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Research Article

Epidemiological and Epizootic Intensity of HEV-Infection in Belarus

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Abstract

Hepatitis E is a viral disease, which has a faecal-oral transmission, a tendency to epidemic spread, is characterized by a predominant liver damage and has a particularly severe course in pregnant women. The aim of this study was determination of the presence and intensity of HEV infection in Belarus, identification and description of clinical cases, isolation of the virus and detection of HEV genotypes, which can circulate on the territory of the country. The material for the study was serum samples from citizens of Belarus, as well as temporarily foreign residents in Belarus, individuals from high-risk groups and patients with acute and chronic hepatitis. Serum samples from domestic pigs, wild boars, deer and rabbits were also analysed to assess the intensity of the epizootic process. Serum samples were tested for anti-HEV IgM and IgG using ELISA, blood and faces samples were analysed in nested RT-PCR for HEV RNA. The prevalence of anti-HEV IgG among the examined citizens of Belarus was 8.4%, 11.8% in blood donors, 6.6% in pregnant women, and 5.2% in foreign residents, among patients with viral pathology of the liver - 15.3%, including patients after orthotropic liver transplantation - 9.1%. Patients with icteric form of acute hepatitis E, including pregnant women and HIV-infected patients, as well as liver recipients have been identified. All cases of hepatitis E in the Republic of Belarus were associated with HEV genotype 3. Pprevalence of anti-HEV IgG among the examined pigs was 33.8%, wild boars - 35.6%, deer - 100%, and rabbits - 20.2%. HEV RNA was detected in 26% faces samples from pigs, 21.2% from rabbits, 3.7% from wild boars and was not detected in faces samples of deer. The circulation of HEV genotype 3 in pigs and genotype 3r in rabbits was demonstrated.

Keywords: *Hepatitis E; ELISA; RT-PCR; Phylogenetic Analysis; Epidemiology; Prevalence anti-HEV IgM and IgG*

Abbreviations

HEV: Hepatitis E; Anti-HEV IgM(G): Immunoglobulins M(G), antibodies against hepatitis E; RT-PCR: Revers-Transcription Polymerase Chain Reaction; ORF: Open Reading Frame

Introduction

Hepatitis E virus (HEV) is a major cause of viral hepatitis. HEV is distributed worldwide and has been responsible for outbreaks in developing countries and sporadic cases in both developing and developed countries [1]. It is estimated that there are 20 million hepatitis E infections worldwide every year, leading to 3.3 million symptomatic cases and more than 56.000 deaths [2].

HEV is highly dangerous for pregnant women, who are at risk of serious illness and mortality. In the third trimester of pregnancy, mortality rates can be as high as 10 - 30% [3].

Nowadays 8 genotypes of HEV have been identified. HEV genotypes 1 and 2 are only found in humans and account for most of the hepatitis E cases in the developing world where transmission occurs by the faecal-oral route via contaminated waterways. HEV genotypes 3 and 4 are found in humans, pigs, wild boars, deer, rabbits, mongoose and other animals across the world [4]. Genotypes 5 and 6 were identified in Japanese wild boars and cluster together with HEV4 isolates on phylogenetic analysis [5]. HEV7 and HEV8 were identified in dromedary and Bactrian camels, respectively [6].

For a long time, it was believed that HEV was a travel-associated disease, endemic in developing countries with poor hygienic standards and unsafe water supply. However, in recent years, an increasing number of sporadic cases, and occasional small foodborne outbreaks related to autochthonous hepatitis E, has been reported from non-endemic regions. Several observations suggest that autochthonous cases in these areas are caused by zoonotic spread of infection from wild or domestic animals [7]. Autochthonous cases of hepatitis E are associated with 3 and 4 genotypes of HEV in developed countries. A recent meta-analysis identified 73 studies of HEV seroprevalence in Europe; estimates of seroprevalence ranged from 0.6% to 52.5%, with rates increasing with age but unrelated to gender. In the USA, seroprevalence for anti-HEV is around 6%, in the UK 3 - 16% and in some regions of France up to 52% [8].

HEV genotype 1 is a common cause of acute hepatitis in Asian countries, whereas genotype 2 is prevalent in Central America, Mexico and Africa. Studies of anti-HEV IgG seroprevalence in endemic regions showed antibodies rate in India 15 - 73%, 46,3% in Iran, 23,5% in China, 42% in Zambia, 19,1% South Africa, 67,6% in Egypt, 36,3% in Mexico [9].

Hepatitis E was previously considered to be a cause of acute hepatitis with no progression to chronic carriage. However, this perception changed with the publication of a case series of 14 solid organ transplant recipients with acute autochthonous HEV3 infection in southern France [10]. In the immunocompromised patients HEV is often difficult to clear, and about 60% of these patients go on to develop chronic HEV infection. Chronic HEV infection may lead to complications such as liver cirrhosis [8,10].

Hepatitis E mostly has no clinical features, but if has, symptoms are nonspecific and include myalgia, jaundice, fatigue, nausea, anorexia. Laboratory tests show high levels of serum bilirubin and liver enzymes. The mean incubation period is about 40 days. Most acute infections resolve spontaneously, with symptoms disappearing within 4 - 6 weeks. However, acute HEV infection can cause acute liver failure, most commonly in pregnant women [8,9]. Clinical manifestations of hepatitis E are similar to hepatitis A, as well as to other acute viral hepatitis. This fact creates certain difficulties with correct diagnosis of sporadic HEV cases, although experienced physicians can differentiate HEV by a combination of small signs.

Diagnosis of HEV in Belarus was not carried out until this study.

The aim of this study was determination of the presence and intensity of HEV infection in Belarus, identification and description of clinical cases, isolation of the virus and revelation of HEV genotypes, which can circulate in the country.

We determined the prevalence of anti-HEV among the relatively healthy population of Belarus, pregnant women, blood donors, HIV-infected patients, hunters, as well as among foreign residents from high-endemic regions of HEV.

The prevalence of HEV markers was assessed among animals, the alleged sources of infection: domestic pigs, wild boars, deer and rabbits, to determine the intensity of the epizootic process in Belarus. Moreover, the description of several clinical cases of hepatitis E was also presented.

Materials and Methods

Serum samples were collected in 2015 to 2020 from 415 healthy citizens of Belarus (mean age 41.5), 144 blood donors with high ALT levels, 227 pregnant women with the symptoms of liver injury, 1457 foreign residents from 41 countries (mean age 23.3), 306 HIV-infected patients, and 17 hunters, who have direct contact with blood and meat of wild boars. In addition, serum samples from 315 patients with acute and chronic viral pathology of the liver were analysed.

Animal serum samples were obtained from 1126 domestic pigs, 101 wild boars, 28 deer and 124 rabbits.

Faecal samples were collected in 2015 - 2019 from 95 patients, 77 domestic pigs, 27 wild boars, 40 deer and 359 rabbits.

Detection of anti-HEV in serum specimens: Human serum specimens were detected for anti-HEV IgM and IgG by using a commercial enzyme-linked immunosorbent assay (RPC Diagnostic Systems, Russia) according to the manufacturer's instructions. Animal serum samples were detected for anti-HEV IgG by using the same ELIZA kit with species-specific conjugates.

RNA extraction and reverse transcription-nested PCR: Total RNA was extracted from 50 **µL** of serum samples or 10% faecal supernatants using the Total RNA Purification Kit (Jena Bioscience, Germany). To detect all four known HEV genotypes a set of universal nested primers was designed to amplify the partial fragment in the ORF2 (6023-6295 nt) of the HEV genome. External primers include: P1 (forward 5′- aay tat gcm cag tac cgg gttg-3′) and P2 (reverse 5′- ccc tta tcc tgc tga gca ttctc-3′) and internal: P3 (forward 5′- gty atg yty tgc ata cat ggct-3′) and P4 (reverse 5′- agc cga cga aat yaa ttc tgt c-3′). The protocol was as follows: 42°C for 60 minutes, 94°C for 5 minutes, followed by 94°C for 30s, 45°C for 30s, and 72°C for 45s, all of them were repeated during 35 cycles. Negative and positive controls were included in all assays. RNA extraction and other pre-PCR amplification steps were performed in a separate clean room to avoid cross-contamination. The final amplicons were analysed by 2% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.

Sequencing and phylogenetic analysis: The target second-round PCR products were purified with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Then, purified PCR products were double-end sequenced by means of the automatic DNA sequencer (3500 Genetic Analyzer), using a BigDye Terminator v 3.1 Cycle Sequencing Kit.

The evolutionary history was inferred by the Maximum Likelihood Method and Tamura-Nei Model. The tree with the highest log likelihood (-6255.80) is shown. The percentage of trees in which the associated taxa clustered together is observed next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with a superior log likelihood value. The tree is drawn to the scale, with the branch lengths measured in the number of substitutions per site. The analysis involved 75 nucleotide sequences. Included codon positions were 1^{st}+2^{nd}+3^{rd}+Noncoding. Totally there were 274 positions in the final dataset. One thousand data re-samplings were used to calculate the percentage of trees containing each branch. Bootstrap values less than 70% were regarded as not providing evidence of phylogenetic grouping. All reference sequences representing different HEV genotypes were retrieved from GenBank.

The obtained results were processed using statistical variance methods.

Results and Discussion

Assessment of 415 serum samples of healthy Belarusian citizens with a median age of 41.5 ± 27.79 revealed the presence of anti-HEV IgG in 8.4% specimens (95% CI, 5.87 - 11.37). The rate of IgG in the age group under 25 years was 4.3% (95% CI 0.11 - 11.73; 1/23), while in the age group over 25 years - 7.9% (95% CI 5.37 - 11.23; 31/392). These data confirmed the circulation of HEV in Belarus with the increase of anti-HEV positive Belarusians in the older age group.

Anti-HEV IgG was detected in 5.2% (95% CI 4.11 - 6.53) serum samples of 1457 foreign residents with a median age of 23.3 ± 7.79. Detection of anti-HEV varied depending on the geographical origin of the foreign citizens with the highest detection rate in the Indians and Turkmen (Figure 1).

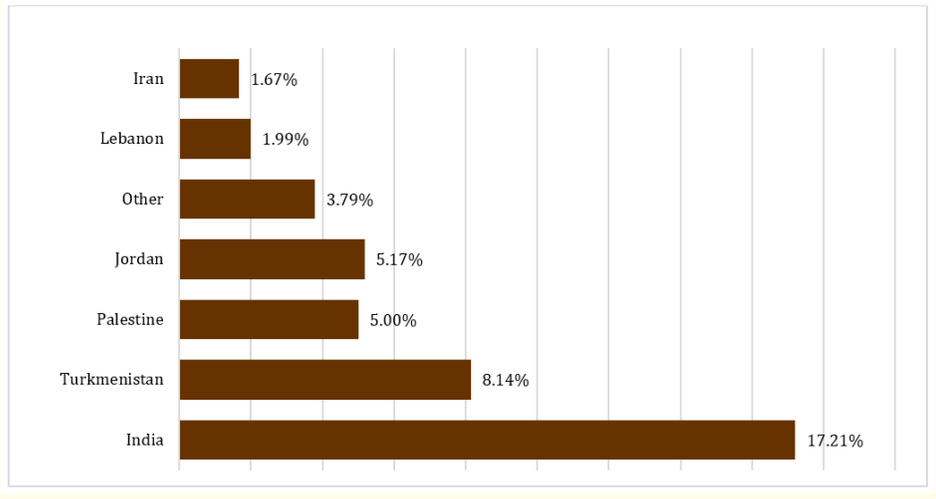

Figure 1: *Frequency of anti-HEV IgG detection among the citizens of other countries residing in the Republic of Belarus.*

Fifteen (1.0%) foreign citizens (95% CI 0.58 - 1.70) with IgM anti-HEV also had symptoms (other than jaundice) of acute hepatitis E including hepatomegaly and the increased level of ALT. The highest quantity of IgM was detected in the citizens of Turkmenistan, India and Jordan (Figure 2).

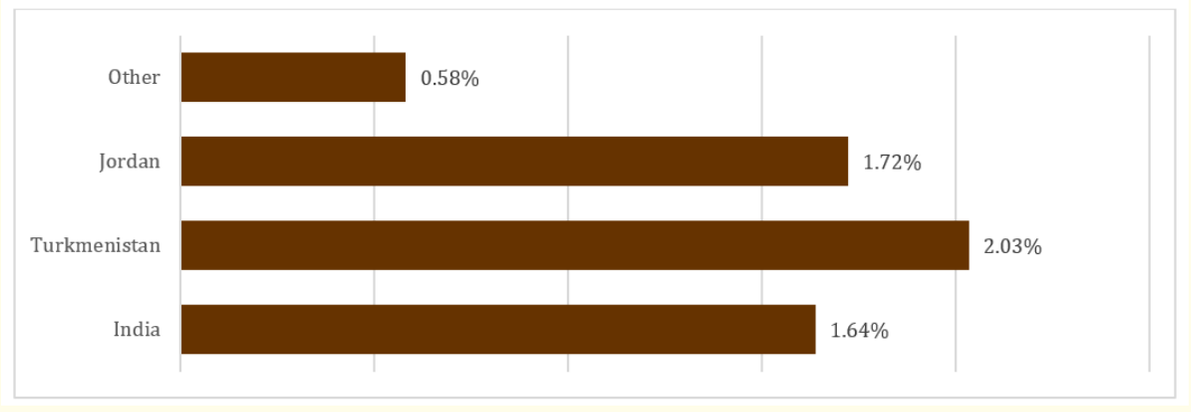

Figure 2: *Frequency of anti-HEV IgG detection among the citizens of other countries residing in the Republic of Belarus.*

A case of acute HEV infection was revealed in a Nigerian student, who lived in a dormitory with other foreign students and did not leave Belarus for 2 years. This fact confirms the possibility of imported HEV cases in Belarus from the endemic regions.

The difference between the anti-HEV prevalence rates in the analysed groups did not reach the level of significance, however, there was a tendency of anti-HEV prevalence in the individuals from the high-risk groups. Anti-HEV IgM was detected both in patients with liver damage symptoms and those who arrived from the endemic regions.

Overall, 17/144 (11.8%; 95% CI 6.88 - 18.90) serum samples from blood donors with the increased level of ALT were positive for IgG anti-HEV and 6/144 (4.2%; 95% CI 1.53 - 9.07) for IgM anti-HEV. Since the presence of HEV RNA was not examined in our study, we cannot draw a conclusion from the obtained data regarding active HEV prevalence in the donors' blood and the risk of HEV transmission via blood transfusion.

Anti-HEV IgG antibodies were revealed in 15 out of 227 pregnant women with clinical and laboratory symptoms of liver damage (6.6%; 95% CI 3.70 - 10.90), 6 women (2.6 6%; 95% CI 0.97 - 5.75) had anti-HEV IgM circulation. Among patients with acute hepatitis 6.3% (95% CI 2.74 - 12.51) were positive for anti-HEV IgG and IgM. Patients with chronic hepatitis and those after orthotropic liver transplantation tested for anti-HEV IgG were positive in 15.3% (95% CI 10.28 - 22.04) and 9.1% (95% CI 1.87 - 26.57) respectively. The rate of anti-HEV IgG and IgM in different groups is shown in figure 3.

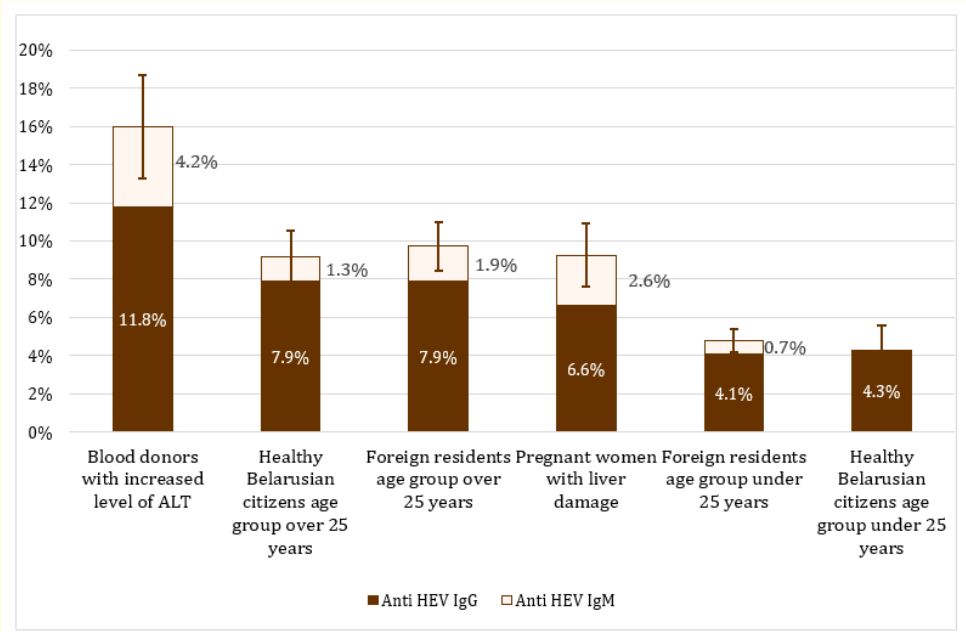

Figure 3: *Frequency of anti-HEV IgG and IgG detection in different cohorts.*

Totally, 17 hunters were involved in the study. 100% of them were males. All of them consumed wild boar meat. 8 individuals (47.1%; 95% CI 2.32 - 92.72) were positive for anti-HEV IgG. No HEV RNA was detected in their serum specimens. A medical history revealed episodes of transient ALT increment and hepatomegaly with the normal level of serum bilirubin.

In the group of HIV-infected patients 4.6% (95% CI 2.50 - 7.68) were positive for anti-HEV (14/306). All patients had an increased ALT level and hepatomegaly. Furthermore, they all (apart from one individual) did not leave the country during the last year.

The prevalence of anti-HEV among domestic pigs from 90 farms was 33.8% (95% CI 30.44 - 37.32; 380/1126). Twenty samples of pig faeces out of 77 (26.0%; 95% CI 18.87 - 40.11) were positive for HEV RNA. All HEV isolates belonged to genotype 3.

To investigate HEV distribution in the wild boars aged 1 year and older 101 serum samples were tested for HEV-specific antibodies, including 27 faecal specimens for HEV RNA. A total of 36 out of 101 serum samples (35.6%; 95% CI 24.96 - 49.35) from wild boars were positive for anti-HEV-IgG and 1 out of 27 faecal specimens (3.7%; 95 CI 0.09 - 20.64) for HEV RNA.

Analysis of biological material taken from 28 deer demonstrated the presence of anti-HEV-IgG in all samples; however, HEV RNA was not detected in the samples of blood and faeces.

The circulation of anti-HEV-IgG in rabbits in the regions of Belarus was 20.2% (95% CI 13.05 - 29.76; 25/124). 21.2% (95% CI 16.68 - 26.50; 6/359) excrement samples of rabbits from the Belarusian regions were positive for HEV RNA. The HEV strain nucleotide sequences, circulating among rabbits in Belarus, are closest to HEV genotype 3.

Clinical case analysis

Acute icteric HEV clinical cases analysis revealed that the median age of the infected patients was 63 (25% - 75% - 40 - 66 years) including 4 males and 2 females. All patients had general malaise, jaundice, dark urine, clay-coloured stool. Four individuals had loss of appetite, abdominal pain, three - nausea, one - joint pain.

Concomitant pathology: A history of myocardial infarction (2/6), arterial hypertension (2/6), type II diabetes mellitus (1/6), hepatitis B (2/6), hepatitis A (1/6), HIV (1/6), anaemia (1/6).

All patients were asked to complete an epidemiological questionnaire. Four patients were abroad prior to the HEV-infection clinical symptoms manifestation (Germany, Lithuania, Uzbekistan, Russia, Thailand and Spain); three of them ate undercooked pork, venison and animal liver.

The dynamics of biochemical parameters of patients with an acute icteric form of HEV is presented in table 1.

Biochemical parameter	Median (25% - 75%)	Median of maximum (25% - 75%)	Median after treatment (25% - 75%)	Change in average during treatment
Total bilirubin (μM/l)	110.43 (55 - 144)	164.06 (144 - 229)	43.98 (39 - 52)	120.09
Direct bilirubin (μM/l)	67.38 (24 - 94)	109.22 (78 - 160)	14.90 (10 - 18)	94.32
AST (U/l)	504.20 (65 - 821)	1239.50 (821 - 2912)	41.68 (27 - 65)	1197.82
ALT (U/l)	756.00 (178 - 1422)	1856.00 (1007 - 2394)	105.20 (56 - 171)	1750.80
Alkaline phosphatase (U/l)	248.40 (174 - 418)	403.50 (280 - 546)	149.50 (140 - 248)	254.00
GGT (U/l)	256.70 (100 - 395)	398.50 (222 - 704)	92.00 (63 - 191)	306.50

Table 1: *Biochemical parameters of patients with acute icteric HEV infection.*

Clinical case #1

Patient S., 66 years old, became acutely ill. The disease has begun from a feeling malaise, nausea, abdominal pain, fever. Later dark urine and growing jaundice appeared. The patient stated staying for 1 month from the onset of the disease in Mallorca, as well as eating venison raw sausage home-made produced in the Republic of Belarus. He had a history of myocardial infarction, stenting of coronary vessels, continuous use of statins and antiplatelet agents.

An objective examination revealed hepatomegaly. In a laboratory study at the peak of the disease an increase in total bilirubin to 110 μM/l, ALT to 1200 U/l, AST to 820 U/l, GGTP to 700 U/l was noted. Dynamics of changes in blood biochemical parameters for the disease period is presented in figure 4.

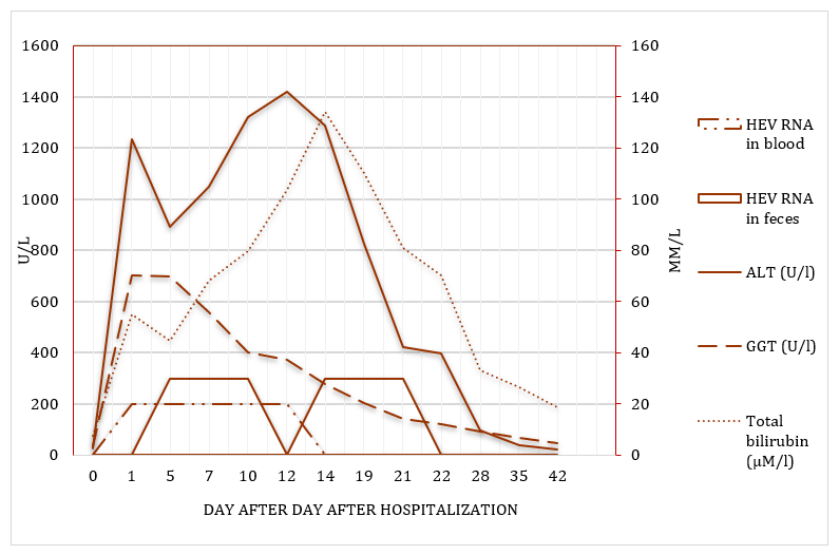

Figure 4: *Dynamics of biochemical parameters and RNA of HEV in patient S.*

Anti-HEV IgM was revealed in blood serum. PCR detected HEV RNA in serum and feces. During virus RNA sequencing the 3f genotype was defined, which is close to that detected in Western Europe (Figure 5).

Clinical case #2

Patient K., 65 years old, complained of skin and sclera jaundice, nausea, moderate skin itching and severe unexplained weakness for 14 days. He had a history of frequent pork liver eating. Objectively: the skin is icteric, no traces of scratching, no rash. Body temperature is 36.8°C. The low border liver is 3 cm below of the right costal arch. The spleen is normal. Feces is acholic.

Biochemical blood analysis data: ALT - 756 U/l, AST - 755.7 U/l, total bilirubin - 326.5 μM/l, bound bilirubin - 159.5 μM/l, GGTP - 533.9 U/l.

Autoimmune hepatitis, hepatitis B and C, yersiniosis, and HIV infection were excluded. Serum markers of autoimmune diseases (ANA, AMA-M2, anti-SLA) were negative, metabolic liver diseases were also excluded.

he ELISA blood serum examination repeated three times, starting from the day of admission, revealed anti-HEV IgM with a gradual decrease in the optical density. Anti-HEV IgG was revealed from the moment of admission. A control study of blood serum 1 month after discharge showed the absence of anti-HEV IgM and retention of IgG class antibodies. In the biochemical blood analysis elevated levels of GGTP, ALT, AST were preserved, the level of bilirubin returned to the norm.

Analysis of the genomic sequence of HEV RNA isolated from the patient demonstrated the virus belonging to genotype 3 and the fact of its similarity to HEV RNA sequences isolated from domestic pigs in the Republic of Belarus, which indicates the probable zooanthroponous nature of the autochthonous HEV in the region. The genetic sequence of HEV RNA genotype 3, isolated in Belarus from patients and domestic pigs, differs from that of virus strains isolated and identified in the Belgorod region, Russian Federation (Figure 5).

The phylogenetic analysis did not reveal any relationship between the HEV of rabbits and verified cases of hepatitis E in Belarus (See figure 5).

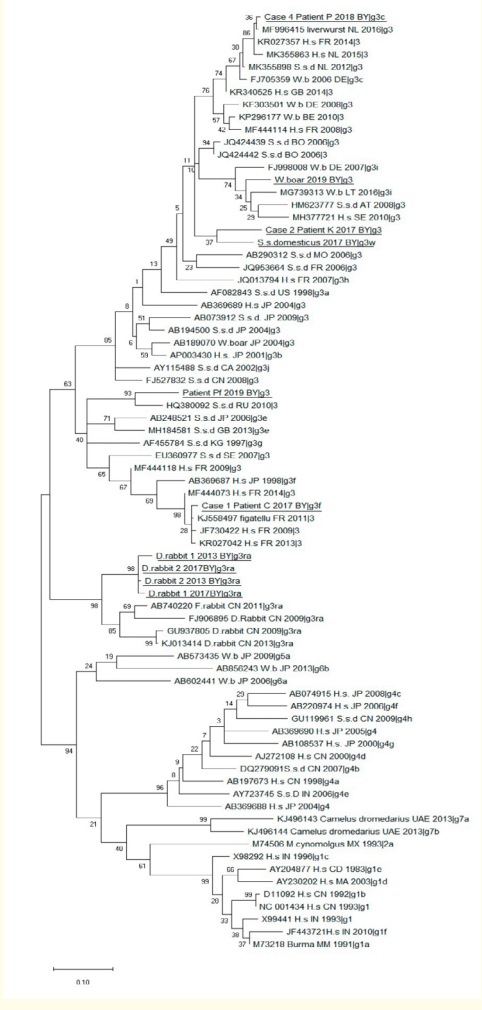

Figure 5: *Phylogenetic tree for the partial sequence of the open reading frame 2 (ORF2) of the hepatitis E virus genome (273 nucleotides, positions 6023–6295, numbering according to the prototype isolate Burma – M73218) The numbers indicate the reliability indicator. On the phylogenetic tree the number in the GenBank database, name of the host species, country and year of isolation, genotype and subgenotype (HEV) are indicated for each isolate. The host organism Ssd is a domestic pig, D. rabbit is a rabbit, H.s is a man, W.b is a wild boar. The names of the sequences isolated on the territory of the Republic of Belarus are underlined.*

Clinical case # 3

Patient D., 34 years old, complaining of severe skin itching and aching pain in the lower abdomen, was hospitalized to the Department of Pregnancy Pathology with the diagnosis "Pregnancy, 246 days. Premature birth threat. Intrahepatic cholestasis of pregnant women. Hepatomegaly". The pregnancy was planned, pregravid preparation was carried out in full volume. The woman had a history of 2 urgent deliveries and 2 medical abortions. During pregnancy, she suffered an acute respiratory viral infection at 30 weeks, starting from 32 weeks there was severe skin itching, worse at night.

In the epidemiological history it was stated that patient D works in the construction industry. She bought pork at a local farm, cooked raw smoked and blood sausages at home. Over the past year, she has not travelled outside the Republic of Belarus. Donation, parenteral manipulation denies. There was no contact with febrile, icteric patients. Her husband and 2 children are healthy. The findings of patient D laboratory assessment in the dynamics are presented in figure 6.

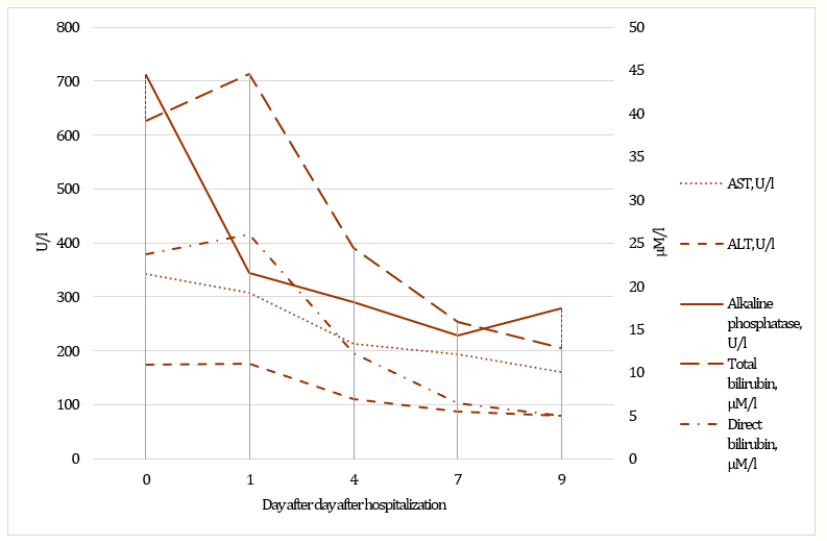

Figure 6: *Dynamics of biochemical blood parameters of patient D.*

Ultrasound of the abdominal cavity revealed moderate hepatosplenomegaly. Markers of viral hepatitis B and C are negative. Anti-HEV IgM and IgG were detected in the blood serum of patient D. on the first day of hospitalization and within 2 months of follow-up, a positive anti-HEV IgG result and a negative anti-HEV IgM result were revealed a year later.

One day after admission, antenatal fetal death occurred. A dead male fetus was born with the body weight of 2990 g and the length of 51 cm.

Clinical case # 4

Patient P., 28 years old. Complaints on admission to the hospital were as follows: general malaise, nausea, discomfort in the right hypochondrium, dark urine, icteric sclera, weight loss.

From the anamnestic data it was found that the patient practices sex with men. From 02/05/2017 to 03/05/2017 he was in Thailand, lived in a bungalow. He drank bottled water, brushed his teeth with tap water. He had unprotected sexual contacts. Parenteral drug not uses. On admission: total bilirubin - 111.01 μM/l; direct - 64.09 μM/l; ALT - 3672 U/l; AST - 3812 U/l; platelets - 129 × 10^9/l.

When examined for markers of HAV, HBV, HEV, HCV and HIV, anti-hepatitis A IgM and anti-hepatitis E IgM+G were detected; anti-HIV, HIV RNA (10 385 copies/ml); $CD4^+$ - 561 cells/μl; CD8 -1051 cells/μl.

Ultrasound examination of the abdominal organs revealed hepatomegaly, diffuse changes in the liver parenchyma. A cyst of the liver left lobe in S_4. Splenomegaly (145 × 60 mm). The patient was diagnosed with acute viral hepatitis mixed A+E (anti-HAV IgM+, anti-HEV IgM+, IgG+); icteric variant, moderate course. Acute HIV infection without secondary diseases. The dynamics during the disease is positive, the patient was discharged in a satisfactory condition. Within 3 months there was a gradual seroconversion of anti-HEV IgM and an increase in anti-HEV IgG.

Conclusion

Thus, clinical cases suggest the presence of HEV circulation both in humans and animals, the existence of autochthonous cases of the disease and hepatitis E possibly imported from other countries to Belarus. An autochthonous case of HEV infection was associated with HEV genotype 3. Domestic and wild pigs and deer are a reservoir of HEV in the region. The possibility of the HEV genotype 3 infection identified in rabbits and transmission to humans needs further study. Significant frequency of anti-HEV IgG detection among various population groups of the Republic of Belarus and the tendency to its increase with age in the absence of indications of a history of icteric hepatitis indicate that erased anicteric forms of HEV prevail in the region.

Tests for identifying hepatitis E virus markers when examining patients with symptoms of liver pathology (primarily patients from the following groups: with the established diagnosis; the history of contact with animals and their raw materials; patients with liver pathology of unspecified etiology; blood and organ donors) seem to be feasible and useful for introducing them into clinical practice.

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Conflict of Interest

The authors declare no conflict of interest.

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