompanying form.

Table 1. Distribution of prospective serum samples by combining criteria

Group/Population	Number (n)
	Prospective
Unspecified acute viral hepatitis (UAVH)	1006
Patients with a working diagnosis of Hepatitis E (HEV)	177
Other liver disease (OLD)	68
Incidentally discovered elevated liver transaminases (ELT)	43
Extrahepatic diseases* (EHD)	199
No diagnosis recorded (ND)	330
TOTAL	**1823**

*individuals with other conditions: renal, oncological, viral infections, etc.

*644 retrospective serum samples from a heterogeneous population, collected during the period 2015–2023, in fulfillment of the objectives set by the research project "Studies on the genotypic diversity and involvement of the polyproline region in the in vitro adaptation of the hepatitis E virus", funded by the Bulgarian National Science Fund under Contract No. KP-06-N-33/2 dated 13.12.2019. Some characteristics of this population are presented in Table 2.

Table 2. Distribution of retrospective samples by combining criteria—diseases, occupation, blood donors

Group/Population	Number (n)
	Retrospective samples
Veterinarians (VET)	75
Blood donors (BD)	94
Patients with liver cirrhosis (LC)	22
Children with hematological diseases (CHD)	16
Hemodialysis patients with renal failure (HP)	218
HIV-positive individuals (HIV)	219
TOTAL	**644**

*serum samples were provided by the biobank of the NRL "Measles, Mumps, Rubella".

The interpretation of the results obtained was compliant with the recommendations of the European Association for the Study of the Liver (EASL, 2018) – Appendix 3

2. Methods

2.1. Enzyme Immunoassays

Enzyme-linked immunosorbent assay (ELISA) for the detection of specific antibodies—anti-HEV IgM/anti-HEV IgG

The detection of anti-HEV IgM/IgG was performed using qualitative enzyme immunoassay kits—anti-HEV IgM/anti-HEV IgG ELISA from Dia.Pro, Italy.

The assay was performed in accordance with the manufacturer's instructions.

2.2. Molecular Biological Methods

2.2.1. Viral RNA Extraction

Viral RNA was extracted from the serum samples using the ExiPrep™ 16 Dx automated nucleic acid extraction system (Bioneer) with the combined ExiPrep™ Dx Viral DNA/RNA extraction kit and the designated viral RNA extraction program. The equipment is available at the NRL "Hepatitis Viruses". The volume of the extract containing the eluted RNA was 50 µl, obtained from an initial serum sample volume of 400 µl.

2.2.2. Reverse Transcription Real-Time Polymerase Chain Reaction for Viral Load Determination (real-time RT-PCR)

Detection and quantification of viral RNA concentration were performed using the RealStar HEV RT-PCR Kit 2.0 (Altona Diagnostics, Germany) according to the manufacturer's instructions. The reaction was carried out in a volume of 50 µl under the following temperature profile (Table 3):

Table 3. Real-time RT-PCR protocol and temperature parameters for quantitative analysis of HEV RNA.

Amplification Stage	Cycle Number	Temperature (°C)	Time (mm:ss)
Reverse Transcription	1	55	20:00
Denaturation	1	95	02:00
Amplification	45	95	00:15
		55 (Signal Detection)	00:45
		72	00:15

2.2.3. Reverse transcription and amplification of complementary DNA (cDNA) from a specific region of the HEV viral genome

For the genotyping of HEV isolates, the extracted HEV RNA was subjected to sequential reverse transcription, amplification, and nested PCR. Reverse transcription was performed using an HEV-specific reverse primer (Table 5) and the smART First Strand cDNA Synthesis Kit for RT-PCR, adhering to the following thermal profile (Table 4).

The reaction was carried out in a total volume of 20 µl using the following reaction mixture: extracted viral RNA (10 ng–5 µg), 5 µM HEV-specific reverse primer, 1 mM dNTPs, 1× cDNA buffer, 0.01 M DTT, 1.25 U/µl RNase inhibitor, 20 U/µl reverse transcriptase (smART), and RNase-free water. To 10 µl of the resulting cDNA, 40 µl of a reaction mixture was added, consisting of: 1.25 U/µl Perpetual Taq DNA polymerase, 1× reaction buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 1 µM forward HEV-specific primer (Table 5), and sterile double-distilled water. The amplification proceeded under the following thermal profile:

Table 4. Temperature cycles for cDNA amplification

Cycle Number	Temperature (°C)	Time (mm:ss)
35	95	00:30
	42*	00:30
	60	00:45

*temperature increase of 0.4°C per second

Table 5. HEV-specific primers used for cDNA synthesis and amplification

Name	Sequence (5' - 3')	Direction	Corresponding position in the HEV genome
HEV-orf2-fo-ch	AAYCARGGiTGGCGYTCiGTiGARAC	F	5885–5910
HEV-orf2-ro-ch	GARAAiGGRCGiGAiGGRGCiGG	R	6510–6488

a – Reference HEV strain (NC_001434) [International Journal of Food Microbiology 257 (2017) 225–231]; F (Forward); R (Reverse) –I=inosine; Y =C and T; R=A and G; W =A and T; S=C and G; M=A and C; K=G and T; N=A and C and G and T

2.2.4. *Nested PCR*

To achieve high specificity, a second PCR (nested PCR) was performed using Perpetual Taq DNA Polymerase Hot Start (EURx, Poland). The reaction was carried out in a final volume of 50 µl and contained: 1 µl of product from the first PCR, 1× reaction buffer, 0.2 mM dNTPs, 0.8 µM forward HEV-specific primer, 0.8 µM reverse HEV-specific primer (Table 6), 1.25 U/µl Perpetual Taq DNA polymerase, and sterile double-distilled water.

Table 6. HEV-specific primers for nested PCR

Name	Sequence (5' - 3')	Direction	Corresponding position in the HEV genome
HEV-orf2-fi-ch	GAGGAGGAAGCTACCTCYGGYYTiGTiATGCTYTGYAT	F	5924–5961
HEV-orf2-ri-ch	GGAGAAGGAGTTGGTCGRTCYTGYTCRTGYTGRTT	R	6489–6455

I=inosine; Y=C and T; R=A and G; W =A and T; S=C and G; M=A and C; K=G and T; N=A and C and G and T

Table 7. Temperature cycles for cDNA amplification

Amplification Stage	Number of Cycles	Temperature [°C]	| Time [min:sec]
Denaturation	1	95	06:00
Amplification	40	95	00:30
		60	00:20
		72	00:15

2.2.5. Detection of amplified products

The visualization of the nested-PCR product was performed using 2% agarose gel electrophoresis. The gel composition consisted of 2% agarose (Cleaver Scientific), 1× TBE buffer (Tris-Borate-EDTA, SERVA GmbH, Heidelberg), and 0.1% ethidium bromide (Carl Roth GmbH, Karlsruhe). Samples were loaded in a final volume of 12 µl, consisting of either 10 µl of nested-PCR product or 3 µl of molecular weight marker, supplemented with 1× sample loading buffer and sterile double-distilled water. Electrophoresis was carried out in 1× TBE buffer (Tris-Borate-EDTA) at a constant voltage of 100 V for 60 minutes. Signal

detection and imaging were performed using a NuGenius XE UV gel documentation system (Syngene, United Kingdom). For fragment alignment and size estimation, a GeneRuler 50 bp DNA Ladder (Thermo Scientific, Lithuania) was utilized, enabling the approximate quantification of double-stranded DNA fragments within a range of 50 bp to 1,000 bp (base pairs). Successful nested-PCR amplification resulted in the generation of an HEV-specific product with an approximate size of 493 base pairs.

2.3. Sequencing

2.3.1. Preparation of samples for sequencing

For sequencing of the positive samples by the Sanger method, the stages of purification, amplification, and capillary electrophoresis are performed.

Purification of the HEV-specific fragment obtained after nested PCR

The purification protocol consists of the following steps:1. Reagent Mixing and Binding:Paramagnetic capturing.Mix 10 µl of the reaction mixture obtained after the completion of the nested PCR with the appropriate volume of AMPure XP reagent, calculated according to the following formula: {Volume of Agencourt AMPure XP per reaction} = 1.8 * {Volume of nested-PCR reaction mixture})During this step, the PCR product binds to the paramagnetic particles. Using a magnetic field, the particles are selectively separated from the remaining components of the PCR mixture.2.Contaminant Removal:Ethanol washes.Remove PCR contaminants (residual primers, dNTPs, and non-specific products) by washing the pellet three times with freshly prepared 70% ethanol.3.Elution:Final collection.Elute the nested-PCR product from the paramagnetic particles by adding deionized water, yielding a final eluate volume of 50 µl.

Amplification - DNA Template Cycle Sequencing (Sequencing PCR)

The purified nested-PCR amplicon was subjected to a sequencing reaction using the Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter, USA). The reaction was performed in a final volume of 20 µl and included the following components: DTCS master mix, an HEV-specific sequencing primer (Table 9), the purified nested-PCR amplicon, and sterile double-distilled water.

The cycle sequencing reaction was carried out according to the thermal profile outlined in Table 8.

Table 8. Temperature cycles for DNA template sequencing PCR

Number of Cycles	Temperature [°C]	| Time [min:sec]
30	96	00:20
	50	00:20
	60	04:00
1	4	∞

∞ indefinitely until the sample is retrieved from the instrument

Table 9. HEV-specific primers for sequencing

Name	Sequence (5' - 3')	Direction	Corresponding position in the HEV genome
HEV-orf2-fs	GAGGAGGAAGCTACCTC	F	5924 - 5940
HEV-orf2-rs	GGAGAAGGAGTTGGTCG	R	6489-6473

- **Sequencing Product Purification**

The sequencing products obtained after the reaction were purified using Agencourt CleanSEQ (Beckman Coulter, USA), a method based on magnetic particle technology. The purification protocol comprises three primary

step 1. Binding:Magnetic capturing.Bind the extended sequencing products to the magnetic particles.

step 2. Contaminant Removal:Ethanol washes.Wash away unbound reaction contaminants (excess dye terminators, residual primers, dNTPs, and salts) using freshly prepared 85% ethanol.

step 3. Elution:Sample resuspension.Elute the purified products by adding 40 µl of Sample Loading Solution (SLS).

- **Capillary Sequencing Electrophoresis**

The purified single-stranded product from the sequencing reaction was subjected to capillary electrophoresis using an 8-capillary GeXP genetic analysis system (Beckman Coulter). The sequencing data was analyzed using the LFR-b method, which is specifically optimized for fragments up to 600 bp in size.

2.3.2 Subtyping and Phylogenetic Analysis

The Sanger sequencing data were processed and analyzed using two software packages: BioEdit and MEGA software version 11 (MEGA11). The raw data obtained from the sequencer consisted of paired-end nucleotide sequences in FASTA format. Using BioEdit, low-quality nucleotides were filtered out and trimmed. Sequence editing and correction were subsequently performed using the complementary strand as a template to assemble a definitive consensus nucleotide sequence. Finally, the consensus sequence was generated. This consensus sequence was compared against a set of HEV reference sequences retrieved from GenBank, the genetic sequence database of the National Center for Biotechnology Information (NCBI). A total of 34 sequences were downloaded from GenBank: 24 HEV reference sequences, 8 sequences from Bulgarian patients with acute viral hepatitis E, and 2 sequences from swine sampled at Bulgarian abattoirs. The characteristics of these sequences are summarized in Table 10.

Table 10. Reference and Bulgarian HEV sequences for phylogenetic tree construction

NCBI Accession No.	Sequence Source	Fragment Size (bp)	Genotype	Subtype	References
AB248521	Reference sequence	7241	3	e	*Inoue*, 2006
AB369687	Reference sequence	7217	3	f	*Li*, 2005
AF455784	Reference sequence	7239	3	g	*Lu*, 2006
FJ906895	Reference sequence	7318	3	ra	*Zhao*, 2009
L08816	Reference sequence	7176	1	b	*Tam*, 1991
FJ457024	Reference sequence	7217	1		*Varma*, 2011
MH809516	Reference sequence	7214	2	b	*Wang*, 2017
KX387865	Reference sequence	7223	8	a	*Woo*, 2016
KJ496143	Reference sequence	7224	7	a	*Woo,* 2014
AB197673	Reference sequence	7257	4	a	*Takahashi*, 2014
AB856243	Reference sequence	7263	6		*Takahashi*, 2014
AB573435	Reference sequence	7267	5	a	*Takahashi*, 2010
AP003430	Reference sequence	7230	3	b	*Takahashi*, 2001
AF082843	Reference sequence	7207	3	a	*Meng*, 1997
AY115488	Reference sequence	7255	3	j	*Pei*, 2002
AB369689	Reference sequence	7215	3	k	*Yano*, 2007
MF959765	Reference sequence	7229	3	-	*De Sabato*, 2018
LC260517	Reference sequence	7242	3	-	*Primadharsini*, 2017
KP294371	Reference sequence	7267	3	-	*Jenckel*, 2021
FJ998008	Reference sequence	7206	3	i	*Adlhoch*, 2009
FJ705359	Reference sequence	7237	3	c	*Schielke*, 2009
JQ013794	Reference sequence	7182	3	h	*Izopet,* 2012
JQ953664	Reference sequence	7238	3	l	*Bouquet*, 2012
KU513561	Reference sequence	7083	3	m	*Muñoz -Chimeno*, 2016
*MH203185	PAVH	355	3	e	*Bruni*, 2018
*MH203169	PAVH	355	3	e	*Bruni*, 2018
*MH203164	PAVH	355	3	e	*Bruni*, 2018
*MH203227	PAVH	345	3	e	*Bruni*, 2018

*MH203186	PAVH	297	3	e	*Bruni*, 2018
*MH203187	PAVH	355	3	e	*Bruni*, 2018
*MH203191	PAVH	355	3	e	*Bruni*, 2018
*MH203166	PAVH	334	3	f	*Bruni*, 2018
MZ555942	collected from pigs at Bulgarian slaughterhouses	636	3	c	*Palombieri*, 2021
MZ555941	collected from pigs at Bulgarian slaughterhouses	621	3	c	*Palombieri*, 2021

** Sequences from Bulgarian patients with acute hepatitis E.*

The consensus and reference sequences were aligned using ClustalW within the MEGA (Molecular Evolutionary Genetics Analysis) software, version 11. Based on this alignment, a phylogenetic tree was generated using 1,000 bootstrap replicates to represent the evolutionary relationships among the taxa.

To confirm the genotype and subtype assignment of the HEV isolates identified in the present work, the online tool Hepatitis E Virus Genotyping Tool Version 1.0 was utilized (https://www.rivm.nl/mpf/typingtool/hev/).

Taxonomic classification was performed via comparative analysis against the latest published consensus on HEV reference strains (*J. Gen. Virol*., 2020).

2.4. Statistical Analysis

The data were processed using Microsoft Office Excel 2010 software. Descriptive statistical analysis was performed to determine the mean age, along with descriptive statistics functions to calculate the 25th and 75th percentiles. Frequency distributions (e.g., gender, seroprevalence) were calculated as percentages. To determine statistical significance (p-value), the online calculator Epitools Epidemiological Calculators (Ausvet) was utilized (Epitools Epidemiological Calculators, Ausvet; http://epitools.ausvet.com.au

III. RESULTS

1. Detection of serological markers of HEV infection in serum samples from different populations.

1.1. Prospective Group

The prospective cohort includes a total of 1823 serum samples. The largest number of prospective samples was received in 2018 (353 samples), whereas the lowest volume was recorded in 2022 (89 samples). During the 2015–2017 period, a relatively stable percentage of submitted samples was observed, which was followed by a peak in the number of incoming samples in 2018 (353 samples) (Fig. 1).

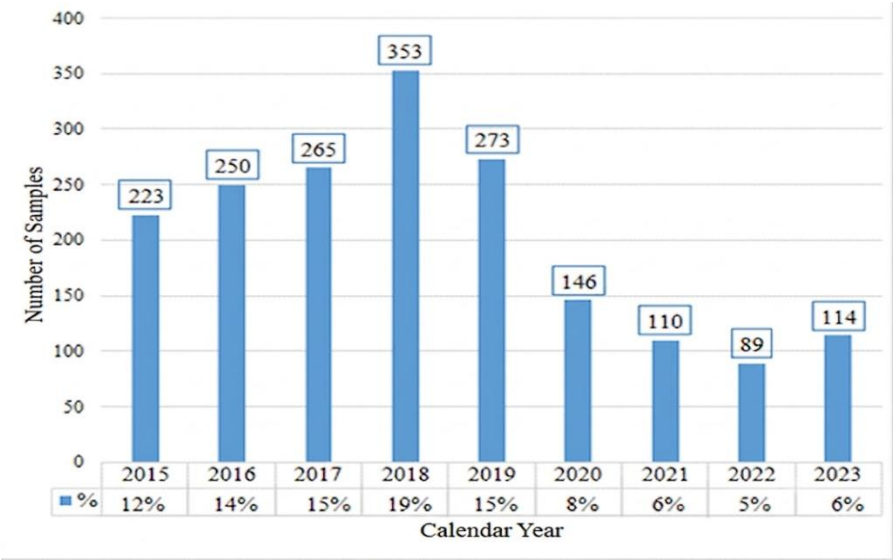


Figure 1. Annual distribution of the analyzed prospective samples.

Half (50%) of the patients were aged 38–63 years, and the median age of the individuals in the prospective cohort was 53 years. Male individuals predominated among the studied population, with a male-to-female ratio of 1.6:1 [1127 (62%) males/ 696(38%) females].The disparity between the two sexes was most pronounced among patients with acute hepatitis E, reaching a ratio of 2:1 [118 (67%) males/59 (33%) females], with this difference being statistically significant ($p < 0.001$). The distribution of patients by gender, age, and nosology is presented in Table 11.

Table 11. Distribution of patients by sex, age, and nosological unit.

Diagnosis/Parameters	Gender	N (%)	Median (IQR)
AVHE (n= 177)	M F	119 (67%) 58 (33%)	53 (41-65)
UAVH (n= 1006)	M F	620 (62%) 386 (38%)	48 (36-63)
Unspecified liver disease (n=68)	M F	44 (65%) 24 (35%)	50 (37-63)
↑AST, ↑ALT (n= 43)	M F	26 (60%) 17 (40%)	49 (36-62)
Extrahepatic diseases (n=199*)	M F	118 (59%) 81 (41%)	44 (31-58)
No diagnosis (n= 330)	M F	200 (61%) 130 (39%)	53 (44-64)
Total (n=1823)	M F	1127 (62%) 696 (38%)	53 (38-64)

* *including renal disease, oncological disease, neurological disease, individuals with diarrhea, colitis, viral infections, etc.*

In 36% (661 of 1,823) of the semples, anti-HEV antibodies were detected – Figure 2.

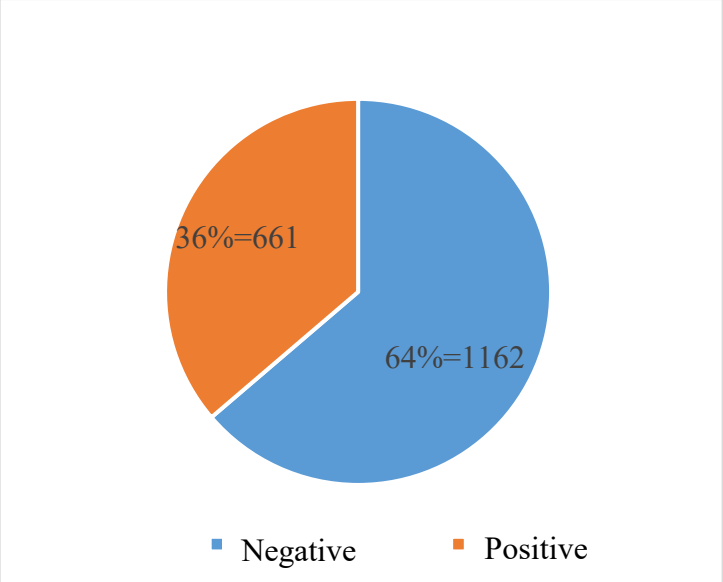


Figure 2. Relative proportion of individuals with detected *anti-HEV*

HEV RNA was detected in 1.7% (32 out of 1,823) of the investigated sera (Figure 3).

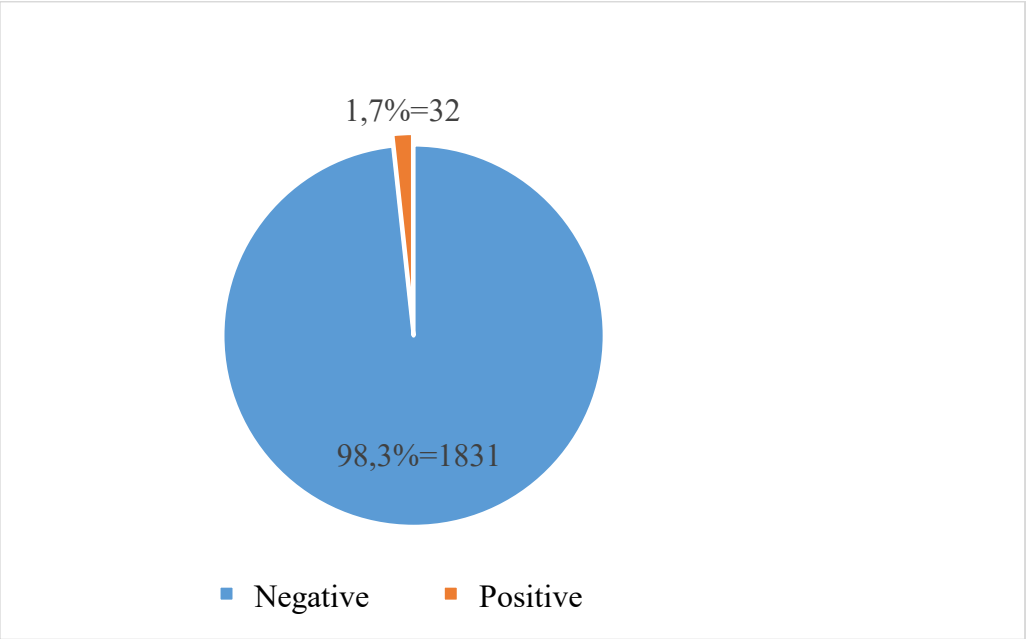


Figure 3. Relative proportion of individuals with detectable HEV RNA.

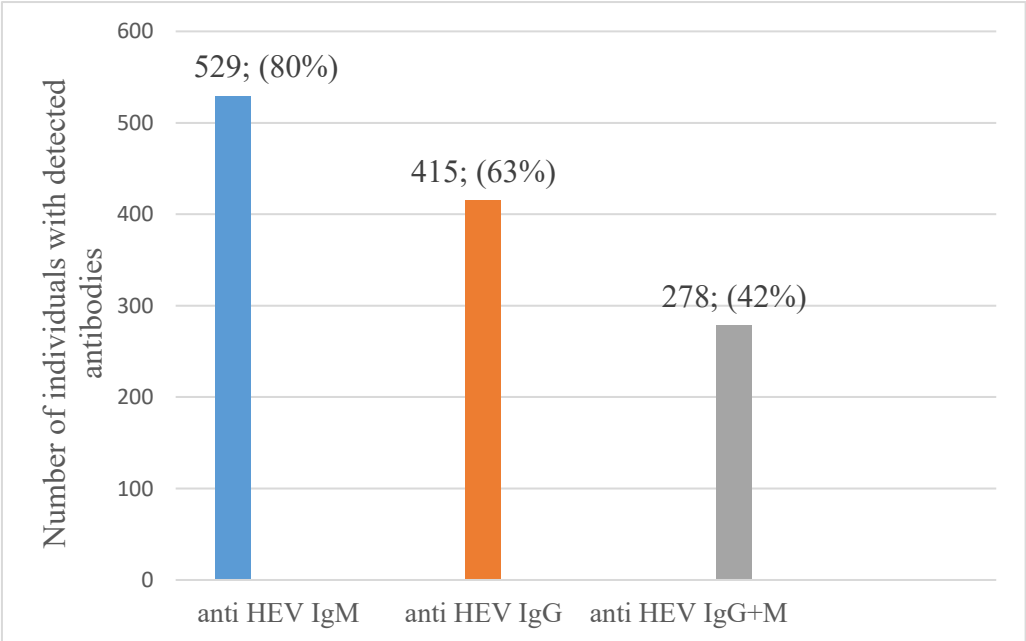


Figure 4. Distribution of seropositive cases by the type of detected antibodies.

Specific antibodies of both classes (anti-HEV IgM, IgG) were detected within the studied cohort. Among the seropositive individuals, 80% were positive for anti-HEV IgM and 63% for anti-HEV IgG, while antibodies of both classes were simultaneously detected in 42% of the tested subjects.

Table 12. Distribution of seropositive individuals by nosological unit

Diagnosis/Parameters	***Anti HEV***		**RNA (+)**
	Result	**N (%)**	
Acute hepatitis E (n= 177)	**(+) Pos.** (-) Neg.	**118 (67%)** 59 (33%)	28 (15%)
UAVH (n= 1006)	**(+) Pos.** (-) Neg.	**343 (34%)** 663 (65%)	
Other liver disease (n=68)	**(+) Pos.** (-) Neg.	**17 (25%)** 51 (75%)	1 (1,4%)
↑AST, ↑ALT (n= 43)	**(+) Pos.** (-) Neg.	**12 (27%)** 31 (72%)	
Extrahepatic diseases (n=199*)	**(+) Pos.** (-) Neg.	**52 (26%)** 147 (71%)	2 (1%)
No diagnosis (n= 330)	**(+) Pos.** (-) Neg.	**119 (36%)** 211 (63%)	1 (0,3%)
Total (n=1823)	**(+) Pos.** (-) Neg.	**661 (36%)** 1162 (64%)	**32 (1,7%)**

1.2. Retrospective Group

The retrospective cohort includes 644 serum samples.

The retrospective samples were received at regular intervals over the years in fulfillment of the objectives of the aforementioned project funded by the Bulgarian National Science Fund (BNSF).

Half of the tested individuals (50%) fell within the 33–59 age range, and the median age was 45 years.

Table 13. Distribution of patients by sex, age, and nosological unit

Diagnosis/Parameters	Gender	N (%)	Median (IQR) in years
HIV (+) (n=219)	M F	184 (84%) 35 (16%)	35 (29-41)
Hemodialysis patients with renal failure (n=218)	M F	124 (57%) 94 (43%)	61 (52-71)
Patients with liver cirrhosis (n=22)	M F	17 (77%) 5 (23%)	56 (50-66)
Children with hematological diseases (n=16)	M F	6 (37%) 10 (63%)	7 (1-13)
Blood donors (n=94)	M F	77 (82%) 17 (18%)	37 (30-44)
Veterinarians (n=75)	M F	63 (84%) 12 (16%)	49 (43-57)
Total (n=644)	M F	471 (73%) 173 (27%)	45 (33-59)

Men also predominated among the individuals in the retrospective group, with a male-to-female ratio of 2.7:1. This difference was most pronounced in certain subgroups, specifically veterinarians, blood donors, and patients with cirrhosis.

Seventeen percent (17%) (110/644) of the tested samples were anti-HEV (+) positive.

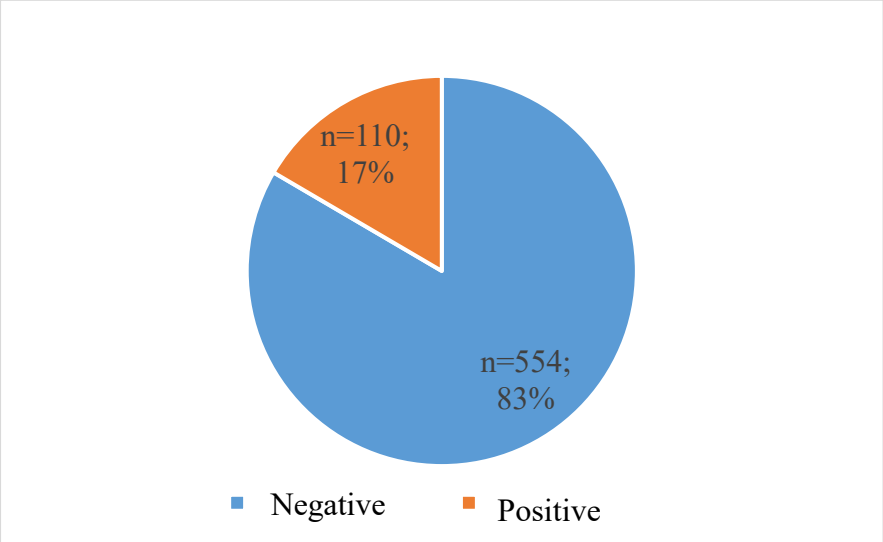


Figure 5. Relative proportion of individuals with detected anti-HEV antibodies.

Table 14. Distribution of seropositive individuals by nosological unit.

Diagnosis/Parameters	***Anti HEV***		**RNA (+)**
	Result	**N (%)**	
HIV (+) (n=219)	**(+) Pos.** (-) Neg.	***30 (14%)*** 189 (86%)	
Hemodialysis patients with renal failure (n=218)	**(+) Pos.** (-) Neg.	***23 (11%)*** 195(89%)	
Patients with liver cirrhosis (n=22)	**(+) Pos.** (-) Neg.	**7 (32%)** 15 (68%)	1 (4,5%)
Children with hematological diseases (n=16)	**(+) Pos.** (-) Neg.	***11 (69%)*** 5 (31%)	
Blood donors (n=94)	**(+) Pos.** (-) Neg.	**29 (31%)** 65 (69%)	
Veterinarians (n=75)	**(+) Pos.** (-) Neg.	**10 (13%)** 65 (87%)	
Total (n=644)	**(+) Pos.** (-) Neg.	***110 (17%)*** ***534 (83%)***	***1 (0,2%)***

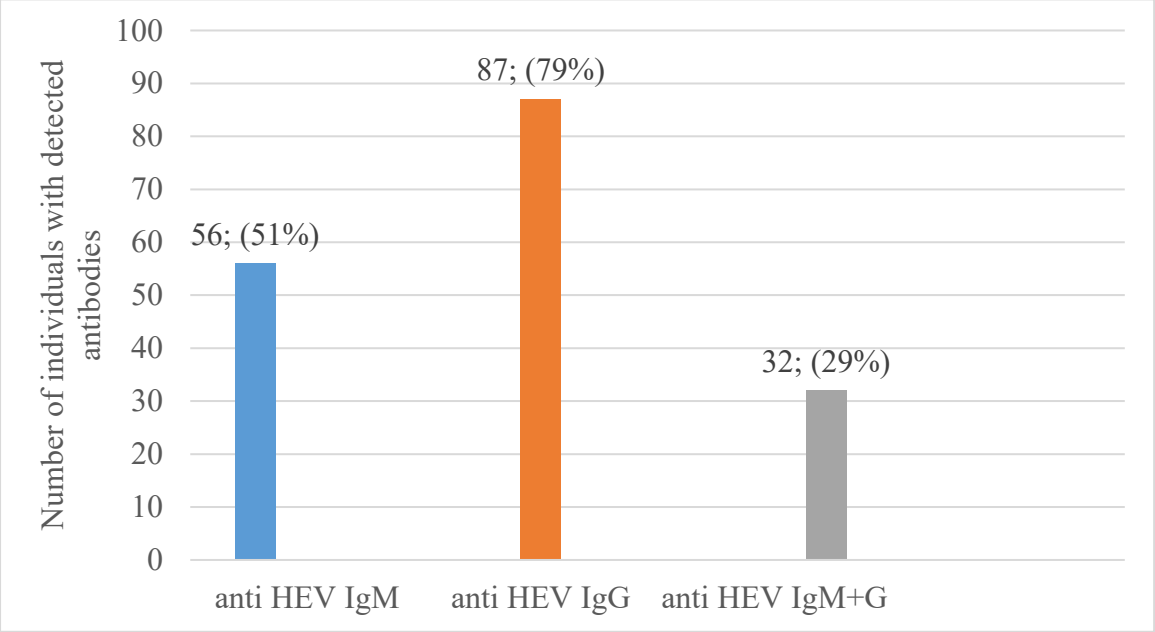


Figure 6. Distribution of seropositive individuals according to the type of detected antibodies.

Within this group as well, specific antibodies of both classes (anti-HEV IgM, IgG) were detected among the tested individuals—either simultaneously or independently. Individuals with anti-HEV IgM accounted for 51% of the seropositive cases, those with detected anti-HEV IgG accounted for 79%, and antibodies of both classes were simultaneously detected in 29% of cases.

2. The significance of gender and age in the prevalence of HEV infection.

2.1. Prevalence of HEV infection across different age groups

The studied individuals are divided into 7 age groups by decade: <20 years, 20–29 years, 30–39 years, 40–49 years, 50–59 years, 60–69 years, and >70 years.

2.1.1. Prospective Group

Within the prospective group, a total of 36% (661/1,823) tested positive for anti-HEV (+). Patient age was specified in 82% (1,501/1,823) of the accompanying forms.

Anti-HEV IgM (29%): Out of 529 individuals found to be anti-HEV IgM positive, age was specified in the accompanying form for 445 of them.

The lowest relative proportion of anti-HEV IgM was observed in the <20 age group. This proportion increased in subsequent decades, reaching a peak of 35% in the 50–59 and 60–69 age groups (Table 15).

Table 15. Age distribution of anti-HEV IgM (+) individuals

Decade	**anti *HEV* IgM (+)**	
	N	**%**
<20 y. (n=122)	13	11%
20-29 y. (n=93)	20	22%
30-39 y. (n=208)	62	30%
40-49 y. (n=242)	61	25%
50-59 y. (n=323)	115	35%
60-69 y. (n=309)	107	35%
>70 y. (n=204)	67	32%

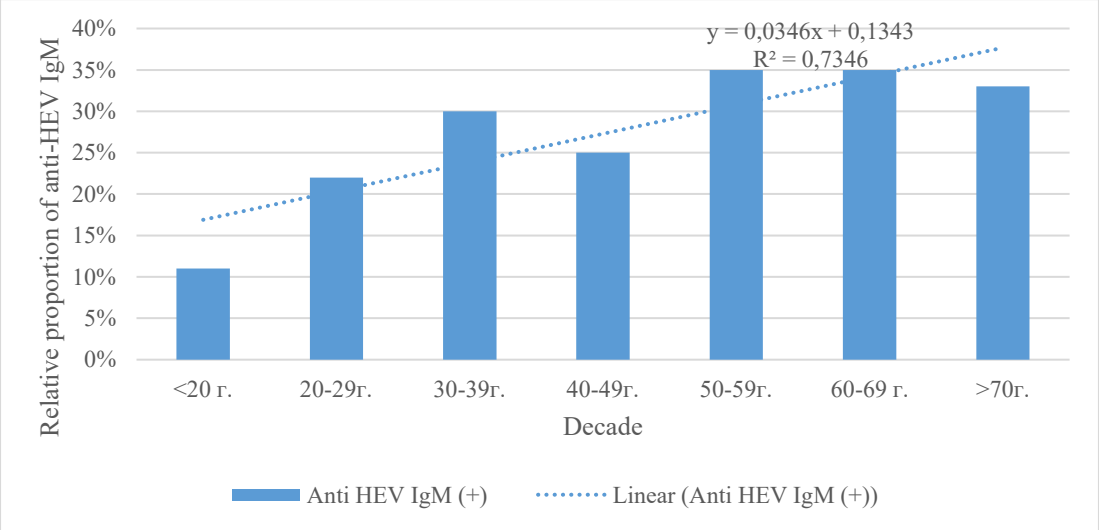
The relative proportion of anti-HEV IgM (+) individuals increased by approximately 0.035% for each decade of life, with a coefficient of determination R^2 = 0.7341 (Figure 7). The established difference across the various age groups is statistically significant ($p < 0.0001$).

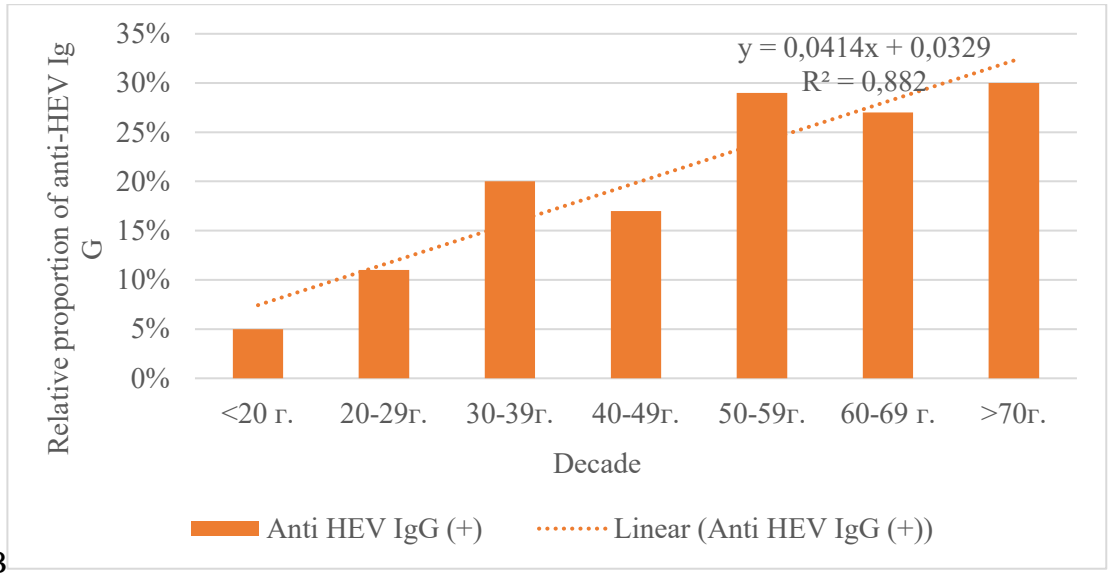
Anti-HEV IgG (22%): Out of 415 individuals found to be anti-HEV IgG positive, age was specified in the accompanying form for 340 of them.The lowest relative proportion of anti-HEV IgG was recorded in the <20 age group (5%). An upward trend was observed across the subsequent age groups, reaching its peak in individuals over 70 years old (Table 16). The relative proportion of anti-HEV IgG (+) individuals increased by approximately 0.041% for each decade of life, with a coefficient of determination R^2 = 0.882 (Figure 7).

Table 16. Age distribution of anti-HEV IgG (+) individuals

Decade	**anti *HEV* IgG (+)**	
	N	**%**
<20 y. (n=122)	6	5%
20-29 y. (n=93)	10	11%
30-39 y. (n=208)	42	20%
40-49 y. (n=242)	42	17%
50-59 y. (n=323)	95	29%
60-69 y. (n=309)	84	27%
>70 y. (n=204)	61	30%

A





B

Figure 7. Distribution of anti-HEV IgM (A) and IgG (B) positive individuals across the different age groups.

2.2.2. Retrospective Group

Of the prospective group, a total of 17% (110/644) were positive for anti-HEV (+).

***Anti-HEV IgM*:**

The highest relative proportion of anti-HEV IgM was found in the <20 age group (38%). With advancing age, this percentage significantly decreased, dropping to 12% for the 20–29 age group and not exceeding 7% in the other age groups. Accordingly, the trendline indicates a decline in the percentage of anti-HEV IgM (+) samples as age increases, where each one-decade increase in age leads to a decrease in positive cases by 0.04%, with a coefficient of determination R^2 = 0.514 (Figure 8).

Table 17. Retrospective samples – Age distribution of anti-HEV IgM (+) positive individuals

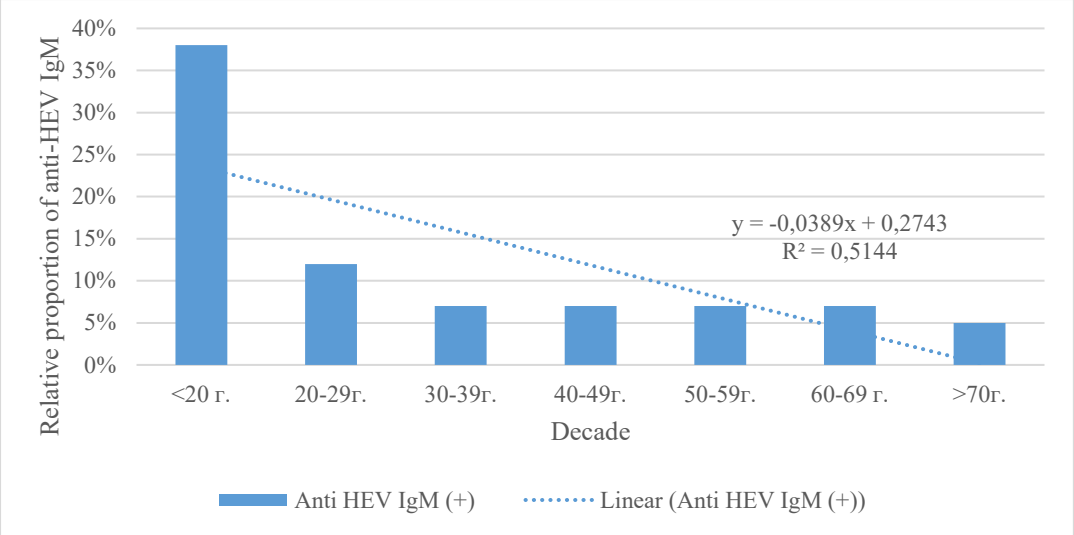
Decade	**anti *HEV* IgM (+)**	
	N	**%**
<20 y. (n=26)	10	38%
20-29 y. (n=78)	9	12%
30-39 y. (n=162)	11	7%
40-49 y. (n=119)	8	7%
50-59 y. (n=99)	7	7%
60-69 y. (n=85)	6	7%
>70 y. (n=75)	4	5%

Anti-HEV IgG: The highest percentage of anti-HEV IgG (+) positive samples was found in the <20 age group (Figure 8). A decrease in positive samples is noted over the next two decades, with no clearly defined linear relationship relative to age.

Table 18. Retrospective samples – Distribution of anti-HEV IgG (+) positive individuals by age

Decade	**anti *HEV* IgG (+)**	
	N	**%**
<20 y. (n=26)	3	12%
20-29 y. (n=78)	6	8%
30-39 y. (n=162)	12	7%
40-49 y. (n=119)	12	10%
50-59 y. (n=99)	7	7%
60-69 y. (n=85)	7	8%
>70 y. (n=75)	8	11%

A



B

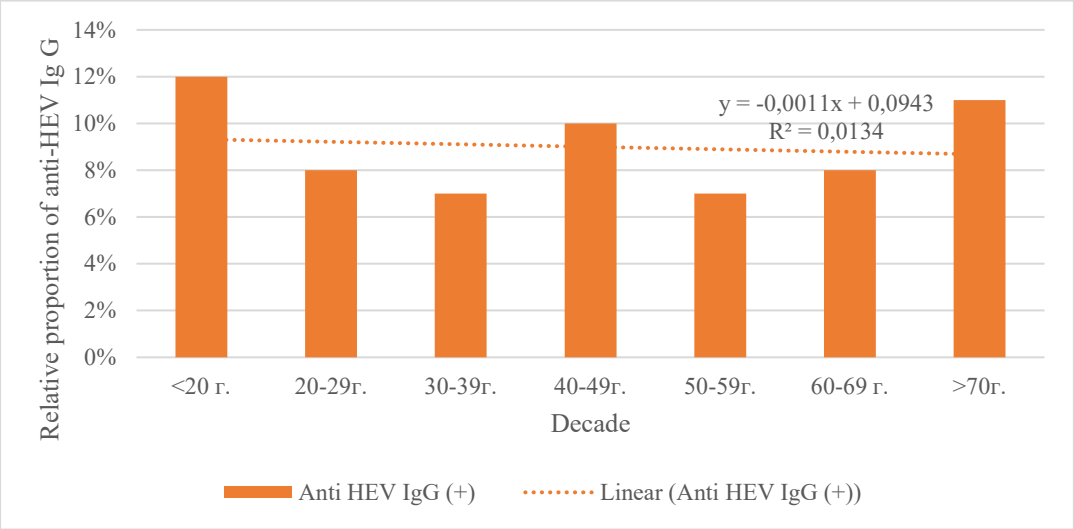


Figure 8. Distribution of anti-HEV IgM (A) and IgG (B) positive individuals in the different age groups

2.2. Gender distribution of seropositive individuals.

2.2.1. Prospective Group

In the prospective group, males predominate; the male-to-female ratio is 1.6:1. Anti-HEV IgM is detected more frequently in males (33%) than in females (23%). Males also predominate among anti-HEV IgG (+) individuals (26% males vs. 17% females) – Fig. 9. The difference between the two sexes is statistically significant ($p < 0.0001$; $z = 4.6$).

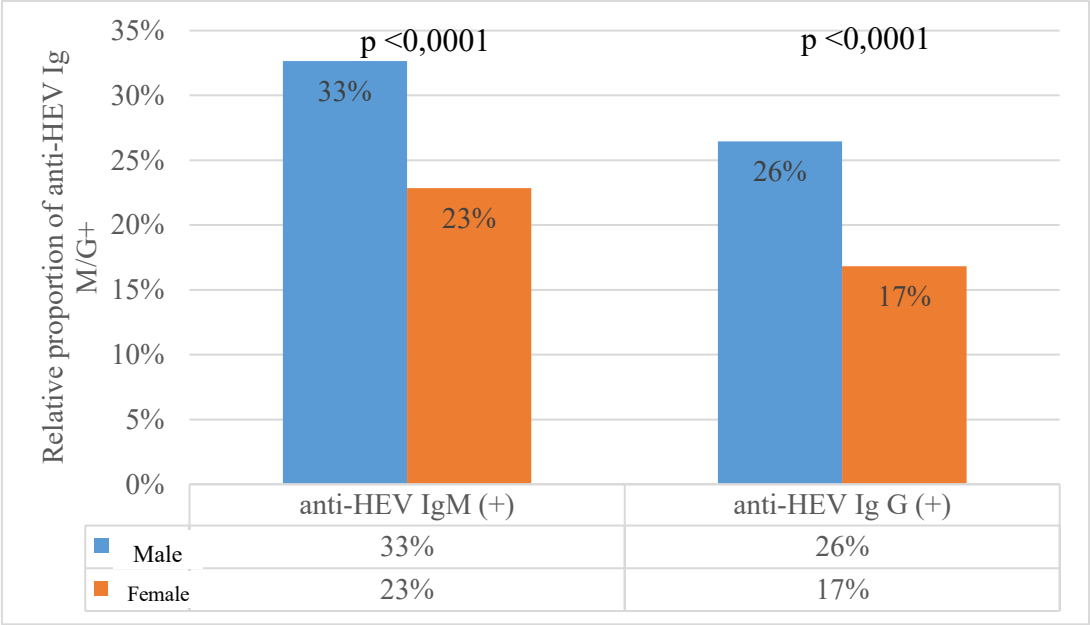


Figure 9. Gender distribution of anti-HEV (+) individuals from the prospective group

2.2.2 Retrospective Group

In this study group as well, the male sex predominates, with a male-to-female ratio of 2.7:1.

Among anti-HEV IgG (+) individuals, males predominate (9% males to 8% females), whereas among HEV IgM (+) individuals, females predominate (9% vs. 8%) (Figure 10); however, the difference is not statistically significant, with p=0.6903 and p=0.683, respectively.

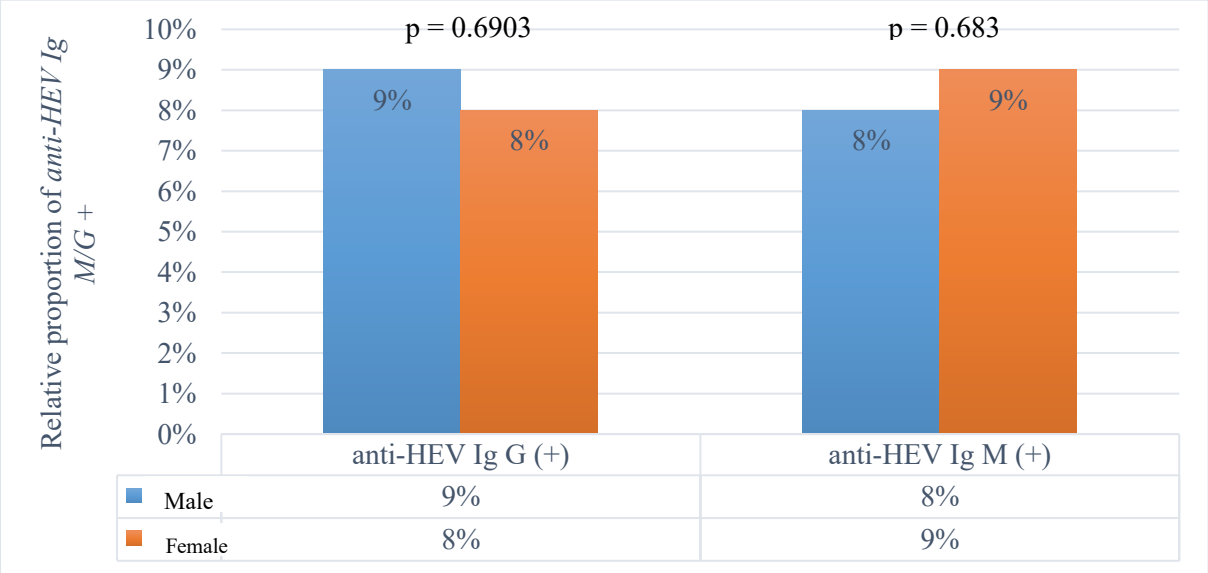


Figure 10. Gender distribution of anti-HEV positive individuals from the retrospective group

2.3. Distribution of anti-HEV IgM (+) samples according to nosology and occupation

2.3.1. Prospective Group

The highest proportion of anti-HEV IgM positive samples was found in individuals with acute hepatitis E (58%), with the difference compared to the other groups being statistically significant ($p \le 0.0001$). Among patients with unspecified hepatitis (UAVH), anti-HEV IgM was detected in 28% of cases (Fig. 11). The lowest percentage of positive samples (19%) was observed in patients with other liver diseases.

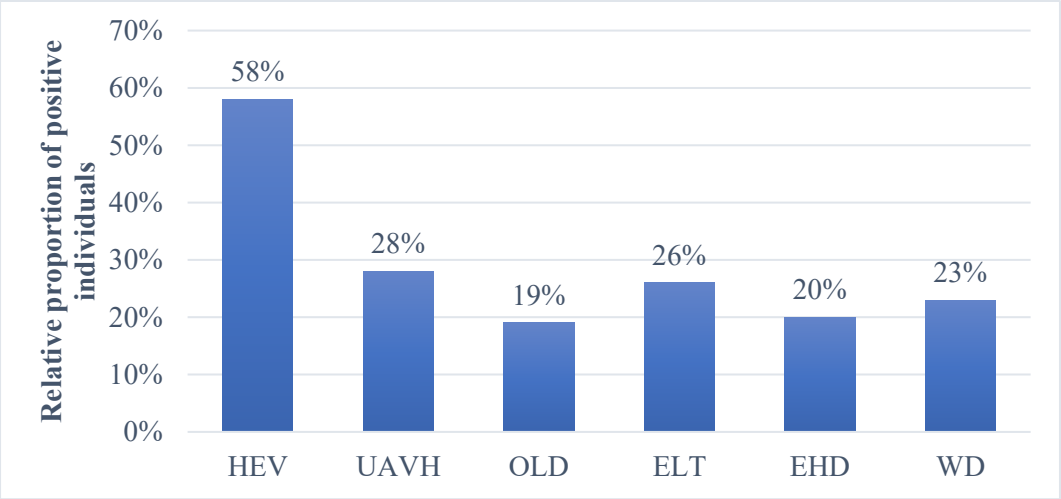


Figure 11. Distribution of anti-HEV IgM (+) individuals according to working diagnosis

Legend: acute hepatitis E (HEV); unspecified hepatitis (UAVH); other liver disease (OLD); elevated liver transaminases (ELT); extrahepatic diseases (EHD); without diagnosis (WD).

2.3.2. Retrospective Group

The highest relative proportion of anti-HEV IgM (+) samples was found among children with hematological diseases (56%), followed by patients with cirrhosis and blood donors. In the remaining retrospective subgroups—HIV-positive individuals, hemodialysis patients (HP), and veterinarians (V)—an identical positive rate of 5% was established.

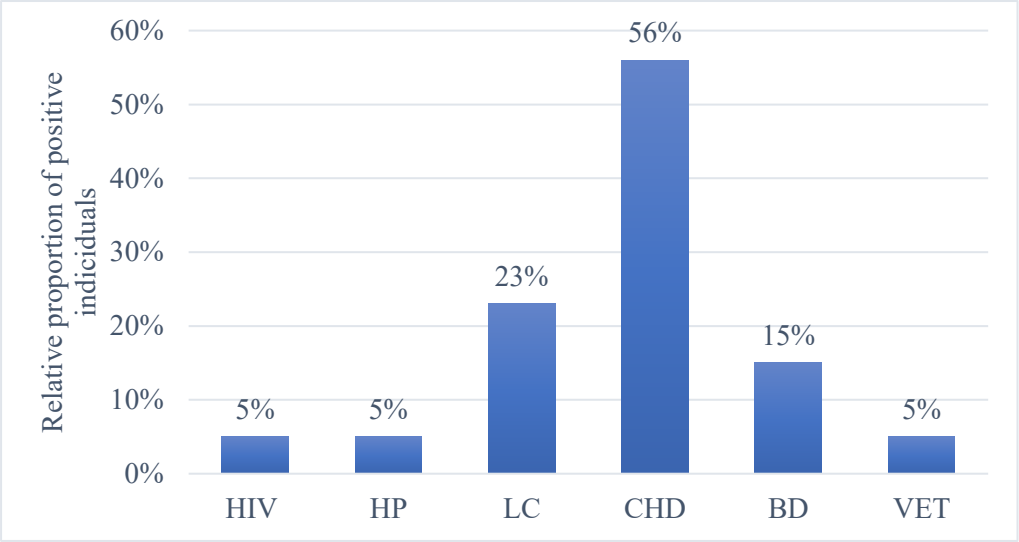


Figure 12. Distribution of HEV IgM (+) individuals according to working diagnosis.
Legend: HIV-positive individuals (HIV); Hemodialysis patients (HP); Patients with liver cirrhosis (PC); Children with hematological diseases (CHD); Blood donors (BD); Veterinarians (VET).

3. Detection of Viral RNA and Viral Load Determination

HEV RNA was detected in 32 (1.8%) of the prospective serum samples.

Among these, 28 (88%; 28/32) belonged to patients with an acute HEV infection. The determined HEV RNA concentration varied widely, ranging from <1,000 IU/ml to >100,000 IU/ml. Table 19 presents the distribution of the RNA-positive samples based on their viral load values.

Among the positive samples 50% (16/32) had a detected viral load exceeding 100,000 IU/ml; Thirteen percent (13%) (4/32)—representing the lowest percentage of the tested samples—had a determined concentration below 1,000 IU/ml.

Table 19. Distribution of RNA-positive samples by viral load [IU/ml]

***HEV* PHK [IU/ml]**	**N**	**[%]**
< 1000	4	13%
1000- 10000	6	19%
10000 - 100000	6	19%
> 100000	16	50%

4. Correlation between serological and molecular markers.

An association was established between the value of the HEV IgM serological index [S/CO] and the detection rate of HEV RNA (Figure 13).At an index value of < 5.0, viral nucleic acid was detected in only about 5% of the tested samples. At values > 5.0, the number of positive samples increased significantly. At index values > 10.0, HEV RNA was detected in 70% of the cases.

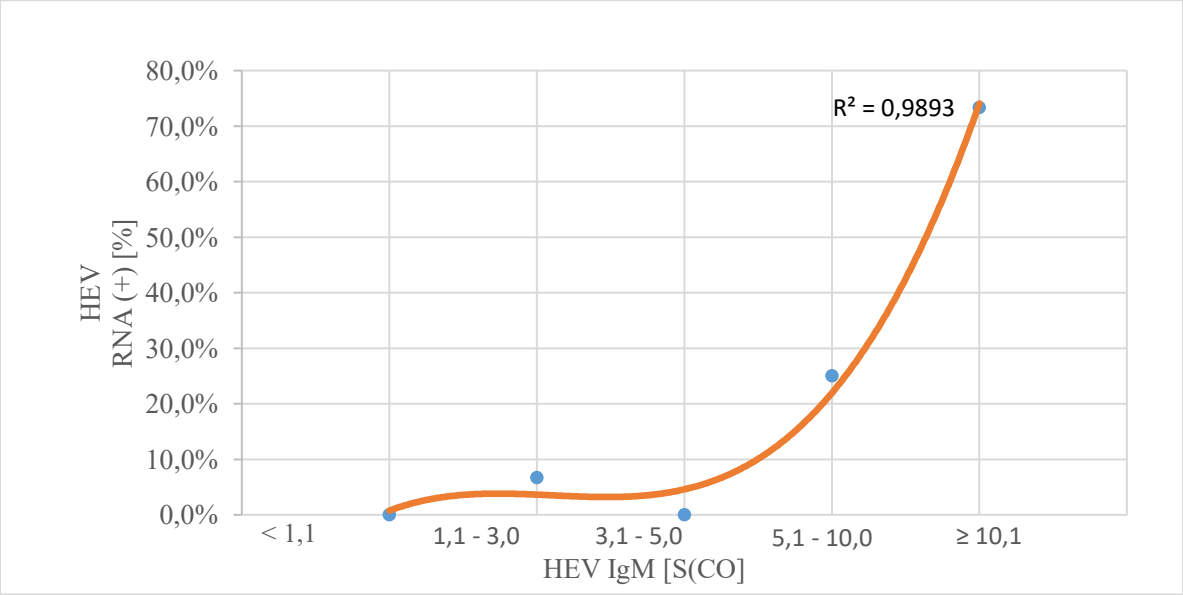


Figure 13. Correlation between anti-HEV IgM levels and the presence of HEV RNA in serum.

5. Molecular-genetic profile of HEV isolates.

For molecular-genetic characterization, RNA from the HEV (+) samples was subjected to nested PCR, followed by capillary sequencing electrophoresis and bioinformatic analysis of the resulting gene-specific sequences to determine the genotype and subtype assignment of each respective HEV isolate.

5.1. *Nested* PCR for obtaining gene-specific amplicons

The selection of HEV isolates for genotyping was performed using nested PCR with gene-specific primers targeting a region within the second open reading frame (ORF2), which encodes the viral structural protein. A positive signal indicating the presence of the gene-specific amplicon was obtained in 25 out of 32 (78.1%) HEV RNA-positive samples (Figure 14).

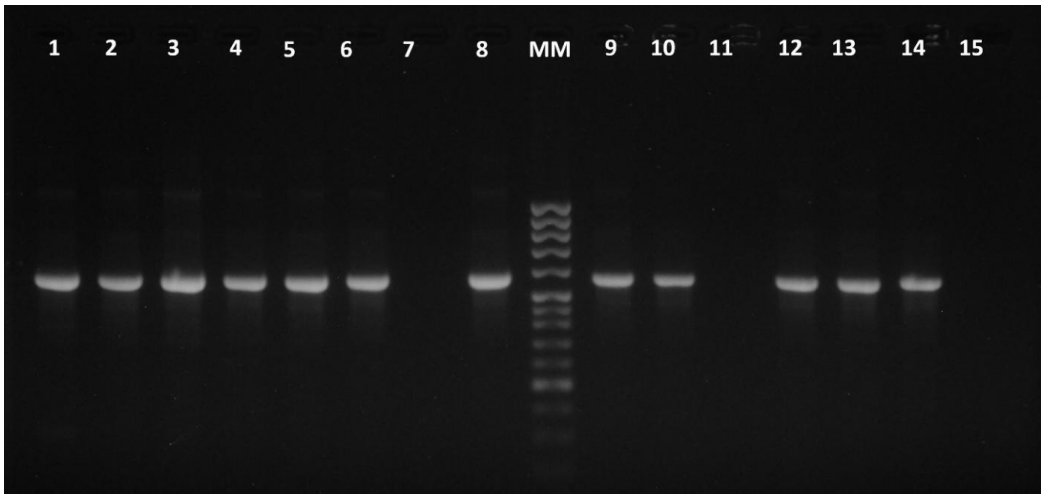
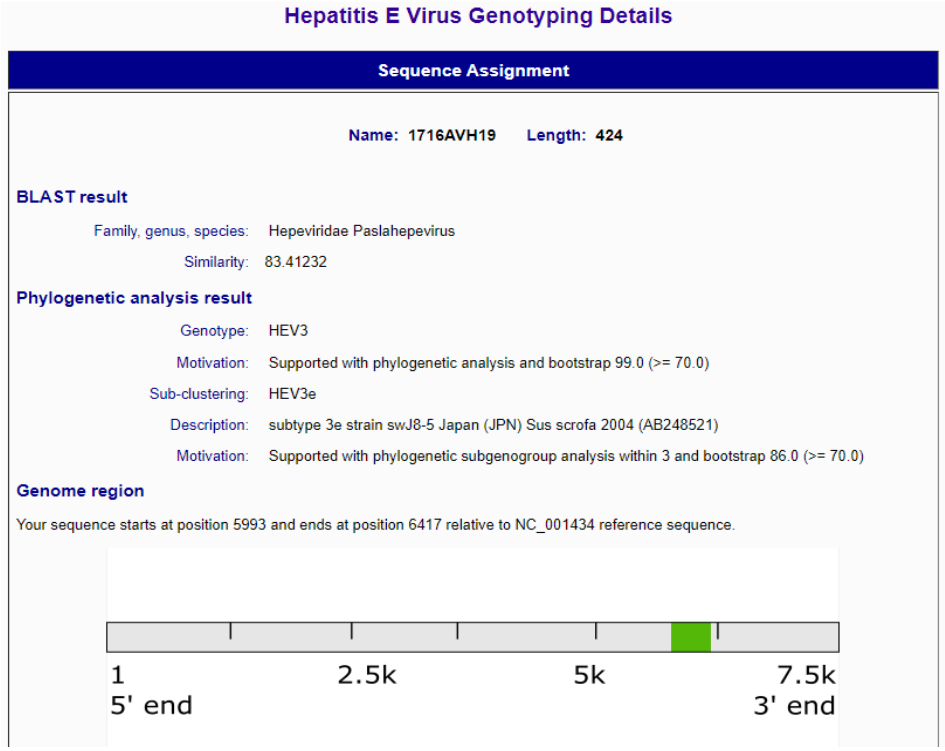


Figure 14. Analytical 2% agarose gel electrophoresis following nested PCR for the detection of a specific 565 bp amplicon. *Legend: MM = molecular marker featuring two prominent, enhanced bands for fragments of 250 bp and 500 bp; No. 1 to No. 15 = investigated HEV isolates.*

5.2. Genotypic characterization of HEV isolates

To determine the genotype and subsequent subtype assignment, the obtained sequences were subjected to phylogenetic analysis using an online genotyping tool (Figure 15). Initial clustering demonstrated that all investigated sequences belong to HEV genotype 3. Following sub-clustering, the sequences were partitioned into 5 distinct subtypes. As a result, 48% (12/25) of the isolates were assigned to subtype HEV-3e, 24% (6/25) to HEV-3f, 12% (3/25) to HEV-3c, 8% (2/25) to HEV-3m, and 4% (1/25) to HEV-3a. One sequence could not be assigned to any known subtype (Figure 16).

A



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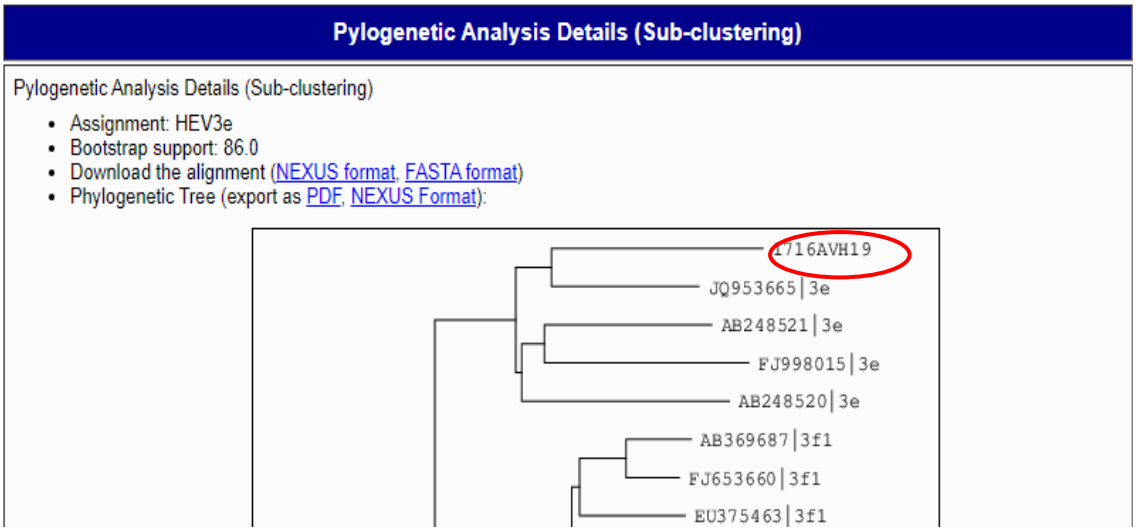

Figure 15. Online genotyping tool (Hepatitis E Virus Genotyping Tool Version 1.0, developed by Harry Vennema and Annelies Kroneman) used for the taxonomic classification of HEV isolates. A) Details of the sequence alignment. B) Details of the phylogenetic analysis for sub-clustering (subtyping). The analyzed HEV isolate is outlined in red.

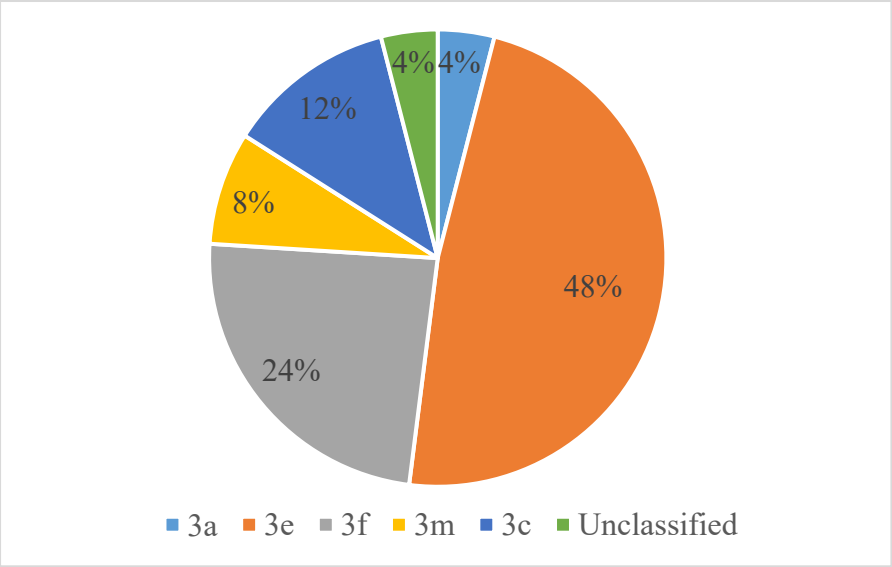

Figure 16. Genotype distribution of the studied HEV isolates.

5.3. Phylogenetic Analysis of the Obtained HEV Sequences

Of the genotyped sequences, 12 were subjected to a detailed cladistic analysis. Table 20 displays the clinical and laboratory characteristics of the serum samples from which these viral sequences were obtained. Ten serum samples originated from patients with laboratory-confirmed acute viral hepatitis E (AVHE), while 2 were obtained from patients with liver cirrhosis.

The quantitative real-time PCR results revealed highly variable viremia levels across the samples. The lowest detected viral load (360 IU/ml) was found in a sample from a patient with acute viral hepatitis E. Conversely, the highest viral load was observed in a sample from a patient with cirrhosis, reaching 4,927,500 IU/ml. The target region of the HEV viral genome utilized for the cladistic analysis was 567 bp in length, spanning nucleotides from position 5,924 to 6,491.

Table 20. Distribution of RNA-positive samples according to genetic characteristics and confirmed diagnosis.

Specimen	*Diagnosis*	*RNA IU/ml*	*Genotype/subtype*	*Genomic region*
224AVH21	VHE	367 375	*HEV*-3f	5923-6488
229AVH18	VHE	1 560	*HEV*-3f	5923-6486
327AVH18	VHE	2 783 750	*HEV*-3e	5960-6444
354AVH18	VHE	13 643	*HEV*-3e	5923 -6491
374AVH19	VHE	2 931 250	*HEV*-3e	5920 -6489
376AVH20	VHE	360	*HEV*-3	5923-6489
1716AVH19	VHE	8 826	*HEV*-3e	5958-6490
324CI19	VHE	4 927 500	*HEV*-3	5923-6487
100CI19	VHE	321 875	*HEV*-3e	5956-6487
2957AVH19	VHE	432 375	*HEV*-3e	5930-6492
780AVH20	VHE	2 872	*HEV*-3	5924-6491
696AVH20	VHE	6 756	*HEV*-3m	5921-6442

To construct the phylogenetic tree, a best-fit nucleotide substitution model selection was initially performed using MEGA11 (Molecular Evolutionary Genetics Analysis) software. An evolutionary analysis evaluating 68 parameters (including branch lengths) was conducted, taking into account the Bayesian Information Criterion (BIC), corrected Akaike Information Criterion (AICc), and maximum likelihood values (Figure 17). The tree topology was calculated automatically, incorporating the 1st+2nd+3rd+non-coding codon positions.

The TN93+G+I model was selected as it exhibited the lowest BIC score and provided the best fit for the nucleotide substitution pattern. For the phylogenetic reconstruction, the 12 Bulgarian HEV sequences were aligned against 14 full-genome reference sequences

representing HEV genotypes 1, 2, 3, 4, 5, 6, 7, and 8, retrieved from the National Center for Biotechnology Information (NCBI) GenBank database. According to the obtained results, HEV genotype 3 is actively circulating in Bulgaria, demonstrating evolutionary relationships with both European and Asian isolates (Figure 18). Through the phylogenetic analysis, the sequenced Bulgarian isolates were partitioned into two well-defined subgroups:Subgroup A (n=8): These sequences showed the highest genetic similarity and shared a common ancestor with a reference sequence for HEV-3 from Mongolia (AB290313).Subgroup B (n=3): These sequences clustered with HEV-3 reference sequences from Italy (MF959764) and Germany (KP294371), sharing their closest common ancestor with the Italian isolate.The remaining sequence, 696AVH20, formed a distinct standalone branch, positioned closest to the European (MK39097) and Japanese (LC260517) genotype 3 reference sequences.

Results

Table. Maximum Likelihood fits of 24 different nucleotide substitution models

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	*f*(A)	*f*(T)	*f*(C)	*f*(G)	
TN93+G+I	68	9259.545	8736.546	-4299.984	0.57	1.76	5.91	0.215	0.250	0.280	0.255	(
K2+G+I	64	9264.449	8772.183	-4321.836	0.58	1.78	6.12	0.250	0.250	0.250	0.250	(
GTR+G+I	71	9271.778	8725.732	-4291.551	0.56	1.73	6.06	0.215	0.250	0.280	0.255	(
K2+G	63	9279.484	8794.902	-4334.203	n/a	0.19	5.93	0.250	0.250	0.250	0.250	(
T92+G+I	65	9279.741	8779.792	-4324.632	0.58	1.84	6.03	0.233	0.233	0.267	0.267	(
TN93+G	67	9282.509	8767.193	-4316.316	n/a	0.21	5.70	0.215	0.250	0.280	0.255	(
HKY+G+I	67	9288.320	8773.004	-4319.222	0.58	1.65	8.08	0.215	0.250	0.280	0.255	(
HKY+G	66	9293.546	8785.913	-4326.684	n/a	0.19	5.68	0.215	0.250	0.280	0.255	(
GTR+G	70	9294.847	8756.483	-4307.936	n/a	0.21	5.86	0.215	0.250	0.280	0.255	(
T92+G	64	9295.312	8803.046	-4337.267	n/a	0.19	5.84	0.233	0.233	0.267	0.267	(
TN93+I	67	9319.740	8804.424	-4334.932	0.60	n/a	5.10	0.215	0.250	0.280	0.255	(
K2+I	63	9328.894	8844.312	-4358.908	0.60	n/a	5.40	0.250	0.250	0.250	0.250	(
GTR+I	70	9333.416	8795.052	-4327.220	0.60	n/a	5.26	0.215	0.250	0.280	0.255	(
HKY+I	66	9335.630	8827.997	-4347.726	0.60	n/a	5.25	0.215	0.250	0.280	0.255	(
T92+I	64	9341.179	8848.913	-4360.200	0.60	n/a	5.36	0.233	0.233	0.267	0.267	(
JC+G	62	10198.798	9721.900	-4798.710	n/a	0.19	0.50	0.250	0.250	0.250	0.250	(
JC+G+I	63	10208.498	9723.916	-4798.710	0.00	0.19	0.50	0.250	0.250	0.250	0.250	(
JC+I	62	10223.984	9747.086	-4811.303	0.61	n/a	0.50	0.250	0.250	0.250	0.250	(
TN93	66	10292.684	9785.051	-4826.253	n/a	n/a	4.58	0.215	0.250	0.280	0.255	(
GTR	69	10304.669	9773.987	-4817.696	n/a	n/a	4.12	0.215	0.250	0.280	0.255	(
HKY	65	10387.112	9887.162	-4878.317	n/a	n/a	4.53	0.215	0.250	0.280	0.255	(
K2	62	10398.451	9921.553	-4898.536	n/a	n/a	4.53	0.250	0.250	0.250	0.250	(
T92	63	10402.802	9918.220	-4895.862	n/a	n/a	4.53	0.233	0.233	0.267	0.267	(
JC	61	11229.578	10760.364	-5318.949	n/a	n/a	0.50	0.250	0.250	0.250	0.250	(

Figure 17. Selection of the best-fit Maximum Likelihood substitution model for nucleotide substitutions in MEGA11.

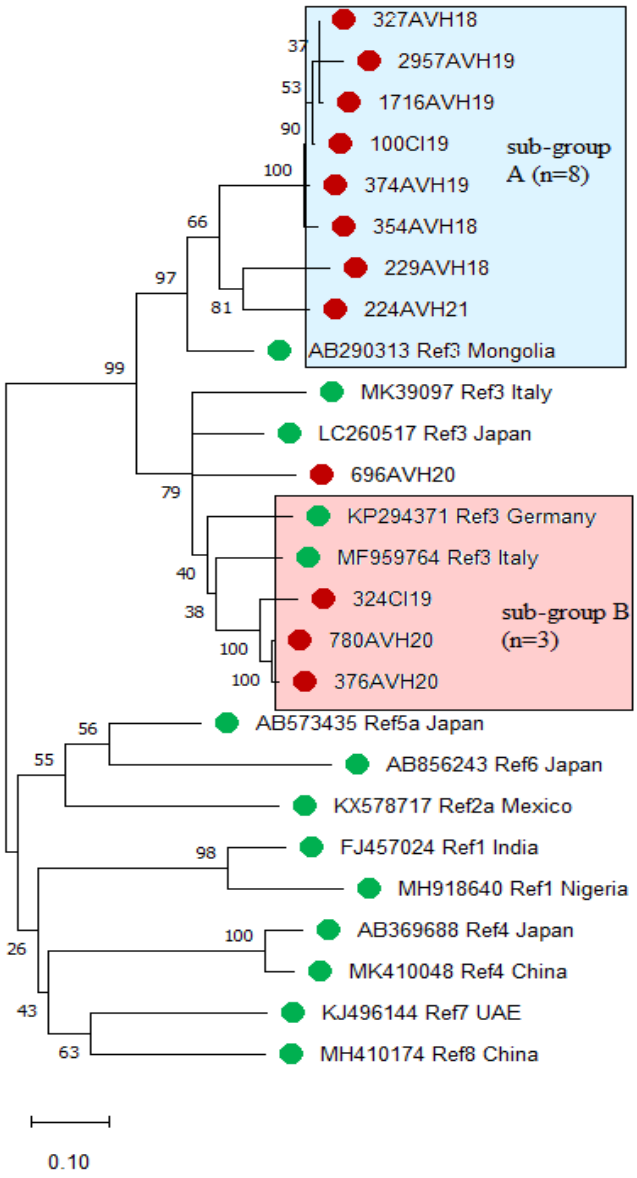

Figure 18. Phylogenetic analysis and genotype distribution of HEV isolates.

Legend: Reference sequences retrieved from GenBank are marked with green circles, while the investigated Bulgarian sequences are indicated with red circles. (AVH = Acute Viral Hepatitis).

To achieve a detailed subtyping of the identified HEV isolates, an additional phylogenetic analysis was performed by constructing a second phylogenetic tree. For comparative purposes, this analysis incorporated the 12 sequences from the present study alongside retrospective sequences from Bulgarian patients with viral hepatitis E (vHEV) (n=8, specifically: MH203185; MH203169; MH203164; MH203227; MH203186; MH203187; MH203191; MH203166), and two isolates from Bulgarian slaughterhouse swine (MZ555941;

MZ555942). The analysis revealed three well-defined clusters—C, D, and E—into which the Bulgarian sequences partitioned:Cluster C (n=12): The majority of the isolates were assigned to this group, originating from a common ancestor shared with the HEV subtype 3e reference sequence (AB248521).Cluster D (n=3): This smaller, yet well-defined cluster consists of three distinct lineages of Bulgarian isolates, which form a separate monophyletic subgroup alongside the HEV subtype 3f reference sequence (AB369687). The third cluster (E, n=3) is statistically well-supported and comprises a group of Bulgarian sequences that could not be assigned to any established HEV-3 subtype. The sequenced isolate 696AVH20, which formed a distinct standalone branch during the initial genotyping phase, shares a common ancestor with the HEV-3m subtype reference sequence (KU513561) upon detailed subtyping. This phylogenetic relationship is highly reliable, backed by a maximum bootstrap value of 100%. Meanwhile, the two HEV sequences isolated from Bulgarian slaughterhouse swine demonstrate genetic similarity to the HEV-3c subtype reference sequence (FJ705359); however, they do not cluster with any of the human HEV isolates. Consequently, the application of detailed phylogenetic analysis enables the precise classification of isolates that initially resisted definitive subtyping.

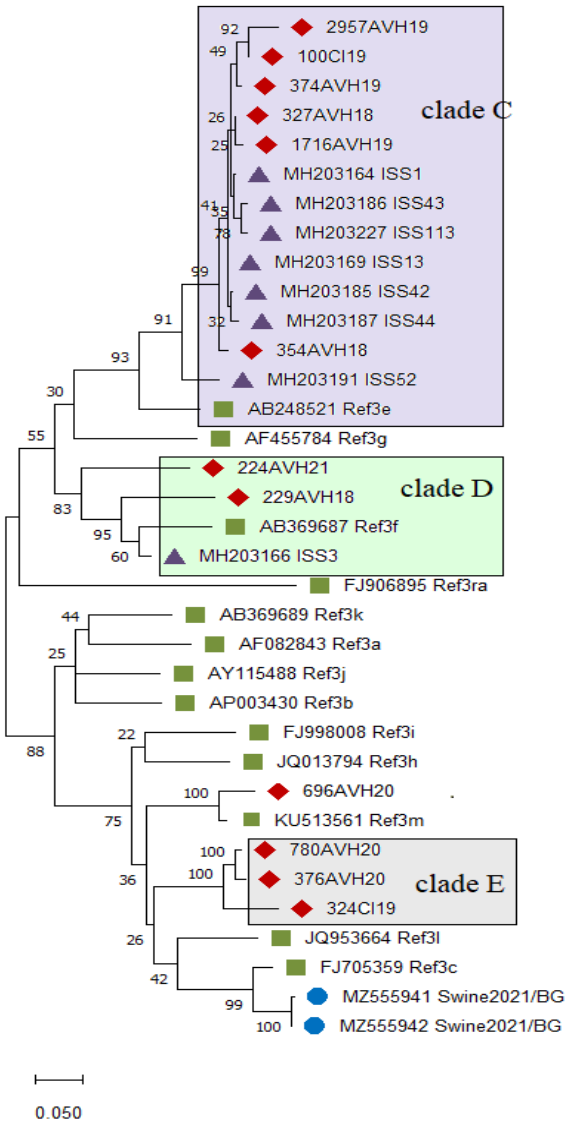

Figure 19. Phylogenetic analysis and subtype distribution of Bulgarian HEV isolates.

Legend: Reference sequences are marked with green squares, sequences investigated in our study with red diamonds, retrospective sequences from Bulgarian HEV patients with purple triangles, and sequences from Bulgarian slaughterhouse pigs with blue circles.

IV. DISCUSSION

The HEV virus and the diseases it causes have been studied for only four decades. During this time, data have accumulated regarding transmission routes, risk groups for infection, and severe courses of the disease; furthermore, prevention and control programs have been developed, a vaccine has been created, and diagnostic tests continue to be refined.

Three decades ago, hepatitis E was considered endemic only to underdeveloped regions and, for this reason, was a neglected disease in Europe and developed nations across other continents. Following the isolation of the virus from swine, interest in the infection increased. Over the past 20 years, publications dedicated to hepatitis E have emerged in Bulgaria as well, including two monographs published on the subject (*Pishmisheva-Peleva*, 2022; *Baymakova*, 2024). For the period 2021–2025, a National Program for the Prevention and Control of Viral Hepatitis was active in Bulgaria, with one of its primary objectives being the reduction of the prevalence of viral hepatitis (A, B, C, D, and E).

1. HEV Circulation

In the present doctoral thesis, the results of studies detecting serological and molecular-genetic markers of HEV infection in 2,467 samples (1,823 prospective and 644 retrospective) are presented. Throughout the designated study period (2015–2023), the National Reference Laboratory "Hepatitis Viruses" received a varying number of samples, with the lowest count recorded during the COVID-19 pandemic. This can be explained, on one hand, by the imposed isolation measures and, on the other, by the anxiety surrounding the coronavirus among both the general population and medical professionals, which led to remote consultations. Frequently, regardless of the patient's symptoms, a diagnosis of COVID-19 was established, meaning that anicteric and mild forms of viral hepatitis likely remained undiagnosed. According to data from the "Epidemiology, Surveillance, and Early Warning of Infections" (ESEWI) Section at the NCIPD, 217 cases of HEV infection were registered in Bulgaria in 2019, representing an incidence rate of 3.10 per 100,000 (*Vladimirova*, 2019). In 2020, 88 cases were reported with an incidence rate of 1.27 per 100,000, and in 2021, only 44 cases were recorded, with an incidence rate of 0.67 per 100,000 (*Vladimirova*, 2020; *Vladimirova*, 2021).

In the literature, the reduced number of registered cases is attributed not so much to direct biological inhibition between the viruses, but rather to other factors: the introduction of

strict anti-epidemic measures and improved personal hygiene, which limited the fecal-oral transmission mechanism of infectious agents (*Qin Y*., 2024), particularly in endemic countries. Alongside this, the pandemic led to the phenomenon of "hidden morbidity" due to a deficit in diagnostic activity for infections other than COVID-19 (*Wingrove et al*., 2021; *ECDC*). In 2019, amendments were made to Ordinance No. 21 on the procedure for registration, reporting, and surveillance of infectious diseases, on the basis of which HEV infection became subject to mandatory notification and registration. Concurrently, the number of laboratories in the country performing detection of serological markers for HEV infection has increased. This higher number of certified laboratories consequently led to a decrease in the volume of samples sent to the NRL "Hepatitis Viruses" for the laboratory diagnosis of HEV infections.

2. Significance of Gender, Age, Occupation, and Risk Factors for HEV Infection.

In the present doctoral thesis, the influence of various criteria—such as age, gender, comorbidities, etc.—on the development of HEV infection was analyzed in a prospective and a retrospective group of patients.

The prospective group included patients with various hepatic and extrahepatic conditions.

The group with liver dysfunction included individuals with a wide range of diseases, among which patients with hepatitis of unspecified etiology were the most numerous. These were patients in whom a disease caused by any of the other known hepatotropic viruses had been ruled out. Over the last decade, tests for the diagnosis of hepatitis E have become widely available, the number of laboratories is sufficient, and the assays themselves do not require substantial financial resources; therefore, it is recommended to perform them for every patient presenting with hepatitis who tests negative for markers of other hepatitides. Furthermore, it is considered that patients from risk groups should be screened for markers of HEV infection even in cases of a confirmed hepatitis caused by another virus (*Kotsev S*, 2023*; Kotsev St,* 2022) due to a significant probability of co-infection.

Patients with unspecified liver disease (ULD) are those in whom elevated levels of cytolytic and/or cholestatic enzymes were detected either due to clinical symptoms or during routine health checkups, or in whom icterus and/or hepatomegaly or hepatosplenomegaly were established during physical examination. This is a heterogeneous group in terms of nosology, and testing for hepatitis E may often be overlooked due to mild and non-specific complaints, the absence of jaundice, or mild elevations in enzyme levels. In a study conducted

in the United Kingdom, 15% of individuals initially diagnosed with drug-induced hepatitis were actually found to have acute hepatitis E (*Dalton et al*., 2007). In the ULD subgroup, seropositive individuals accounted for 19%, presenting with antibodies of either one or both immunoglobulin classes

The largest relative share of seropositive individuals was observed in the acute hepatitis E subgroup, reaching 58%. Concurrently, the proportion of individuals with confirmed viremia (detected HEV RNA) within the same group was only 1.7%. This low percentage is associated with both the transient nature of viremia and the specific timing of sample collection for testing. Comparison with the aggregated data from the Regional Health Inspectorates (RHI) and the National Center of Infectious and Parasitic Diseases (NCIPD) for the study period indicates that the officially registered incidence is lower than the actual prevalence established in our study. This confirms the hypothesis that a significant proportion of HEV cases across various regions in Bulgaria remain undiagnosed or are registered as "hepatitis of unspecified etiology" or as another liver disease that is not subject to mandatory notification

In recent years, an association has been established between HEV and certain extrahepatic diseases, and the number of publications in this field continues to grow (*Wu J*, 2021; *Kanda T*, 2025; *Fousekis FS*, 2020; *Baymakova*, 2024). Extrahepatic manifestations may be the sole clinical presentation of the infection; therefore, screening is highly recommended, particularly for individuals from risk groups (*EASL*, 2018). According to our results, serological evidence of HEV infection was detected in 20% of patients with other diseases. This was a heterogeneous group that included individuals presenting with diarrhea, fever, infectious mononucleosis, pancreatitis, neurological disorders, and thrombocytopenia, among others. To date, the link to HEV has been confirmed in cases of thrombocytopenia, acute pancreatitis, and Guillain-Barré syndrome (GBS), as reported by numerous authors (*Pishmisheva*, 2024; *Lhomme*, 2020). Our obtained results must be interpreted with caution—while for some of the patients these conditions were not casually linked to HEV infection, the high proportion of seropositive individuals confirms the wide prevalence of this infection. Further research is necessary to fully clarify all clinical manifestations of the disease.

Patients without a specified diagnosis in the accompanying referral letter were also tested. Testing was performed for them despite the reasons for the request being unstated; in this group, seropositive individuals accounted for 36%, though these findings cannot be

thoroughly discussed due to the lack of clinical patient data. Nonetheless, this high relative share of seropositivity indicates a wide prevalence of HEV infection.

One of the transmission routes of the virus is associated with the transfusion of blood products, and blood donors were consequently included in the retrospective group. The objective was to determine the prevalence of HEV infection within this subgroup and to assess the risk of transmission to recipients. To date, two such studies have been conducted in different regions of Bulgaria (*Baymakova*, 2021; *Kotsev*, 2023). The authors reported positivity rates among blood donors of 25.9% and 40%, respectively. According to the results of the present study, the seroprevalence is 31%. Currently, donor blood in Bulgaria is not screened for markers of HEV infection, meaning that during the period of viremia, a recipient could be infected. Blood recipients are typically individuals with one or more comorbidities (oncological conditions, chronic hemodialysis patients, patients with cirrhosis, or those presenting after acute blood loss or trauma), and acquisition of HEV could worsen their condition, leading to an uncertain outcome. We consider it necessary that donor blood testing positive for antibodies should also be screened for HEV RNA. In the study by Kotsev (2023), anti-HEV-positive samples were evaluated, and viral nucleic acid was not detected in any of them.

Veterinarians were also included in the retrospective group as individuals at high risk for HEV infection; 13% of them tested positive for anti-HEV IgM. Studies in this direction should be continued and expanded to include slaughterhouse workers, animal care professionals, and individuals employed on mixed-species livestock farms. It is recommended that a larger cohort of individuals be tested, and that screening encompass both anti-HEV IgG and the detection of HEV RNA in seropositive individuals. In our study, HEV RNA was not detected in any of the positive samples obtained from the veterinarians. In a study conducted among veterinarians in Korea, 28.2% were found to be seropositive (*Lyoo*, 2019). According to the results of that same study, 5% of domestic dogs were also seropositive. Although the role of dogs as a direct source of infection for humans has not been definitively proven and remains under debate, some authors have reported cases of complete genetic identity between viral isolates from domestic dogs and their owners. This elevates the theoretical possibility of transmission to a proven epidemiological risk, albeit with low frequency, and highlights the need for further research in this direction (*Okamoto*, 2004; *Zeng*, 2017).

Several studies have been conducted on domestic and slaughterhouse animals in Bulgaria, establishing a high prevalence of HEV among them: 60.05% among domestic pigs and 40.0% among slaughter pigs (Baymakova, 2021; Takova, 2020; Pishmisheva, 2018).

In the literature, conflicting data exist regarding the prevalence of HEV infection among patients undergoing dialysis treatment. According to the results of some studies, the proportion of seropositive individuals does not differ from that of the general population (Tavakoli A, 2021), whereas others report that anti-HEV antibodies are detected more frequently in these patients. In Bulgaria, three studies have evaluated the prevalence of anti-HEV IgG in this population, reporting seropositivity rates of 6.2% (*Kevorkyan*, 2023), 11% (in the present study), and 14.7% (*Pishmisheva*, 2020), respectively. In Croatia, a high relative share of anti-HEV IgG-positive individuals was registered, reaching 27.9% among this cohort (*Mrzljak*, 2020). These substantial differences in prevalence could be explained by local endemicity at the population level (*Kevorkyan*, 2023).

Children with oncohematological diseases were also included in the retrospective group. Although they are not a primary target for infection by the genotypes circulating in Bulgaria, the studies were conducted to evaluate the role of drug-induced immunosuppression in acquiring the infection. The relative share of anti-HEV IgM among them was high, reaching 69%. It is advisable that research continues by incorporating anti-HEV IgG testing, the detection of HEV RNA in positive samples, and screening for EBV and CMV markers, given the cross-reactivity with these viruses of up to 33% at this age (Hyams C., 2014). In collaboration with clinicians, it would be beneficial to investigate the course of symptomatic hepatitis E infection and chronic infection within this group, as well as the overall prevalence of the infection in the pediatric population.

Regarding gender distribution, specific antibodies were detected significantly more frequently in men than in women: within the prospective group, anti-HEV IgM was present in 33% of men compared to 23% of women, while anti-HEV IgG was found in 26% of men versus 17% of women. Our findings align with literature data indicating that males are more heavily affected by this infection (*Faber et al*., 2018; Gay, 2021; *Jacobsen, M*., 2021; *Golkocheva-Markova E*., 2023; *Teoharov P*., 2014).

At present, the underlying reasons for this disparity remain fully unclarified; however, the influence of both behavioral and biological factors is postulated. These include distinct dietary habits (such as the consumption of larger quantities of meat and a preference for raw-

cured delicacies), a higher prevalence of preexisting liver damage, and greater alcohol consumption (Lhomme et al., 2016; EFSA, 2017).

In contrast, no statistically significant difference in anti-HEV prevalence was observed between genders within the retrospective group. These findings correspond with the results of a study conducted among outpatients in the Plovdiv region during the 2012–2013 period (Teoharov, 2014). Research within this cohort should also be extended by categorizing the studied subjects by age, risk habits, and comorbidities across each age group.

Age-specific prevalence of hepatitis E has been reported in numerous studies, which have established a statistically significant higher seroprevalence of HEV-3 among individuals over the age of 50 compared to younger cohorts (Teoharov, 2014; van Gageldonk-Lafeber, 2017; Mansuy, 2016; Faber, 2012). This pattern is further corroborated by our own findings, where the highest relative share of anti-HEV IgM-positive individuals within the prospective group was observed in subjects over 50 years of age.In contrast to the prospective group, an atypically high relative share of anti-HEV IgM (38%) and anti-HEV IgG (12%) was observed within the retrospective group among individuals under 20 years of age. The absence of the characteristic age-dependent trend and the predominance of positive results among children with oncohematological diseases highlight the distinct clinical profile of this cohort. Due to the limited sample size (n=26), the state of immunosuppression, and the potential risk of cross-reactivity (with EBV and CMV), these results do not permit the formulation of generalized conclusions regarding either the broader pediatric population or the retrospective study group as a whole. Nonetheless, we believe it is highly appropriate for immunosuppressed children to be screened for HEV infection alongside other potential pathogens, and that this diagnostic protocol should routinely include the detection of hepatitis E virus nucleic acid.

The prevalence of anti-HEV antibodies varies significantly across different studies. According to Hartl et al. (2016), the primary factors underlying this variability (heterogeneity) include the characteristics of the studied population, the geographic region, and the specific serological assay utilized by the laboratory.In the present study, we established a high percentage of seropositive individuals among patients with cryptogenic cirrhosis (32%; $p = 0.1243$). Similar findings have been reported by other authors (*Akyüz F.*, 2019). Individuals with liver cirrhosis are at an increased risk for a severe course of the disease; in these patients, cytolytic enzymes are often only moderately elevated, which can cause the involvement of HEV to remain unrecognized. Consequently, we consider it appropriate that individuals from risk groups, even those presenting with minimal hepatic abnormalities, be routinely screened for hepatitis E virus infection. The prevalence of HEV among HIV-positive individuals varies

considerably across different studies. According to our data, anti-HEV antibodies were detected in 14% of the evaluated subgroup. In another study conducted by the NRL "Hepatitis Viruses" among HIV-infected individuals, a 10% anti-HEV positivity rate was reported (*Golkocheva-Markova*, 2022). In Greece, the relative share of HEV-positive individuals within the HIV population stands at 7.3%, which mirrors that of the general population, leading to the assumption that HIV infection is not an independent risk factor for HEV (*Politou*, 2015). Conversely, data from Portugal show a different trend, where seropositivity reached 25% in the evaluated subgroup compared to 18% in the general population (*Filipe*, 2022). Other European countries, such as Spain and Denmark, report positivity rates of 26% and 23%, respectively, among HIV-infected individuals (*Harritshøj*, 2019; *Pineda*, 2014). Because HIV-infected individuals may fail to produce specific antibodies, it is recommended that both this cohort and other immunosuppressed individuals be screened via the detection of HEV RNA in serum or HEV antigen in stool samples (*Keane F.*, 2012).

3. HEV RNA in Serum Samples with Confirmed Anti-HEV Antibodies.

Since 2019, the concentration of HEV RNA has been determined using real-time RT-PCR at the National Reference Laboratory "Hepatitis Viruses". The detection of HEV RNA in blood or stool is indicative of active HEV infection. All antibody-positive samples from both study groups were tested for HEV RNA, which was detected in 1.4% (33/2,467) of cases. Among them, two clinical cases belonging to the group of patients with extrahepatic manifestations stand out: a patient with Guillain-Barré syndrome and a patient with lymphoma. These cases illustrate the potential of HEV to provoke acute neurological complications, as well as the clinical significance of the virus in individuals with malignant hematological diseases.

The low relative share of RNA-positive samples—even among individuals with clinical, paraclinical, and serological evidence of acute hepatitis E—is explained by the transient nature of viremia *(Balaban*, 2022). The timing of biological sample collection (blood, serum) is critical to the result obtained. For this reason, a negative nucleic acid test result does not rule out acute hepatitis E. Routine diagnosis of the infection is based on serological testing—namely, the detection of specific antibodies. In immunocompetent individuals, this is usually sufficient. However, for immunocompromised patients and individuals who fail to produce anti-HEV IgM (approx. 10%), the use of molecular biological methods is highly recommended for proper diagnosis.

4. Correlation Between Serological and Molecular Markers

In addition to qualitative diagnostic tests for HEV, quantitative assays were also employed to determine antibody levels and HEV RNA concentrations. According to the obtained results, a directly proportional relationship exists between the anti-HEV IgM levels and the frequency of HEV RNA detection. Specifically, when the anti-HEV IgM serological index—expressed as the sample-to-cutoff signal ratio (S/CO)—exceeded 5, the number of HEV RNA-positive samples increased. When this index exceeded 10, the proportion of RNA-positive cases reached 70%.

In a study of sporadic cases of acute HEV infection in China, HEV RNA was detected in 43% of cases. The study demonstrated that the cumulative probability of detecting HEV viremia drops rapidly to approximately 10% within 32 days after the onset of clinical symptoms, subsequently declining more gradually to 5% by day 41, and reaching zero by day 131 (*Lu*, 2014). Furthermore, evaluations of blood donor samples have shown that anti-HEV IgM antibodies become detectable at an average of 33 days after the initial detection of viremia (ranging from 7 to 108 days), reaching peak values at an average of 36 days (range 14–115 days). The maximum viral load was observed on day 22, preceding the peak of anti-HEV IgM (*Plümers*, 2024).

Consistent with data from other publications (*Tavakoli A*., 2010), HEV RNA is detectable early in the course of the infection, primarily during the initial days of clinical manifestation. For this reason, it is highly appropriate for the exact timing of sample collection to be recorded on the referral letter accompanying blood samples sent for laboratory confirmation of hepatitis E infection. A comprehensive understanding of the viral life cycle remains essential for the accurate clinical interpretation of these laboratory findings.

5. Molecular-Genetic Characterization of HEV Isolates

In the present doctoral thesis, a taxonomic classification of Bulgarian HEV isolates was performed. According to the obtained results, HEV Genotype 3 is prevalent in Bulgaria. This aligns with data from an earlier study in the country, which performed HEV genotyping based on a different specific region within the first open reading frame (ORF1) of the viral genome (*Teoharov*, 2014).

The majority of the isolates identified in the current study belong to subtypes 3e (48%), 3f (24%), and 3c (12%). These same subtypes were also detected in a retrospective study of

samples from acute hepatitis E patients evaluated at the NRL "Hepatitis Viruses" during the 2013–2015 period (*Bruni*, 2018).

The confirmed Genotype 3 and subtypes 3e, 3f, and 3c are zoonotic and represent the most widely distributed strains in Europe, with Bulgaria being no exception to this trend. Although the hepatitis E virus exhibits substantial genetic diversity, subtypes 3f, 3c, and 3e remain the most frequently isolated strains among infected individuals across Europe, including Bulgaria (*Zahmanova*, 2023).

In 2020, a study conducted in France established a correlation between the causative subgenotype and the clinical course of the disease (*Lhomme S.*, 2020). According to the results, subtype HEV-3f is associated with a more severe clinical presentation, higher serum bilirubin levels, and a higher rate of patient hospitalization compared to subtype HEV-3c, which follows a more favorable course. In Hungary, subtypes 3e and 3f have been detected in patients with acute hepatitis E (*Balázs*, 2023; *Ulber*, 2022). Subtype 3c was identified in infected blood donors from Croatia (Gorski, 2023). Furthermore, phylogenetic studies of HEV genotype diversity in Germany identified 3c as the predominant subtype (67.3%), followed by 3f, 3e, and 3i at 14.3%, 9.7%, and 4.0%, respectively (*Schemmerer*, 2022).

The HEV sequences obtained in the present study are grouped into two major clades, which are homologous to sequences isolated in Europe (originating from Italy and Germany) and Asia (originating from Mongolia). These data are in agreement with the spatiotemporal reconstruction conducted by Zehender et al. regarding the currently circulating HEV genotypes in Europe. According to the authors, HEV-3 is subdivided into two main clades, one of which groups together the less common European subtypes with the common ancestor of the Asian subtypes (Zehender, 2014).

Concurrently, Palombieri and co-authors (*Palombieri A.*, 2021) reported high nucleotide identity (92.0–93.9%) between sequences subtyped as HEV-3c isolated from swine and humans in Bulgaria (*Bruni*, 2018), which is further corroborated by the subtyping performed in the present work. The detection of HEV-3c in stool samples from swine in Germany, Italy, the Netherlands, and Poland led the authors to hypothesize that this subtype was introduced and spread in Bulgaria through trade in infected animals from other European countries.

The zoonotic nature of HEV-3 is widely accepted as the primary driver behind the high heterogeneity of viral strains circulating in the human population. This mechanism accounts for the shift in circulating strains observed in Belgium, France, and the United Kingdom, where subtypes HEV-3f and 3e were replaced by HEV-3c. Over the course of our

investigations, we also identified several viral isolates that cannot currently be assigned to any of the known types or subtypes.

Due to the high heterogeneity established among the HEV strains circulating in Bulgaria, further research in this direction is warranted. Collaborative efforts with clinicians are essential to investigate the clinical manifestations of disease caused by these unclassified isolates, alongside animal studies across various species to identify their reservoirs.

A limitation of the present doctoral thesis is the relatively small number of genotyped and subtyped samples. This constraint stems from the transient nature of viremia and a general lack of clinical documentation regarding the exact timeframe of the illness at which the samples were collected. Similar limitations have been reported by other authors; for instance, a study evaluating organ transplant recipients in Turkey established a viremia rate of only 0.6% (*Balaban*, 2022). Similarly, in the Netherlands, only 2.4% of anti-HEV-positive patients with hematological malignancies were found to be HEV RNA-positive. Furthermore, a large-scale screening of 225,000 blood donors in the United Kingdom identified HEV viremia in just 0.035% of the cohort (*Hewitt*, 2014).

V. Conclusion

Hepatitis E is also prevalent among viral hepatitides in Bulgaria. This necessitates a comprehensive understanding of the disease's clinical manifestations, transmission routes, risk groups, as well as reliable diagnostic tests. Patient complaints in hepatitis E cases mirror those of other viral hepatitides, as do the abnormalities in laboratory parameters; thus, laboratory diagnostics—both serological and molecular-biological—frequently play a decisive role in establishing an etiological diagnosis.

Hepatitis E should be considered in individuals from risk groups, for whom screening is recommended even in cases of minimal liver function abnormalities, as well as in individuals presenting with extrahepatic manifestations associated with this infection. Although rare, the infection can also be transmitted via blood product transfusion (31% seropositivity rate), which warrants the screening of donor blood to prevent the infection of recipients.

The isolated genotypes and subtypes are consistent with European variants, while the identified untyped isolates provide a baseline for more extensive research.

Hepatitis E virus, with its diverse manifestations and currently unclarified potential, poses a challenge to both clinicians and biologists, necessitating further continuous collaborative efforts.

The present study may contribute to the improvement of targeted prevention and management strategies for HEV infection in our country.

VI. CONCLUSIONS

1. Hepatitis E virus is a major etiological agent of acute hepatitis in Bulgaria, with an overall seroprevalence of 31% among the 2,467 individuals tested, reaching 36% in the prospective group and 17% in the retrospective group.
2. Consistent with literature findings, our study confirms that age over 50 years and male sex are associated with an increased risk of developing symptomatic HEV infection.
3. Due to transient viremia, the detection of HEV RNA in patient serum is only possible during the early stages of infection.
4. A negative molecular result does not rule out the presence of an acute infection.
5. A high anti-HEV IgM value (serological index ≥ 10) serves as an indicator of the potential presence of HEV RNA in the tested sample.
6. HEV genotype 3, which is typical for Europe, circulates in Bulgaria. The most prevalent subtypes identified are 3e (48%), 3f (24%), and 3c (12%).
7. One subtype, which remains unclassified to date, has been identified.
8. There is significant heterogeneity among the viral variants circulating in Bulgaria.

- VII. Scientific and Applied Contributions

Declaration of Originality

From Chiydem Baarieva Ismailova……..

In connection with the procedure for the
DEFENSE OF A DISSERTATION FOR THE ACQUISITION OF THE EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR" (PhD)
at the National Center of Infectious and Parasitic Diseases, I hereby declare:

1. The results and contributions in the scientific works presented by me are original and have not been borrowed from research and publications in which I have no participation.
2. The information contained in the documents, references, and publications required for conducting the procedure is accurate and corresponds to the objective truth.

Original Contributions

1. A correlation has been established between the serological index (anti-HEV IgM value) and the molecular marker (HEV RNA), providing a foundation for validating a diagnostic algorithm to confirm acute HEV infection.
2. Genetic heterogeneity of the studied HEV isolates in Bulgaria has been demonstrated, serving as a basis for further phylogenetic studies on the viral variants circulating in the country.
3. Unidentified viral variants have been detected, which establishes a prerequisite for further genetic investigations.
4. Our study further confirms that demographic and behavioral factors—including sex, age, occupation, and specific risk factors—play a significant role in HEV transmission. Identifying these high-risk groups is key to enhancing prevention and public health surveillance within the country

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IX. Publications Related to the Thesis

1. **Ch. Ismailova**, A. Stoyanova, V. Yoncheva, T. Tenev, L. Nikolaeva Glomb, E. Golkocheva-Markova. Phylogenetic analysis of twelve Bulgarian sequences based on partial open reading frame 2 genome fragment of hepatitis E virus. Problems of Infectious and Parasitic Diseases. 2024;52(1):5-12. doi:10.58395/y45wev44 **Q4**
2. **Исмаилова Ч.,** Голкочева-Маркова Е., Тенев Т., Л. Николаева-Гломб. "Хепатит Е вирусна инфекция. Биологична характеристика и молекулярна епидемиология", МедИнфо. 2021, 3: 82-87.
3. Elitsa Golkocheva-Markova, **Chiydem Ismailova**, Tencho Tenev, Lubomira Nikolaeva-Glomb „ "Studies on the epidemiological and molecular characteristics of the hepatitis E virus in Bulgaria: a comprehensive review: HEV in Bulgaria" Problems of Infectious and Parasitic Diseases. 2021; 49(3):27-34. doi:10.58395/pipd.v49i3.75 **Q4**
4. Komitova R, Kevorkyan A, Golkocheva-Markova E, Atanasova M, Rangelova V, Raycheva R, **Ismailova C**, Stoyanova A, Tenev T. Clinical and virological profile of locally acquired acute hepatitis E in South Bulgaria. The Journal of Infection in Developing Countries, 2024; 18:136–144. https://doi:10.3855/jidc.18341, SJR (2022) 0.47, IF (2022) 1.9 **Q3**
5. Elitsa Golkocheva-Markova, Ani Kevorkyan, Ralitsa Raycheva, **Chiydem Ismailova**, Viliana Yoncheva, Tencho Tenev, Radoslava Emilova, Lyubomira Grigorova, Ivan Baltadzhiev, Radka Komitova. Assessment of hepatitis E seropositivity among HIV-infected patients in Bulgaria. The Brazilian Journal of infectious diseases, 2022;26(1):102329; https://doi.org/10.1016/j.bjid.2022.102329 ; IF (2021) 3.257, , SJR 0.67 **Q3**

X. Participation in International and National Scientific Forums Related to the Doctoral Dissertation

1. **Ismailova, Ch**., Tenev, T., Genova-Kalu, P., Golkocheva-Markova, E. "Prevalence of hepatitis E virus antibodies among veterinarians." Online International Scientific Conference "Traditions and Modernity in Veterinary Medicine", Faculty of Veterinary Medicine, University of Forestry, April 16–18, 2021. [Poster]
2. Yontcheva, V., **Ismailova, Ch**., Tenev, T., Golkocheva-Markova, E. "Animal models for studying hepatitis E virus infection." XII Workshop with International Participation "Experimental Models and Methods in Biomedical Research", Sofia, Bulgarian Academy of Sciences (BAS), June 9–11, 2021. [Poster]
3. **Ismailova, Ch**., Yontcheva, V., Tenev, T., Golkocheva-Markova, E. "Replication of hepatitis E virus in cell cultures." XII Workshop with International Participation "Experimental Models and Methods in Biomedical Research", Sofia, Bulgarian Academy of Sciences (BAS), June 9–11, 2021.
4. **Ismailova, Ch**., Yontcheva, V., Tenev, T., Golkocheva-Markova, E. "Hepatitis E virus co-infection in patients infected with other hepatitis viruses." XIX National Congress of Microbiology and Infections of the BAM, September 14–16, 2021. [Poster]
5. **Ismailova, Ch**., Yontcheva, V., Tenev, T., Golkocheva-Markova, E. "Impact of the COVID-19 pandemic in 2020 on testing for viral hepatitis." VIII National Conference "The Effect of the COVID-19 Pandemic on HIV/AIDS and Other Infectious Diseases", Medical University of Plovdiv, September 16–19, 2021, Tsigov Chark, Bulgaria. [Poster]
6. **Ch. Ismailova**, V. Yontcheva, T. Tenev, A. Stoyanova, E. Golkocheva-Markova: Determination of the molecular-epidemiology characteristics of the hepatitis E virus by Sanger sequencing technique. International Scientific Conference Kliment’s Days 2022, 4 May 2022, Sofia, Bulgaria
7. **Ismailova, Ch.,** Yontcheva, V., Tenev, T., Kevorkyan, A., Komitova, R., Kostadinova, T., Tsekov, V., Chardakova, Ts., Sariyan, S., Golkocheva-Markova, E. "Hepatitis E virus markers and other hepatitis co-infections in hemodialysis patients." *Jubilee XX National Congress of Clinical Microbiology and Infectiology of the BAM*, September 16–18, 2022, Plovdiv, Bulgaria.

8. Yontcheva, V., **Ismailova, Ch**., Tenev, T., Golkocheva-Markova, E. "Evaluation of the prevalence of hepatitis E antibodies in pooled plasmas – a preliminary study and literature review." Jubilee XX National Congress of Clinical Microbiology and Infectiology of the BAM, September 16–18, 2022, Plovdiv, Bulgaria
9. Kotsev, S., Pishmisheva-Peleva, M., Stoeva, V., Golkocheva-Markova, E., **Ismailova, Ch.**, Naseva, E., Vateva, N. "Prevalence of hepatitis E virus infection among blood donors from Pazardzhik region." *Jubilee National Scientific Conference "HIV and Exotic Parasitic and Infectious Diseases in the Post-Pandemic Period of COVID-19"*, May 25–28, 2023, Tsigov Chark, Bulgaria.
10. **Ismailova, Ch**., Stoyanova, A., Yontcheva, V., Tenev, T., Kotsev, St., Pishmisheva-Peleva, M., Komitova, R., Nikolaeva-Glomb, L., Golkocheva-Markova, E. „Detection and diversity of Hepatitis E Virus genotype 3 in patients with Acute Viral Hepatitis E from Bulgaria“ за V. BALKAN AGRICULTURAL CONGRESS, September 20-23, 2023, Edirne, Turkey"
11. Golkocheva-Markova, E., **Ismailova, Ch**., Tenev, T., Nikolaeva-Glomb, L.. Evolution of the hepatitis E signal-to-Cutoff positive score among patients with acute viral hepatitis. P063. 23rd Annual Conference of the European Society for Clinical Virology (ESCV 2021), September 15-17, 2021, virtual.
12. **Ismailova, Ch**., Stefanova, R., Tenev, T., Miteva, P., Krumova, S., Golkocheva-Markova, E.: Seroprevalence of antibodies against hepatitis E virus and Parvovirus B19 among hematology pediatric patients. International Scientific Conference 40th Annual Meeting of the ESPID. 9-13 May 2022, Athens & online"

XI. Projects Related to the Dissertation

1. „Project „Studies on the genotypic diversity and the involvement of the polyproline region in the in vitro adaptation of Hepatitis E virus", funded by the National Science Fund of Bulgaria" (Grant No KP-06-N33/2)
2. „Improving National Diagnostic Capabilities for the Detection of Hepatitis E Virus in Pigs and Pork Products" (BUL5017), funded by the International Atomic Energy Agency (IAEA)
3. Project BG05M2OP001-1.002-0001 'Fundamental, translational and clinical research in the field of infections and infectious immunology', funded by OP 'Science and Education for Smart Growth 2014-2020' and the European Regional Development Fund.

XII. Appendix

Appendix 1. Interpretation of markers of HEV infection.

Duration of HEV infection	Positive markers
Current ACUTE	• HEV RNA • HEV RNA + anti-HEV IgM • HEV RNA + anti-HEV IgG • HEV RNA + anti-HEV IgM + anti-HEV IgG • Anti-HEV IgM + anti-HEV IgG (rising values) • Fecal HEV antigen
Current CHRONIC	• HEV RNA (± anti-HEV) ≥ 3 months • Fecal HEV antigen
Past infection	• Anti-HEV IgG

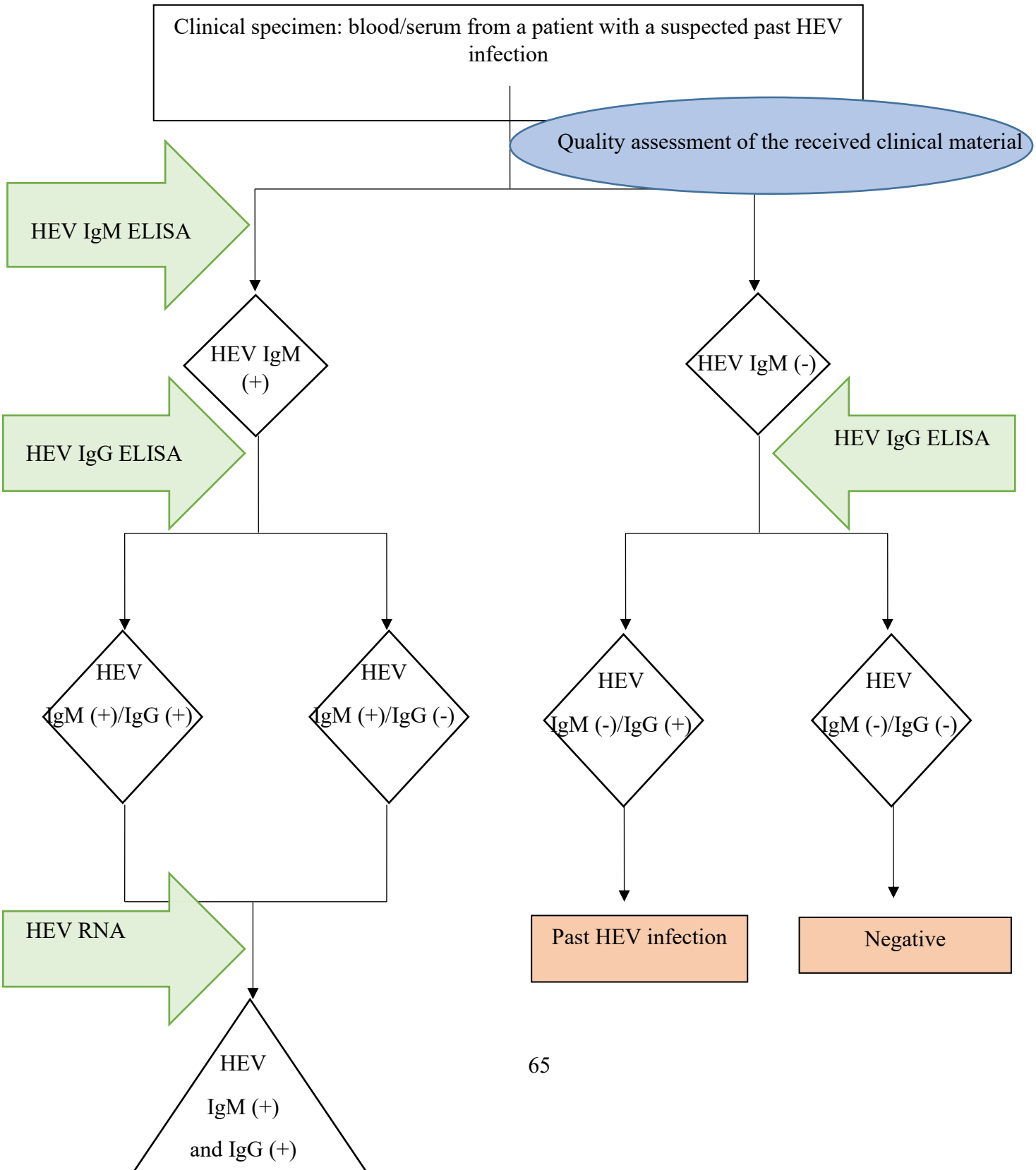
Appendix 2. Healthcare institutions in the country from which the samples included in the doctoral dissertation were obtained.

- SBALPFZ EOOD, Sofia; UMBAL "Alexandrovska" EAD, Sofia; UMBAL "Tsaritsa Yoanna - ISUL" EAD, Sofia; UMBAL "St. Anna" AD, Sofia; MBAL "Knyaginya Klementina" EAD, Sofia; UMBALSMP "N. I. Pirogov" EAD, Sofia; MBAL NKB EAD, Sofia; SBAL for Children's Diseases “Prof. Ivan Mitev”, Sofia; UMBAL ACIBADEM CITY CLINIC EOOD, Sofia; Second MBAL Sofia EAD, Sofia; SBALIPB “Prof. Ivan Kirov” EAD Sofia; MBAL "Dr. Stamen Iliev" AD Montana; UMBAL Lozenets EAD, Sofia; MBAL "Dr. Bratan Shukerov" AD Smolyan; UMBAL Burgas AD, Burgas; MBAL Dobrich AD, Dobrich; UMBAL "Kanev" AD Ruse; UMBAL "St. Marina" Pleven Varna; UMBAL "Dr. Georgi Stranski" EAD, Pleven; MBAL "Dr. Tota Venkova" AD, Gabrovo; MBAL "Dr. Asen Velev" EOOD, Razlog; MBAL Shumen AD, Shumen; MBAL "St. Ivan Rilski" EOOD, Dupnitsa; MBAL Targovishte AD, Targovishte; SBALBB Pernik EOOD, Pernik; MBAL Silistra AD, Silistra; MBAL "St. Ivan Rilski – 2003" OOD, DUPNITSA; MBAL

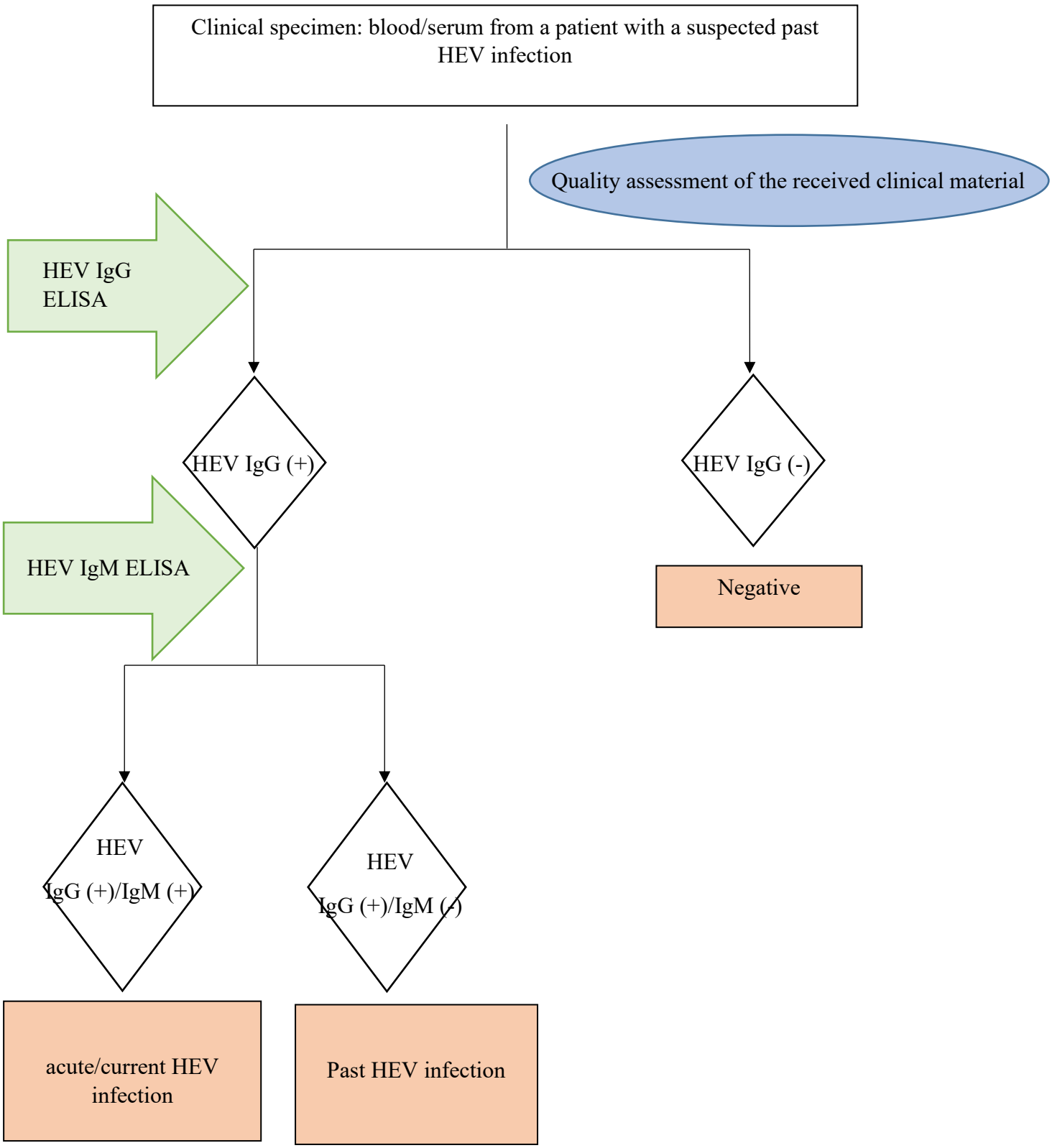
Blagoevgrad AD, Blagoevgrad; UMBAL "St. George" EAD, Plovdiv; MBAL "Rahila Angelova" AD, Pernik; MBAL Samokov EOOD; MBAL Pazardzhik AD, Pazardzhik; MBAL "St. Panteleimon" AD Yambol.

Appendix 3. Diagnostic algorithms for HEV infection (EASL, 2018).

A) Algorithm for laboratory confirmation of acute/current HEV infection



Б) Algorithm for laboratory confirmation of past HEV infection



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