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Hepatitis E in patients with hepatic disorders and HIV-infected patients in Croatia – is one diagnostic method enough for hepatitis E diagnosis?

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Abstract:

We assessed hepatitis E virus (HEV) seroprevalence in patients with hepatic disorders as well as in HIV-infected patients and emphasised the issue of possible non-specific anti-HEV seroresponse and need for combining diagnostic methods for hepatitis E diagnosis.
Over a two-year period, from March 2011 to February 2013, we determined anti-HEV IgM and IgG by enzyme-immunoassays (EIA) (Mikrogen, Germany) in 504 hepatitis patients negative for acute viral hepatitis A-C. Furthermore, 88 samples from randomly selected consecutive HIV-infected patients were also analysed. All EIA reactive samples were additionally tested by line-immunoblot assays (LIA; Mikrogen, Germany). HEV nested RT-PCR was carried out in 14 anti-HEV IgM LIA positive patients.
Anti-HEV IgM or IgG were detected in 16.9% patients by EIA and confirmed by LIA in 10.7% (95% CI 8.3-13.7%) of hepatitis patients. HEV RNA was detected in 5 patients. The agreement between EIA and LIA assessed by Cohen's Kappa was for IgM 0.47 (95% CI 0.55-0.75) and for IgG 0.83 (95% CI 0.78-0.93). Anti-HEV IgM and IgG seroprevalence in HIV-infected patients was 1.1%, respectively.
Our findings show a rather high HEV seroprevalence in patients with elevated liver enzymes in comparison to HIV-infected patients. Discordant findings by different methods stress the need to combine complementary methods and use a two-tier approach with prudent interpretation of reactive serological results for hepatitis E diagnosis.

Key words: hepatitis E, HEV seroprevalence, serodiagnosis, enzyme-immunoassay, line-immunoassay, two-tier testing, PCR, Croatia

Introduction

Hepatitis E infection is an emerging disease displaying hyperendemic outbreaks in developing countries and yet seems to be underestimated in industrialised regions. The cause of hepatitis E is a small non-enveloped RNA virus with 4 genotypes recognized in humans [1]. Hepatitis E virus (HEV) genome contains short non-coding regions and three discontinuous and partially overlapping open reading frames (ORF) for viral non-structural proteins (ORF1), viral capsid protein (ORF2) and proteins with still unclear functions from ORF3 [2, 3]. In developing regions outbreaks are generally caused by human specific genotypes 1 and 2 which are recognized as travel-associated HEV infections in developed countries. Genotypes 3 and 4 are considered to be causes of endemic infections with zoonotic potential since they were isolated from humans but also from different mammalian species and marine animals [4-10]. HEV is acquired through the fecal-oral route, although sources of HEV frequently stay unrecognized [4-12]. Hepatitis E clinical presentation in healthy individuals is usually mild and self-limiting and patients may even not seek medical attention [4, 5]. In contrary, in severely immunosuppressed patients chronic HEV infection could be a cause of persistent alanin aminotransferase (ALT) elevation. So, HEV persistence may be a potential risk of disease progression also in HIV-infected patients [13-17]. HEV infection needs to be considered as a possible cause of unexplained increase in liver enzymes when common viral causes of hepatitis are excluded.

The diagnosis of HEV infection is based on serological determination of anti-HEV IgM and IgG antibodies and/or nucleic acid-based testing [5, 18-20]. Viremia is limited to the acute phase of illness, and both anti-HEV IgM and IgG are generally detectable at the onset of disease. Anti-HEV IgM decline rapidly and 3-6 months after the occurrence of the first symptoms are undetectable while a rise in IgG antibody titre is observed [21]. Acute hepatitis E is defined when anti-HEV IgM and IgG in serum or HEV RNA is documented. The finding of anti-HEV IgM when IgG and HEV RNA remain undetectable could not be considered as an acute hepatitis E infection. Serological HEV diagnostic is not standardized. Diagnostic performances and results differ in specificity and sensitivity of used commercially available assays. Serological findings depend on the principle of enzyme-immunoassays (EIA; indirect or μ-capture method) and the used antigen selections and conformations [5, 14, 18-24]. Although HEV is antigenically consistent and only one serotype has been identified, recombinant antigens used for antibody

testing are not optimized. New generation assays based on HEV-ORF2 genotype 1 and 3 (Wantai/Fortress, Mikrogen) represent a significant step towards establishing serological standards [20-23].

Hepatitis E is a notifiable disease in Croatia since 1994. Until 2012 only a few travel associated HEV cases per year were registered [25, 26]. Even so, HEV has been detected in domestic swine and wild boars [8, 27] resulting in a possibility of viral spread to humans [28]. Therefore, accurate and quick diagnosis is needed to distinguish HEV infection from other causes of acute viral hepatitis. The aim of this study was to assess HEV seroprevalence in patients with hepatic disorders as well as HIV-infected patients and to evaluate the serological diagnosis of hepatitis E using EIA followed by LIA and RT-PCR.

Patients and methods

Over a 2-year period, from March 2011 until February 2013, sera from 504 patients (age range 16 to 81 years) with elevated liver enzymes (ALT and AST >2xULN) were serologically tested at the University Hospital for Infectious Diseases in Zagreb for viral hepatitis E. Furthermore, 88 sera samples from randomly retrospectively selected consecutive HIV-infected patients (age range 20 to 80 years) were also tested for the presence of anti-HEV IgM and IgG. Moreover, CD4 cell count was available for 85 of the HIV-infected patients included in the study with a median of 279 cells/mm^3 (interquartile range, 129-488 cells/mm^3). The number of the latter samples represents approximately 10% of all registered HIV-infected patients in Croatia.

Acute viral hepatitis A-C were excluded according to specific markers: anti-HAV IgM, anti-HAV total (DiaSorin, Italy), HbsAg (Murex/DiaSorin, Italy), anti-HBs, anti-HBc, anti-HBc IgM, anti-Hbe, HbeAg (DiaSorin, Italy), anti-HCV (HCV Ag/Ab Biorad, France). EBV and CMV infections were serologicaly defined with assays for anti-CMV IgM and IgG (DiaSorin, Italy), anti-VCA EBV IgM and IgG respectively, anti-EA EBV IgG and anti-EBNA IgG (DiaSorin, Italy). The testing included detection of anti-HEV IgM and IgG by EIA with recombinant HEV ORF2 (genotype 1 and 3) antigens (recomWell HEV IgG, IgM; Mikrogen GmbH, Germany). All samples with primarily positive or borderline EIA findings were additionally tested by LIA (recomLine HEV IgG/IgM, Mikrogen GmbH, Germany). LIA was

used as a supplemented method and the standard for result interpretation. All procedures were managed according to the manufacturer's instructions.

Positive or borderline anti-HEV EIA results were defined according to a follow-up serological testing by LIA. Only LIA positive results were assessed as positive anti-HEV antibodies. LIA confirmed anti-HEV IgM and IgG findings in sera were considered as indicators of an acute hepatitis E. Anti-HEV IgM positive and IgG negative findings were interpreted as indicators of a possible recent HEV infection. Positive anti-HEV IgG accompanied with negative IgM was considered as an indicator of a past infection.

For confirmation of acute and recent HEV infection a nested RT-PCR was carried out in 14 patients by a protocol described by van der Poel [7]. HEV RT-PCR was conducted on sera with positive anti-HEV IgM. A fragment of 287 bp within the metal-transferase gene region of ORF1 was the amplification target. RNA was isolated by *QIAamp® Viral RNA Kit* (Qiagen, Germany), according to the manufacturer's instructions. The reverse transcription was carried out using *GoScript Reverse Transcription System* for RT-PCR (Promega, USA) in a *Gene Amp PCR System 9700* machine (Applied Biosystems, USA). Thirty-five PCR cycles were conducted by the use of 2 pairs of primers (nested PCR). Electrophoresis was done in a 1.5% agarose gel. All samples with a band of 287 bp were considered as HEV positive.

The agreement between EIA and LIA for IgM and IgG anti-HEV assays was quantified with the Cohen's Kappa coefficient. Statistical analysis was performed using the Statistica 8.0 (StatSoft).

Results

Anti-HEV reactive antibodies determined by EIA were found in 85 of the 504 (16.9%) patients with hepatic disorders. Of the 85 anti-HEV EIA positive patients 42 (49.4%) were males, with a median of 48 years of age. Anti-HEV IgM and IgG were reactive by EIA in 46 (54.1%) and 51 (60.0%) of the 85 patients, respectively (Fig 1a). Anti-HEV antibodies were confirmed by LIA in 54 of the 85 (63.5%) EIA-reactive patients. Anti-HEV IgM and IgG were positive by LIA in 16 of 46 (34.8%) and 44 of 51 (86.3%) EIA-reactive patients, respectively. Unconfirmed EIA

reactive anti-HEV IgM or IgG antibodies had 31 (6.2%) patients. Anti-HEV antibodies were confirmed by LIA in 10.7% of all hepatitis HIV-negative patients.

Even though 11 (12.5%) HIV-infected patients displayed EIA positive anti-HEV IgM, only in one patient (1.1%) the positive EIA result was positive also by LIA. However, this patient had no clinical signs or elevated liver enzymes, so a possible false positive LIA finding could not be excluded. Of the 88 HIV-infected patients, one (1.1%) had confirmed anti-HEV IgG antibodies as a sign of past infection (Fig 1b).

Different serological IgM and IgG profiles were found by EIA in comparison to LIA (Table 1). Borderline LIA findings, 5 for IgM and 2 for IgG, were considered as negative. In 12 patients with combined EIA reactive IgM and IgG, anti-HEV were confirmed by LIA in 6 for IgM and IgG, 4 for only IgG and 1 for IgM while one patient had IgM LIA borderline and IgG negative. Anti-HEV antibodies stayed unconfirmed for 30 of 46 (65.2%) IgM and 7 of 51 (13.7%) IgG EIA reactive results. The agreement between EIA and LIA assessed by the Cohen's Kappa coefficient was for IgM 0.47 (95% CI 0.55-0.75) and for IgG 0.83 (95% CI 0.78-0.93).

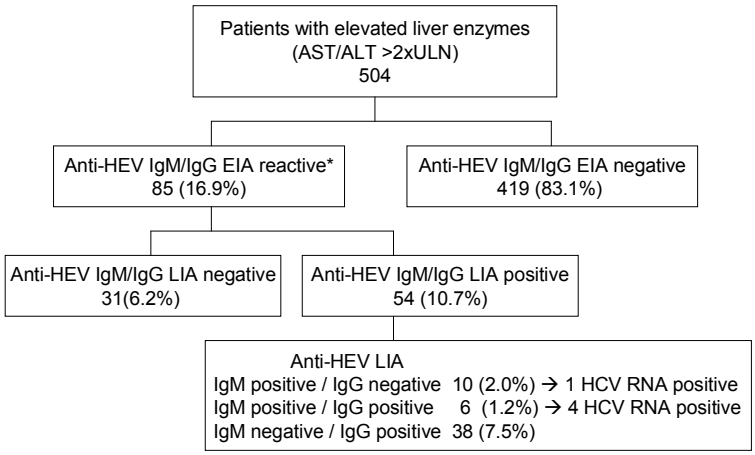
HEV RT-PCR was conducted in patients with positive anti-HEV IgM and HEV RNA was found in 5 of 14 (35.7%) tested patients.

To determine the influence of other hepatotropic viruses, except for viral hepatitis A-C patients were also tested for EBV and CMV. Among patients with anti-HEV antibodies, 42 (49.4%) had serological signs of past HAV, HBV or HCV infection. Anti-HAV was most commonly detected (Table 2).

From 16 patients with positive and 5 with borderline LIA anti-HEV IgM, hepatitis was caused by CMV in 4 (19.0%) and EBV in 1 (4.8%). One patient with EIA reactive anti-HEV IgM that was not confirmed by LIA had an acute EBV infection. Among 8 HIV-infected patients with EIA positive anti-HEV IgM acute CMV infection was found in 3 (37.5%), EBV in 1 (12.5%), and 1 (12.5%) HIV-infected patient had sifilis.

Specific antibodies against different HEV antigens are displayed by LIA of which the most represented were anti-HEV IgM for O2N antigen (47.8%) and IgG for O2C antigen (86.3%) (Fig 2). The reactivity to the HEV O3 antigen was high for both antibody classes, while low against O2M antigen. The differentiation between HEV genotypes 1 and 3 according to the bands reactivity might be only assumed but was not reliable. Further validation is needed.

a)



b)

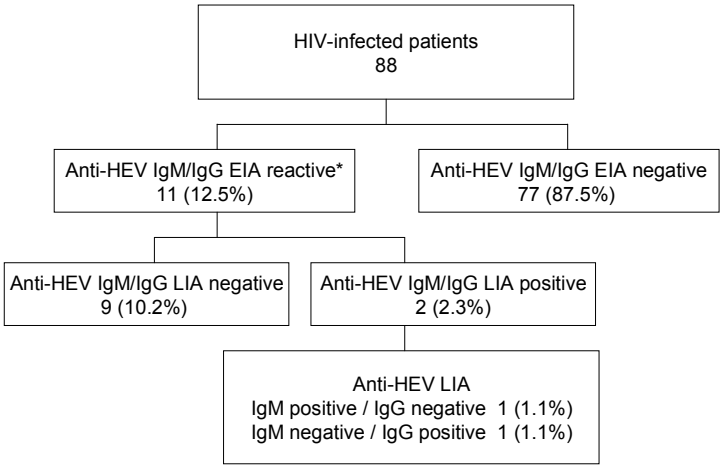


Fig. 1 Anti-HEV IgM and IgG antibodies in patients with elevated liver enzymes (AST/ALT >2x ULN) and excluded acute viral hepatitis A-C (a) and in HIV-infected patients (b) (EIA, enzyme immunoassay; LIA, line-immunoassay; *EIA reactive, positive and borderline results included)

Table 1 Anti-HEV IgM and IgG enzyme (EIA) and line-immunoassay (LIA) discordant results in hepatitis and HIV-infected patients

Anti-HEV IgM	EIA* positive		EIA borderline		Total IgM (%)	
	Hepatitis patients	HIV- patients	Hepatitis patients	HIV- patients	Hepatitis patients	HIV- patients
LIA** positive	16	1	-	-	16 (3.2%)	1 (1.1%)
LIA borderline	4	1	1	-	5 (1.0%)	1 (1.1%)
LIA negative	14	6	11	3	483 (95.8%)[#]	86 (97.7%)
Total IgM (%)	34 (6.7%)	8 (9.1%)	12 (2.4%)	3 (3.4%)	504	88
Anti-HEV IgG	EIA positive		EIA borderline		Total IgG (%)	
	Hepatitis patients	HIV- patients	Hepatitis patients	HIV- patients	Hepatitis patients	HIV- patients
LIA positive	38	1	6	-	44 (8.7%)	1 (1.1%)
LIA borderline	2	1	-	1	2 (0.4%)	2 (2.3%)
LIA negative	3	-	2	-	458 (90.9%)[#]	85 (96.6%)
Total IgG (%)	43 (8.5%)	2 (2.3%)	8 (1.6%)	1 (1.1%)	504	88

*EIA, enzyme immunoassay; **LIA, line-immunoassay; [#] negative EIA serum was not determined by LIA

Table 2 Results in 42 patients with anti-HEV antibodies and serological evidence of past HAV, HBV and HCV infection

HAV / HBV / HCV Past infection N = 42	Anti-HEV				
	LIA IgG positive	LIA IgM positive	LIA IgM+IgG positive	LIA IgM borderline	LIA IgM+IgG negative EIA IgM+IgG positive
HAV	8	2	1	-	9
HAV / HBV	12	-	-	1	-
HAV / HCV	2	-	-	-	-
HBV	1	-	2	-	2
HCV	-	1	-	-	1
Total %	23 54.8%	3 7.1%	3 7.1%	1 2.4%	12 28.6%

*LIA, line-immunoassay; EIA, enzyme immunoassay

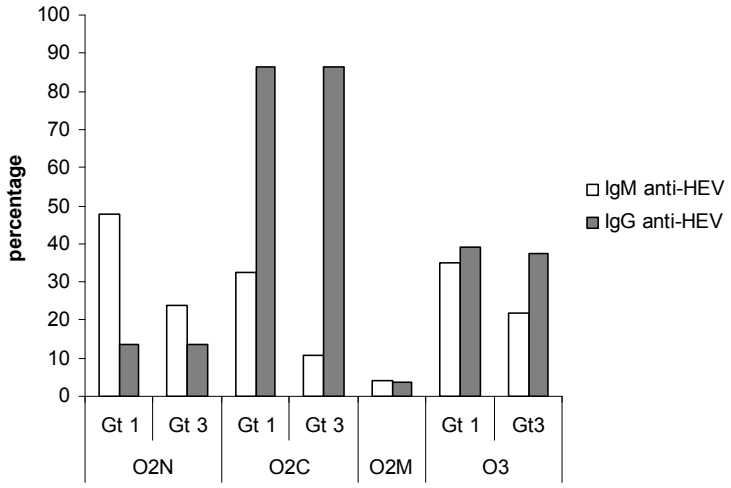


Fig 2. Specific anti-HEV IgM and IgG reactivity in line-immunoassays in serum samples: 46 for IgM, 51 for IgG (O2N, N-terminal part of ORF2 protein (HEV capsid-protein); O2C, C-terminal part of ORF2 protein (HEV capsid-protein); O2M, middle part of ORF2 protein (HEV capsid-protein); O3, ORF3 protein of HEV; Gt 1, genotype 1; Gt 3 genotype 3)

Discussion

Even though Croatia is considered as a country of low HEV incidence with sporadic, mostly imported HEV cases [25], the average seroprevalence in patients with non-specific elevation of liver enzymes and negative for acute viral hepatitis A-C assessed by EIA followed by LIA was 10.7%. HEV seroprevalence gained by EIA only was even higher (16.9%). The HEV IgG seroprevalence in HIV-infected patients was lower (1.1%) than in patients with hepatic disorders, and might presume the seroprevalence of the adult population in general while HIV-infected patients according to literature are not at increased risk of acquiring HEV infection [5]. Our results show discrepancies related to the used methods and reinforce previous reports on difficulties in HEV diagnostic. The strength of agreement between EIA and LIA (Mikrogen) for anti-HEV IgG was very good but only moderate for IgM.

The data on HEV seroprevalence gained by different testing approaches are demanding for analysis although in general our results may be comparable with others [10, 13, 20, 22, 29-33]. There is a high probability that our patients were HEV infected in Croatia since there is no recognition of their recent travel to endemic regions and all HEV RNA determined were genotype 3 that was documented in swine although a direct connection of viral transmission cannot be defined. Therefore the sources of infection remain unrecognized [8, 27]. According to EIA anti-HEV IgM even 46 patients could be considered as acute HEV cases but in only 8 of them recent HEV infection was confirmed by RT-PCR or the rising IgG titre in a convalescent serum. All patients with positive HEV RNA showed a high anti-HEV IgM titre and had detectable IgG when they requested medical attention. Only one HEV RNA positive patient had anti-HEV IgM without IgG, however the patient seroconverted to IgG very soon. The findings support previous reports that only anti-HEV IgM detected in one serum sample is not sufficient for establishing HEV diagnosis [5, 20, 24, 29, 34-37]. Reactive anti-HEV IgM may be the result of polyclonal B cell activation by EBV or other infections which can induce B cells [24, 34-37]. False positive IgM anti-HEV due to acute EBV, CMV and syphilis were documented also in our patients. Overdiagnosis of acute hepatitis E due to possible false positive results should not be neglected and needs to be recognized and reduced. One possible solution may be the use of a two-tier approach with combining anti-HEV IgM and IgG screening by EIA with additional

testing by line-immunoassay (LIA). Detection of HEV RNA is always recommended especially in recent illness [18].

The limitations of the study need to be noted. We used LIA and EIA assays based on the same antigens from the same manufacturer (Mikrogen). However, similar two-tier testing approaches are used for borreliae and HCV to show specific reactions against separate antigens and to exclude unspecific EIA reactions. According to published data for patients with positive HEV RNA the specificity and sensitivity of newer serological tests show very good performances [5, 20-23], but in routine clinical practice patients with different simultaneous diagnoses may gain unspecific ambiguous anti-HEV results, so the seroprevalence rates may differ significantly according to used diagnostic protocols. Low positive predictive values, even for high sensitive EIA due to low prevalence, imposes the need to check all positive and borderline results [20]. This supports the importance of combining at least two assays to assess the result. A two-step testing approach with anti-HEV IgM and IgG EIA and LIA, which has a positive impact regarding to the specificity, could be recommended. A further solution could also be combining two EIAs with high sensitivity and specificity [14, 21, 22].

LIA was criticized for its low sensitivity [23], even though it is not intended for screening purposes (high cost, labour complexity) but for confirmation. According to our clinical practice patients with unconfirmed LIA anti-HEV IgM were unlikely to be HEV infected; some of them had other hepatitis infections that emphasizes the importance of differential diagnosis. In our study, positive anti-HEV antibodies were found in 49.4% of patients with a past HAV, HBV or HCV infection and the clinical impact of HEV in these patients is yet to be defined since co-infection with HEV could pose as a severe health risk among persons with pre-existing chronic liver disease [13, 14, 17, 32].

From our limited experience, immunocompetent patients with acute hepatitis E when seeking medical attention mostly have detectable antibodies by EIA and LIA. Viremia seems to be documented as long as IgM exceeds the IgG titre. Acute HEV infection with only anti-HEV IgM findings and without HEV RNA seems to be rarely possible. When HEV RNA decreases to undetectable levels anti-HEV IgM decline as well, while anti-HEV IgG titres increase [21]. In the subacute phase of infection, HEV RNA may already be undetectable and the antibody kinetic needs to be monitored in a follow-up testing. Unfortunately, a consecutive serum sample is often unavailable. We managed to collect paired sera from only 2.4% of tested patients. Furthermore,

the diagnosis of HEV infection solely by serological testing may result in false negative findings when HEV is recently acquired. Therefore, diagnosis in such cases needs to be assessed by further testing of a new serum and/or with HEV RT-PCR.

Whether serotyping is even possible via different specific antibodies against genotype 1 or 3 by LIA, and if these antibodies are cross protective, remains to be elucidated. The current data set by Pas et al [21] show cross reactivity between known genotype 1 and 3 sera. We found high reactivity against the N-terminal (O2N) and C-terminal parts (O2C) of the capsid protein for IgM and IgG, respectively, similar as the specific LIA bands findings in Germany [2, 33]. Further follow-up studies during HEV disease are needed to reveal the expression of different antigens in HEV pathways and the role of specific antibodies.

In conclusion, our findings show a rather high HEV seroprevalence in patients with hepatic disorders comparing with HIV-infected patients in Croatia. Discordant findings by different methods (16.9% and 10.7% anti-HEV by EIA and LIA, respectively) stress the need to combine complementary methods and use a two-tier approach with prudent interpretation of reactive serological results for hepatitis E diagnosis. Combining EIA as a screening method with LIA or other sensitive and highly specific EIA for additional evaluation, as well as a follow-up anti-HEV IgG to document possible seroconversion or IgG titre rising and also HEV RT-PCR, could be recommended for definite diagnosis because false positive IgM and IgG findings should not be neglected.

Conflict of interest

The authors declare no conflict of interest.

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