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Acute non-A, non-B, non-C hepatitis differences and similarities between hepatitis E virus infection and autoimmune hepatitis, with phylogenetic analysis of hepatitis E virus in humans and wild boars

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Abstract

Background Hepatitis E virus (HEV) infection incidence is increasing in Europe, accounting for the majority of acute hepatitis cases. We investigated the prevalence and clinical characteristics of acute hepatitis E (AHE) in patients with acute non-A/B/C hepatitis from central Greece, their differences from acute autoimmune hepatitis (AIH) patients and the molecular similarity of human strains to local HEV strains in wild boars.

Methods Sera from 20 patients with non-A/B/C acute hepatitis (2015-2017) were tested prospectively for anti-HEV IgM, IgG antibodies and HEV-RNA. Sera from patients diagnosed with acute AIH (2000-2015; n=56) were tested retrospectively. Liver tissue samples from 40 wild boars were tested for HEV-RNA. Positive wild boar and patients' samples were sequenced and phylogenetically analyzed.

Results Twelve of the 76 (16%) patients were diagnosed with AHE: HEV-RNA $11.5x10^4$ (38.7-$39.7x10^6$) IU/mL; 11/20 (55%) acute non-A/B/C hepatitis and 1/56 (2%) AIH patients. Patients with AHE were older than those without, predominately men, with higher alanine aminotransferase but lower IgG levels (P=0.005 and P=0.002, respectively), and had high titers of smooth muscle antibodies. Liver biopsies, performed in 6/12 patients with HEV infection, revealed histology compatible with AIH. HEV strains from both patients and wild boars belonged to genotype 3.

Conclusions Approximately one sixth of patients with acute non-A/B/C hepatitis had autochthonous HEV infection with AIH features. Therefore, a careful workup to exclude HEV should be carried out in all acute hepatitis cases before a definite diagnosis of AIH is established. Wild boars seem to be an important reservoir of HEV in Greece.

Keywords Hepatitis E virus, acute autoimmune hepatitis, genotyping, wild boars

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Introduction

Hepatitis E virus (HEV) infection has traditionally been regarded as the leading cause of acute hepatitis in adults in the developing world, bearing a significant morbidity and mortality burden [1]. However, over the past decades, HEV has been re-introduced in Europe, mainly as an endemic zoonotic disease [1-3], and it is now considered the most common cause of acute viral hepatitis in many European countries [3-5]. The European Center for Disease Control has estimated that endemic HEV accounts for approximately 2 million silent infections annually [4].

The HEV strains include 8 genotypes [6]: genotypes 1 and 2 infect only humans and are the most prevalent genotypes in low-income countries, transmitted through contaminated water; genotypes 3 and 4 infect several mammalian species and are more frequent in the developed countries, transmitted through contaminated food consumption, mainly undercooked porcine meat [1-5,7,8]. HEV genotypes are further divided

into subtypes, with HEV-3c, HEV-3f and HEV-3e being the commonest circulating subtypes in Europe [4].

Wild boars seem to be a significant reservoir of HEV in Europe (predominately HEV-3), since the seroprevalence in these animals ranges between 10% and 60% [8-11]. Therefore, zoonotic transmission from wild boars may be more common than anticipated.

HEV-3 infections usually run a subclinical course [4,5,12,13], in which 5% of the affected patients present with acute self-limited viral hepatitis [13-15]. However, chronic infection, with rapid progression and dismal prognosis, can occur in immunocompromised individuals [13,14,16].

On the other hand, hepatitis viruses have long been suggested as potential triggers for the development of autoimmune hepatitis (AIH), a chronic liver disease of unknown etiology [17-19]. In this context, a large German study [20] reported that the seroprevalence of HEV was higher in AIH patients than in blood donors and controls. However, a similar study from The Netherlands [21] failed to reproduce similar results. Furthermore, HEV infection has been misdiagnosed in many cases as AIH because of the detection of autoantibodies during its acute phase [22-24]. In fact, Terziroli Beretta-Piccoli *et al* [25] showed that half of 48 patients with acute HEV had at least one autoantibody.

Accordingly, the aim of the present study was to investigate the prevalence, clinical, laboratory and histological characteristics of acute HEV infection in patients with acute non-A/B/C hepatitis in central Greece and to compare the characteristics of patients with acute HEV infection with those who presented with the acute form of AIH. At the same time, we intended to determine the genotype of human strains and find their molecular similarity to strains from the local wild boar population.

Patients and methods

Patients

Seventy-six patients with acute non-A/B/C hepatitis, defined as the presence of transaminases above 10 times the upper limit of normal (ULN) with or without jaundice, were included in the study. The patients were divided in 2 groups: a) group 1 included 20 patients who presented in our department with acute non-A/B/C hepatitis between 8/2015 and 12/2017, studied prospectively; and b) group 2 included 56 patients who presented between 1/2000 and 7/2015, were analyzed retrospectively and had been diagnosed with acute AIH according to the established AIH criteria [26,27]. Before 2015, determinations of IgM antibodies against HEV (anti-HEV IgM) and HEV-RNA were not available in our hospital.

In all patients (n=76), other causes of acute hepatitis—namely other viral and non-viral infections (hepatitis A, B and C virus, Epstein-Barr, cytomegalovirus, herpes simplex virus, leptospirosis), alcoholic hepatitis, drug-induced liver injury, genetic diseases (Wilson's disease, hemochromatosis) and acute vascular disease—were excluded by appropriate testing.

Serum samples collected during the acute phase and stored at -80°C were tested for the presence of IgM antibodies against HEV (anti-HEV IgM) as well as for HEV-RNA either prospectively (group 1) or retrospectively (group 2).

All subjects agreed to the use of their data after anonymous analysis by written consent at the time of initial evaluation. The ethical committee of the General University Hospital of Larissa approved the study protocol, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki as revised in Brazil in 2013, as reflected in *a priori* approval by the institution's human research committee (2258/21-3-2016).

Anti-HEV IgM antibodies detection

The detection of anti-HEV IgM was performed using commercially available enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Wantai Diagnostics, China). The Wantai ELISA was chosen because of its proved higher sensitivity compared to other commercial ELISAs [12].

HEV-RNA quantification

HEV-RNA was purified from the patients' sera (stored at -80°C), using the QIAamp Viral RNA extraction kit (Qiagen, CA, USA). Viral RNA was transcribed into cDNA using a specific primer-mediated reverse transcription step followed immediately in the same tube by real-time polymerase chain reaction (RT-PCR) using the FTD Hepatitis E RNA kit (Fast Track Diagnostics, Luxembourg). The presence of HEV sequences in the reaction was detected by an increase in fluorescence observed from the relevant dual-labeled probes and was reported as a cycle threshold value by Lightcycler 96 Thermocycler (Roche, Basel, Switzerland). The cycling conditions were: 1 cycle at 50°C for 15 min and 94°C for 1 min, and 40 cycles at 94°C for 8 sec and 60°C for 1 min. The total volume of the reaction was 25 μL and each run included a negative control and 3 quantification standards—quantified according to the 1st World Health Organization International Standard for HEV-RNA (PEI code 6329/10). The quantitative results obtained by the standard curve were reported in International Units (IU/mL) for HEV and dilution factors were calculated as recommended for every experiment. The lower limit of detection of the reaction was determined at 188 IU/mL, with a linear detection range up to 188

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× 108 IU/mL. The assay uses *Streptoccocus equi* as an extraction control—the internal control—introduced into each sample at the lysis buffer stage of the extraction process according to the manufacturer's instructions.

HEV-RNA detection in wild boar liver tissue samples, HEV genotyping and phylogenic analysis, and autoantibody testing in human serum samples

HEV-RNA detection in liver tissue samples from wild boars and the primers that were used (Supplementary Table 1) as well as ethical approval, HEV genotyping and phylogenetic analysis, and autoantibody testing in human serum samples are shown in the Supplementary material.

Liver histology

Liver biopsy was available in 57/76 (75%) patients. In the remaining 19 patients, liver histology was not available, either because liver biopsy could not be performed because of the acute/severe mode of presentation, with significant coagulation impairment, or because patients refused the procedure. The histological evaluation was assessed using the Knodell histological/activity index score [28]. According to our previous publications [29-33] and for statistical reasons, patients were divided into 2 groups according to inflammation: minimal-mild (score: 0-8) and moderate-severe (score: 9-18); and according to fibrosis: none-mild-moderate (score: 0-2) and severe fibrosis-cirrhosis (score: 3-4). A detailed description (presence of interface hepatitis, lymphoplasmacytic infiltrate, hepatocellular rosette formation, emperipolesis, and lobular inflammatory activity) of the biopsies was included in the analysis.

Statistical analysis

Data were analyzed using SPSS 19 software. The Kolmogorov-Smirnov test was used to assess the normality of the distribution of variables. Normally distributed values are expressed as mean ± standard deviation (SD), and non-normally distributed as median (range). Data were analyzed using the *t*-test, Mann-Whitney *U* test, χ^2 (2x2 with Yate's correction), or Fisher's exact test, as applicable. Two-sided P-values <0.05 were considered statistically significant, and 95% confidence intervals (CI) were calculated by the Wilson procedure with a correction for continuity.

Results

Twelve of 76 (15.8%, 95%CI 8.7-26.3%) acute non-A/B/C hepatitis patients had acute HEV. None of the patients reported traveling abroad during the past 6-12 months, transfusion with blood products, occupational engagement with swine or consumption of raw meat. In 6 cases (50%) infection occurred during the winter. The remaining 64 patients were diagnosed with AIH (n=63) or drug-induced liver injury (DILI, n=1).

More specifically, 11/20 (55%, 95%CI 32-76%) patients of group 1 tested positive for IgM anti-HEV antibodies as well as for HEV-RNA: 18×10^4 ($38.7\text{-}39.7\times10^6$) IU/mL. Interestingly, one of the patients of group 1 who proved to have acute HEV had been diagnosed with AIH of insidious onset 6 months before (the diagnosis of AIH had been established by excluding all known viruses including HEV) and was under treatment with prednisolone (10 mg/day) and mycophenolate mofetil (1.5 g/day). In addition, another patient was diagnosed with low grade non-Hodgkin lymphoma (NHL) concurrently with the diagnosis of acute HEV. The remaining 9/20 patients, who tested negative both for IgM anti-HEV and HEV-RNA, were diagnosed with AIH (8/9) or DILI (1/9).

The retrospective serological testing in group 2 (n=56), patients with acute non-A/B/C hepatitis who had been diagnosed with acute AIH according to established criteria [26,27] but who had not been tested for HEV markers, revealed that 1/56 (1.8%, 95%CI 0.3-9.4%) was misdiagnosed, since he proved retrospectively to be IgM anti-HEV and HEV-RNA positive (4.1×10^4 IU/mL). The frequency of acute HEV among non-A/B/C acute hepatitis cases in group 2 was significantly higher compared to group 1 (P<0.001).

Three of 12 patients with acute HEV infection (25%) were already cirrhotic at the time of the episode of acute hepatitis. The diagnosis of cirrhosis was based on clinical or laboratory findings, including transient elastography performed after the convalescence from acute hepatitis, as we have described previously [34-37]. In 2 patients the cirrhosis was due to alcoholic liver disease, while the third patient was diagnosed during follow up with primary biliary cholangitis (PBC) based on persistent cholestasis that existed before the acute HEV infection and the presence of anti-mitochondrial antibodies (AMA). The characteristics of the 12 patients with acute HEV infection at the time of initial evaluation are shown in Tables 1 and 2.

Comparison between patients with acute HEV infection (n=12) and those with acute AIH (n=63)

Patients with acute HEV infection were predominately male and older compared to the patients with acute AIH (Table 1). Most of the patients with either acute hepatitis E (10/12, 83%, 95%CI 55-95%) or acute AIH (43/63, 68%, 95%CI 56-78%) had symptomatic disease at presentation, with jaundice and constitutional symptoms (Table 1). However, patients with acute HEV infection had more frequent fever (Table 1). In addition, patients with acute AIH presented more frequently with acute severe hepatitis—transaminases >10× ULN with international normalized ratio (INR) ≥1.5 and bilirubin ≥4 mg/dL at any time during the acute course [33]—compared to patients with acute HEV infection (38/63, 60.3%, 95%CI 48-71% vs. 2/12, 16.6%, 95%CI 4.7-45%; P=0.014) (Table 1). In terms of comorbidities, patients in the HEV group suffered more commonly from diabetes type II (P<0.001), alcohol

Table 1 Demographic and clinical characteristics of patients with acute hepatitis E (HEV) (n=12) and acute autoimmune hepatitis (AIH) (n=63)

Characteristics	Acute HEV n=12	Acute AIH n=63	P-value
Age (years)	64 (50-76)	53 (6-80)	0.005
Sex (male/female)	11/1	22/41	0.001
Symptomatic presentation (yes/no)	10/2	43/20	ns
jaundice	8/10 (80%)	34/43 (79%)	ns
fever	3/10 (30%)	2/43 (4.7%)	0.041
abdominal pain	1/10 (10%)	3/43 (7%)	ns
diarrhea	1/10 (10%)	1/43 (2.3%)	ns
arthralgia/myalgia	1/10 (10%)	3/43 (7%)	ns
constitutional symptoms	3/10 (30%)	8/43 (19%)	ns
Acute severe hepatitis (%)	2 (16.7)	38 (60.3)	0.014
Diabetes (%)	7 (58)	3 (4.8)	<0.001
Alcohol abuse (>30 g/day) (active or past) (%)	7 (58)	10 (16)	0.004
Concomitant chronic liver disease (%)	5 (42)	1 (1.6)	<0.001
ALD	2	0	
AIH	1	na	
PBC	1	0	
NASH	1	0	
Chronic HBV	0	1	
Autoimmune diseases (%)	3 (25%)	19 (30.2%)	ns
ITP	1	0	
RPF	1	0	
Hashimoto thyroiditis	1	6	
MS	0	5	
Sjs	0	1	
Psoriasis	0	1	
Scleroderma	0	2	
UC	0	1	
Biermer's anemia	0	3	
Cirrhosis (%)	3 (25%)	9 (14%)	ns

ALD, alcoholic liver disease; AIH, autoimmune hepatitis; PBC, primary biliary cholangitis; NASH, nonalcoholic liver disease; HBV, hepatitis B virus; ITP, idiopathic thrombocytopenic purpura; RPF, retroperitoneal fibrosis; MS, multiple sclerosis; Sjs, Sjögren's syndrome; UC, ulcerative colitis; ns, nonsignificant

abuse (P=0.004), and the coexistence of other chronic liver diseases (P<0.001) than patients of the AIH group (Table 1). Interestingly, the prevalence of other autoimmune diseases did not differ between groups (Table 1).

Regarding liver biochemistry, patients with acute HEV infection had significantly higher alanine aminotransferase (P=0.009, Table 2) but lower IgG levels (P=0.002, Table 2). Liver autoimmune serology showed high rates of positive smooth muscle antibodies (SMA) in both groups (Table 2), but patients with acute AIH had higher titers compared to those with acute hepatitis E: SMA titer ≥1/320: 39/60 (65%) vs. 4/12 (33%), P=0.05. However, patients with acute AIH more frequently had positive antinuclear antibodies (ANA) (P=0.003, Table 2). The prevalence of the other autoantibodies tested did not differ between groups.

Liver histology grading and staging did not differ between the 2 groups (Table 3). Interestingly, 2/6 (33%) liver biopsies from patients with acute HEV infection were typical for AIH, 3/6 (50%) were compatible, and 1/6 had atypical features. A detailed description of the liver histology from patients with acute HEV infection and acute AIH is shown in Table 2.

In terms of the simplified AIH score, 2/12 (16.7%) patients with acute HEV infection had ≥7 (definite AIH) while 4/12 (33.3%) had 6 (probable AIH). However, overall, patients with acute AIH had a higher score than patients with acute HEV infection (Table 2).

Disease course and outcome of patients with acute HEV infection

The course of the disease was benign in all patients with acute HEV infection. The mean duration until normalization of liver biochemistry was 35 (21-180) days. Acute kidney injury was documented in 1 patient (estimated glomerular filtration rate declined 46% from baseline) but it was restored with intravenous hydration during hospitalization without requiring further interventions. No other extrahepatic manifestations were documented in any patient. During the acute phase and the long-term follow up over 14 (1-36) months, no cases of acute liver failure, cirrhosis decompensation, liver-related death or need for liver transplantation were observed. However, 3 patients were lost to follow up and the patient with NHL died from complications of chemotherapy 1 year after the acute episode of HEV infection.

Table 2 Laboratory and histologic characteristics of patients with acute hepatitis E (HEV) (n=12) and acute autoimmune hepatitis (AIH) (n=63)

Characteristics	Acute HEV n=12	Acute AIH n=63	P-value
AST (ULN=40 U/L)	1123 ± 470	959 ± 1054	ns
ALT (ULN=40 U/L)	1713 ± 717	1026 ± 821	0.009
γGT (ULN=37 U/L)	362 ± 204	229 ± 222	ns
ALP (ULN=120 U/L)	185 ± 92	179 ± 98	ns
Bilirubin (ULN=1.1 mg/dL)	9.08 ± 9.3	9.17 ± 16	ns
Albumin	3.58 ± 0.88	4.27 ± 5.33	ns
INR	1.12 ± 0.13	1.31 ± 0.60	ns
IgG (ULN=1400 mg/dL)	1275 ± 550	2250 ± 1000	0.002
Liver autoimmune serology			
ANA (%)	0	28 (44.4%)	0.003
SMA (%)	12 (100%)	60 (95.2%)	ns
Anti-SLA/LP (%)	0	2 (3.2%)	ns
Anti-LKM (%)	0	3 (4.8%)	ns
Anti-LC1 (%)	0	0	ns
Simplified score for AIH	5.5 (4-8)	7 (6-8)	0.024
Liver biopsy	n=6	n=51	
Stage: F0-F2/F3-F4	6/0	40/11	ns
Grade: minimal-mild/moderate-severe	3/3	7/44	ns
Interface hepatitis (yes/no)	5/1	38/13	ns
Lymphoplasmacytic infiltrate (yes/no)	2/4	32/19	ns
Emperipolesis (yes/no)	2/4	11/40	ns
Hepatocyte rosettes (yes/no)	2/4	14/37	ns
Lobular inflammation (yes/no)	5/1	37/14	ns

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; INR, international normalized ratio; ANA, antinuclear antibodies; SMA, smooth muscle cell antibodies; anti-SLA/LP, antibodies against soluble liver antigen/liver pancreas; anti-LKM, liver-kidney microsomal antibodies; anti-LC1, liver cytosolic antibodies; ns, nonsignificant; ULN, upper limit of normal

Table 3 Accession numbers of 902 base pair sequences retrieved from 4/12 patients and 6 wild boars

Accession number	Nucleotide sequence
OM654048	GRE-Human1
OM654049	GRE-Human2
OM654050	GRE-Human3
OM654051	GRE-Human4
OM654052	GRE-Wild boar1
OM654053	GRE-Wild boar2
OM654054	GRE-Wild boar3
OM654055	GRE-Wild boar4
OM654056	GRE-Wild boar5
OM654057	GRE-Wild boar6

Repeat HEV-RNA testing 3 and 6 months after the acute infection was performed in the case of the 2 immunocompromised patients (the patient with AIH under immunosuppression and the patient with NHL) and in another 3 patients with persistently abnormal liver biochemistry after the acute phase and for up to at least 6 months. HEV-RNA in months 3 and 6 were negative in all 5 patients, so no case of chronic infection was documented.

Interestingly, 1 female patient with acute HEV infection exhibited persistent elevation in γ-glutamyl transferase and periodically alkaline phosphatase for the following 2 years after the acute hepatitis. AMA seropositivity by ELISA and compatible liver biopsy 2 years after the convalescence from acute hepatitis E supported the diagnosis of PBC.

Phylogenic analysis of HEV strains

A 197-base pair (bp) sequence of HEV *ORF2* gene was detected in 6 wild boars and its presence was further confirmed by nucleotide sequence analysis. Positive HEV-RNA samples from 12 patients and 6 wild boars were tested with a set of 7 primer pairs. Sequences of 902 bp were retrieved from 4/12 patients and 6 wild boars and were deposited in GenBank under the accession numbers OM654048-OM654057 (Table 3). The nucleotide similarity among all Greek sequences varied from 87.8-97.3%. Furthermore, human nucleotide sequences showed 88.0-97.3% similarity, and those from wild boars 87.8-96.4%.

Phylogenetic analysis confirmed that Greek HEV strains, from both patients and wild boars, belonged to genotype 3 and were further divided into the sub-genotypes 3e and 3f. Sub-genotype 3e formed a group that included a Greek human strain (OM654048) that was closely related to a French human strain (97.5%), as well as to 3 Greek strains from wild boars (OM654054, OM654055, OM654056) (91.7-93.3%). Moreover, within the cluster of sub-genotype 3f, 3 Greek human strains (OM654049. OM654050,

OM654051) were closely related not only to a French human strain (93.6-95%), but also to strains from 3 Greek wild boars (OM654052, OM654057, OM654053) (94.7-96.6%) (Fig. 1).

Discussion

The epidemiology of HEV infection in Europe has changed over the past decades. A rising incidence of acute infections highlighted HEV as the main cause of acute hepatitis during 2015-2016 in many European countries [4,5]. Three main points arise from our study: a) more than half (55%) of non-A/B/C acute hepatitis cases treated in a single tertiary center in central Greece during a 2-year period were caused by autochthonous HEV infection; b) wild boars seem to be an important reservoir of the disease in Greece, as in other European countries; and c) acute HEV shares many similarities in terms of clinical, serological and histological characteristics with acute AIH and for this reason should always be excluded before a definite diagnosis of AIH is made.

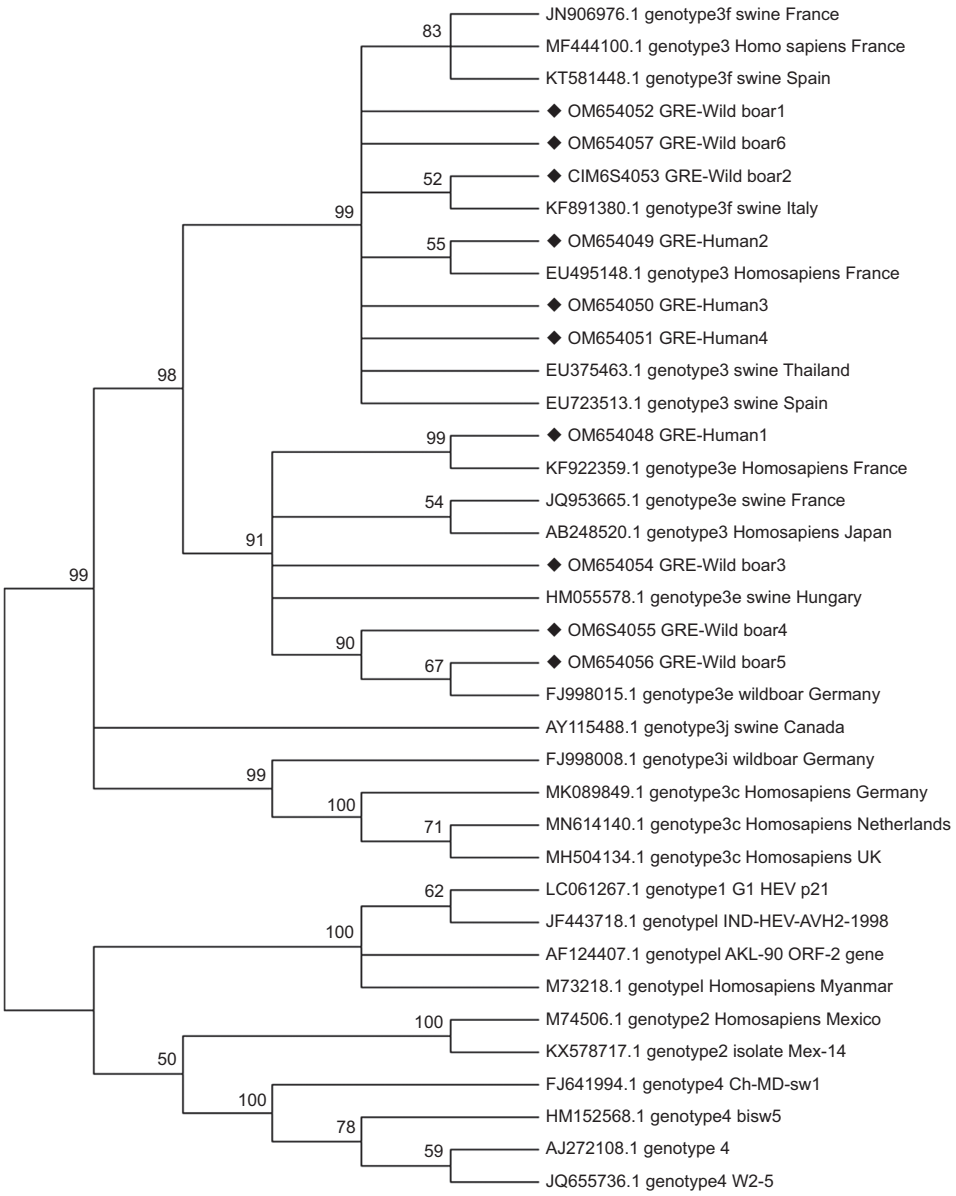

Figure 1 Phylogenetic tree constructed with maximum-likelihood method. Analysis was performed by using 10 nucleotide sequences of Greek strains of hepatitis E virus (HEV), 4 from patients (OM654048-51) and 6 from wild boars (OM654052-57), and 27 HEV sequences of genotypes 1,2,3 and 4 retrieved from the GenBank database. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points; only values over 50% are indicated. Greek strains are marked with ♦

Although the awareness of HEV infection has increased in recent years, surveillance for the disease remains problematic for several reasons: HEV reporting is not mandatory in all countries, including Greece; HEV testing is not uniformly included as a first-line virologic investigation in all centers; the largest amount of data was extrapolated from retrospective seroprevalence studies that do not involve a representative sample of the population; and, finally, there are important discrepancies in reproducibility and accuracy between the diverse diagnostic assays used [4,12,13,38]. Using a method that has proved to be very sensitive for the detection of IgM anti-HEV antibodies [12] and confirming the results with an RT-PCR for HEV-RNA, we found that at least half of the acute non-A/B/C hepatitis cases hospitalized in our department were due to acute HEV infection. Notably, we observed a significant increase in the frequency of HEV infections between the 2 periods of the study (2000-2015 and 2015-2017). This is in agreement with the 10-fold increase in the total number of reported symptomatic cases in Europe between 2005 and 2015 [4] and the fact that HEV infections have become the most frequent cause of acute viral hepatitis in other European countries, such as Germany and the UK [39,40].

We found that more than 90% (11/12) of the patients with acute HEV infection were older males (median age 64), almost 60% were diabetics, 60% consumed a large amount of alcohol, and 40% had a concomitant chronic liver disease. This clinical profile has already been recognized in previous studies [4,40,41], although a definite explanation for the observed increased risk of symptomatic disease among these subjects is not currently available. However, these groups are more likely to have chronic liver disease and consequently symptomatic and/or more severe acute hepatitis [13].

Furthermore, since many centers do not include HEV testing in their first-line virologic assessment for acute hepatitis, acute HEV infection must frequently be differentiated from the acute form of AIH. The problem becomes even more striking when non-sensitive methods for IgM anti-HEV antibodies are used, and taking into account that the seroprevalence of HEV may be higher among AIH patients [20,42]. For this reason, we tested all patients diagnosed with acute AIH retrospectively, with the sensitive Wantai ELISA, and we found that 1 patient (of 56 patients; 2%) had acute HEV rather than acute AIH, although he fulfilled the criteria for AIH diagnosis (simplified score 7; definite AIH). In addition, 1 patient of the prospective group, with well-established AIH and remission under immunosuppression, presented with an acute hepatitis flare resembling AIH relapse, which proved to be acute HEV infection.

We have chosen to compare acute HEV infection with the acute form of AIH, because the AIH diagnosis is based on the exclusion of all other causes, while there is no specific test for its establishment. All other causes of acute liver diseases can be excluded either by history or by specific testing. Furthermore, as we have shown in the current study, the acute presentation of AIH did not differ histologically from that of acute HEV, while on the other hand patients with acute HEV had detectable autoantibodies, making the differential diagnosis very challenging and difficult, if anti-HEV and/or HEV-RNA are not tested. Since the distinction between acute AIH and acute HEV infection is urgent, we tried to detect characteristics that could differentiate the 2 entities. As expected, patients with acute AIH were predominately female and younger than patients with acute hepatitis E. In addition, patients with acute AIH more frequently had acute severe disease than patients with acute HEV infection; however, surprisingly, the prevalence of autoimmune diseases did not differ between the 2 groups. In accordance with Terziroli Beretta-Piccoli *et al* [25], SMA were detected with the same frequency and in comparable titers in both diseases. However, ANA were present only in patients with acute AIH. In addition, liver histology could not differentiate the 2 types of acute hepatitis. Nevertheless, IgG levels and the simplified score could reliably distinguish acute hepatitis E from acute AIH, emphasizing that elevated IgG is a quite distinct feature of AIH, even in its acute form.

Notably, one female patient was diagnosed with PBC 2 years after the acute HEV episode. The presentation or induction of autoimmunity after a viral infection is not new in the literature, being in line with the model suggesting that viral infections can initiate autoimmune reactions through molecular mimicry [17-19]. For this reason, a watchful follow up after the acute HEV infection has subsided might be prudent.

Furthermore, we have shown that, in Greece, HEV circulates among humans and wild boars. Phylogenetic analysis showed that HEV strains isolated from both patients and wild boars were of genotypes 3f and 3e, revealing their close genetic relationship. This observation suggests that wild boar might serve as a reservoir or a potential source of infection to humans and is in accordance with previous studies [43]. Considering that the human and wild boar isolates had different regions of origin, we could suggest that 3f and 3e are the predominant genotypes circulating in Greece.

In the last 2 decades, the wild boar population has increased sharply in Greece, resulting in an increased frequency of contacts between wild boar and humans, even in semi-rural and rural regions. Thus, wild boar hunting and game meat consumption has become a popular practice in several regions, so the handling of carcasses (i.e., evisceration) and consumption of undercooked meat products could be potential zoonotic risk factors for HEV infection of humans. Since only 10 sequences were included in this paper, further studies, based on a larger sample size, are required to confirm these results and clarify the molecular epidemiology of HEV in Greece.

Our study had some limitations, such as the retrospective testing of patient group 2, the lack of data about the incidence of other cases of acute hepatitis in the same period of time, and the absence of a documented connection between human infections and wild boar infections. The retrospective testing of sera, although stored at -80°C, always entails a risk of detection failure, especially regarding HEV-RNA. The prevalence of other types of acute hepatitis, especially viral, would be of importance, in order to compare it with the prevalence of HEV at the same time, but unfortunately data on that were not available. Finally, although the population of the rural regions of Central Greece is known to consume wild boar meat, we could not document from the medical history any wild boar contact or meat consumption by our patients with acute HEV infection.

In conclusion, autochthonous HEV infection as a cause of acute hepatitis is not rare in Greece; therefore, it should be part of the initial diagnostic workup in all patients with an acute episode of hepatitis, regardless of travel history. Wild boars seem to be an important reservoir of the virus in Greece, as in other European countries. Patients of male sex, older age and with coexisting liver disease are more susceptible to develop the acute symptomatic form of hepatitis E. In this series, the clinical course was benign, regardless of comorbidity. Special consideration should be given to patients who present with autoimmune features, since the diagnosis of acute AIH is not safe until acute HEV infection has been ruled out.

Summary Box

What is already known:

- The incidence of hepatitis E virus (HEV) infection is increasing in Europe
- Wild boars seem to be a significant reservoir of HEV in Europe (predominately HEV-3)
- HEV infection may lead to or unmask autoimmune hepatitis (AIH) in susceptible individuals

What the new findings are:

- More than half of the non-A/B/C acute hepatitis cases hospitalized in a single tertiary center in central Greece during a 2-year period were caused by autochthonous HEV infection
- Wild boar also seem to be an important reservoir of the disease in Greece
- Acute HEV shares many similarities in terms of clinical, serological and histological characteristics with acute AIH

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Supplementary material

Supplementary Table 1 Primers used for amplification of a part of the *ORF2* region of hepatitis E virus RNA from strains isolated from liver tissues of wild boars

Primer	Sequence (5' to 3')	Position according to the Burmese strain	Reference
F2	GCTCACGTCATCTGTCGCTGCTGG	5572-5595	2
R2	GGGCTGAACCAAAATCCTGACATC	5837-5859	
3156-EF	Aaytatgcmcagtaccgggttg	5687-5708	1
3157-ER	Cccttatcctgctgagcattctc	6395-6417	
3158-EF	Gtyatgytyygcatacatggct	5972-5993	1
3159-IRS	Agccgacgaaatyaattctgtc	6298-6319	
ORF 2-s1	Gacagaattratttcgtcggctgg	6298-6321	1
ORF 2-a 1	Cttgttcrtgytggttrtcataatc	6470-6494	

HEV-RNA detection in wild boars' liver tissue samples

From 2010-2014, liver tissue samples from 40 wild boars (*Sus scrofa*) originating from Northern and Western Greece were submitted to the Laboratory of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Thessaly and were stored at -80°C for HEVRNA examination. Viral RNA was isolated from 50 mg of liver tissue samples using Trizol reagent protocol (Thermofisher Scientific). A primer pair targeting at a 197-nucleotide part of *ORF2* gene (40) was used for amplifying HEV-RNA. An OneStep RT-PCR protocol (Qiagen, Germany) was performed in a final volume of 50 μL, according to the manufacturer's instruction. Following RT-PCR, 10 μL of amplification products were analyzed by electrophoresis in a 2% agarose gel stained with 0.5 mg/mL ethidium bromide. Product sizes were determined with reference to 100 bp DNA ladder. Amplicons were sequenced bidirectional by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

HEV detection in wild boars is part of the European Union Seventh Framework Programme (2007–2013), a large collaboration project under grant agreement no. 222633 (Novel Technologies for Surveillance of Emerging and Re-emerging Infections of Wildlife—WildTech). All samples used in this study represent material collected by partners and other organizations for other purposes than this project as specified in deliverable D4.5/5.5 titled "Guidelines for ethical sample collection" submitted to the European Commission (26 February 2010, Dissemination Level: PP, restricted to other program participants, including Commission Services). The wild boar liver tissue samples were collected opportunistically (no active capture, killing, and sampling of wild animals specifically for this study were performed) from animal hunter-harvested by members of hunting federations. Thus, special approval was not necessary, and steps to ameliorate suffering were not applicable to this study. Research on animals as defined in the EU Ethics for Researchers document (European Commission, 2007, Ethics for Researchers—Facilitating Research Excellence in FP7, Luxembourg: Office for Official Publications of the European Communities, ISBN 978-92-79-05474-7) is not applicable to this study.

HEV genotyping and phylogenic analysis

HEV-RNA from patients and wild boars was used for partially amplification of the ORF2 gene using four overlapping primer pairs (Supplementary Table 1) [1,2]. An OneStep RT-PCR protocol provided by the manufacturer (Qiagen, Germany), was performed for each primer pair. RT-PCR products of expected sizes were analyzed by a 2% agarose gel electrophoresis and underwent bidirectional sequencing using the fluorescent BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Evolutionary analyses were conducted in MEGA X [3] among 37 HEV strains including those described in this study. Twenty-seven HEV sequences of genotypes 1-4 were also retrieved from the GenBank database. Nucleotide sequences were aligned by CLUSTAL W and evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei substitution model [4]. One thousand bootstrap re-samplings were performed to calculate confident value of internal nodes.

Autoantibody testing in human serum samples

Antinuclear antibodies (ANA), smooth muscle antibodies (SMA), antibodies against liver kidney microsomal (anti-LKM), and against liver cytosol type-1 (anti-LC1) were initially detected by indirect immunofluorescence on 5-μm fresh frozen sections of in-house rodent kidney, liver, and stomach tissue substrates. Anti-LKM1, anti-LKM3 anti-LC1, and antibodies against soluble liver antigen/liver pancreas (anti-SLA/LP) were also evaluated by immunoblotting using rat liver microsomal or cytosolic extracts. Commercially available enzyme-linked immunosorbent assay kits using recombinant SLA/LP/tRNP (Ser) Sec (Inova Diagnostics, San Diego, CA, USA), cytochrome P450 2D6 (INOVA) and formiminotransferase cyclodeaminase (Euroimmun Medizinische Labordiagnostika, Lubeck, Germany) were also used for anti-SLA/LP, anti-LKM and anti-LC1 determination respectively, according to the manufacturer's instructions.

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