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FULL PAPER

Diagnostic performance of hepatitis E virus antigen assay in hepatitis E virus acute infection

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SUMMARY

The aim of this study was to analyze the sensitivity of hepatitis E virus antigen (HEV-Ag) to determine acute E hepatitis.
Ninety-four serum samples resulting anti-HEV IgM by DIA.PRO assay were analyzed with Wantai assay to check for HEV-Ag. Thirty samples were anti-HEV IgM positive and HEV-RNA positive, 19 samples harbored genotype 3, whereas 11 samples were genotype 1. Overall, 16% of anti-HEV IgM samples resulted HEV-Ag positive and 33.3% of HEV-RNA positive were also HEV-Ag positive. Among 64 HEV-RNA negative samples, 5 (7.8%) were HEV-Ag positive.
The concordance of HEV-RNA and HEV-Ag was low (Cohen's Kappa=0.36). The Bland-Altman plot revealed a low agreement between HEV-RNA viral load and HEV-Ag, confirmed by a not significant Spearman's correlation coefficient (rho=0.137, p>0.05). Moreover, the HEV-Ag showed 100% specificity. In genotype 3f samples with a viral load >800 cp/ml HEV-Ag was positive in 80% of samples, whereas all patients harboring genotype 3e were HEV-Ag-negative irrespective of HEV-RNA viral load. Among genotype 1, HEV-Ag positivity was observed only in 27.7% patients and in all samples the viremia was >2000 cp/ml. These data suggest that anti-HEV IgM positivity represents the main biological marker of hepatitis E acute infection in clinical real life settings in developed countries.

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INTRODUCTION

Hepatitis E virus (HEV) represents the main cause of enterically transmitted hepatitis worldwide, being responsible for more than 50% of the cases of acute hepatitis in endemic countries (Lapa *et al*., 2015).
Although several laboratory assays for diagnosis of hepatitis E acute infection are available, including both indirect tests (serological assay) and direct assays based on nucleic acid detection or viral protein detection, at present the detection of immunoglobulin M (IgM) represents the main parameter to assess acute HEV infection.
Anti-HEV IgM antibodies appear in serum a few days after jaundice onset, they remain at a high level for eight weeks, falling below the cut-off level after 32 weeks, thus their presence suggests a recent HEV infection in subjects who had an acute HEV infection and who just recovered from the jaundice phase (Kamar *et al*., 2014). Several assays are available to detect anti-HEV IgM antibodies against the open reading frame viral capsid (ORF2) protein or against open reading frame 3 (ORF 3) protein (Aggarwal, 2013). However, the sensitivity and specificity vary corresponding to the assay used in sample testing. In a comparative analysis of six assays in which anti-HEV IgM testing of samples HEV-RNA positive samples were used, the sensitivity ranged from 72% to 98% and the specificity from 78.2% to 95.6% (Drobeniuc *et al*., 2010).
In another study carried out among blood donors including people with suspected acute hepatitis, the concordance among five commercial assays was 70% (Norder *et al*., 2016). False negative results could be produced in patients infected with genotype (GT)1 strains. In fact, many diagnostic methodologies are based on HEV genotype-specific antigens, that limit the detection of all HEV genotypes (Herremans *et al*., 2007). In addition to the inconsistent performance of sensitivity and specificity among available immunoassays, false reactivity for anti-HEV IgM towards other hepatotropic virus such as Epstein Barr virus (EBV) and cytomegalovirus (CMV) has been reported (Fogeda *et al*., 2009; Hyams *et al*., 2014)
HEV-RNA detection is considered the main direct assay to assess HEV acute infection. It may be detected during the incubation period, it becomes undetectable in blood three weeks after the onset of symptoms, but it can be detected in feces for another two weeks (Kamar *et al*., 2015). Moreover, the low viremia that can occur in acute phase (serum HEV-RNA concentration in acute phase can falls to 2.1 Log copies/ml in immunocompetent patients) (Abravanel *et al*., 2013) can yield false negative results. Therefore, undetectable viral RNA does not rule out HEV acute infection.
A second direct assay is available to detect the HEV antigen. The antigen became detectable in serum almost simultaneously with the appearance of HEV-RNA in feces,

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but disappeared earlier than HEV-RNA (Zhao *et al*., 2009). In a previous study the authors demonstrated that HEV-RNA and HEV antigen were detected in 100% of patients in samples collected within one to three days of illness, and the positivity rate of older samples collected from the fourth day onwards. Detection of HEV antigen declined in samples collected after seven days, whereas positivity where observed for anti-HEV IgM, representing an indicator of acute infection (Majumdar *et al*., 2013). Recently it has been demonstrated that the sensitivity of HEV-Ag is related to the GT 3 subtype considered (Trémeaux *et al*., 2016), but no conclusive results concerning the main subtypes circulating in Europe have been reported. In this study we analyzed the sensitivity of HEV-Ag in samples belonging to patients with acute infection, anti-HEV IgM and HEV-RNA positive or negative and tried to verify the correlation between HEV-RNA viral load, HEV subtypes and HEV antigen positivity.

MATERIAL AND METHODS

Samples. Patient's samples were retrospectively analyzed. Samples can be classified into three category groups:

1) Samples drawn from patients with HEV acute hepatitis (n=94) resulting anti-HEV IgM and immunoglobulin G (IgG) positive, recent <4 weeks onset of jaundice, elevated aminotransferase (ALT) at least four times above the normal value.
2) Samples with a resolved HEV infection, anti-HEV IgG-positive and HEV IgM-negative, all immune competent (n=73).
3) Samples resulting anti-HEV IgM and anti-HEV IgG-negative collected from human immunodeficiency virus (HIV)-positive (n=38) patients.

Furthermore, to check the potential cross-reactivity between HEV-Ag and other viral antigens related to hepatitis acute infection, we tested an additional 46 serum samples, 15 of them were positive for HCV core antigen (HCV-Ag) (mean value: 4807.70 IU/ml range, 168.49-19168.91) (Abbott Diagnostics, Wiesbaden, Germany), five samples were positive for anti-hepatitis B virus (HBV) IgM-positive and for HbsAg, 14 samples were anti-hepatitis A virus (HAV) IgM-positive and HAV-RNA-positive, and 12 samples were anti-HAV IgM, anti-HCV IgG-negative and anti-HBs antibody-positive. For each patient ALT values were collected, when available using data from the Hospital repository. The day of HEV-RNA detection by PCR and HEV-Ag detection was defined as day 0 (T0).

Sera were tested for the presence of IgG and IgM antibodies to HEV using a commercial enzyme-linked immunosorbent assay (ELISA) (DIA.PRO, diagnostic Bioprobes, Milan, Italy) IgM antibodies to HAV, HBV and hepatitis B surface antigen (HbsAg) and antibodies to HCV (anti-HCV) were measured using Abbott ARCHITECT system (Abbott Diagnostics, Wiesbaden, Germany). The study was designed and performed according to the Helsinki declaration.

Anti-HEVAg. HEV-Ag was measured using the Wantai HEV-Ag ELISA assay kit (Wantai Biological Pharmacy Enterprise Co, China) according to the manufacturer's instructions. It is an ELISA for the qualitative detection of hepatitis E virus antigen in human serum or plasma samples. Results were expressed as signal to cut-off ratios (S/CO). Samples with a S/CO>1.1 were considered positive, whereas samples with S/CO antigen value between 0.9 and 1.0 were considered "borderline results".

HEV-RNA detection. HEV-RNA was extracted using the QIASYMPHONY automated extraction system (QIAGEN, Hilden, Germany). It was reverse transcribed and cDNA was amplified by nested polymerase chain reaction (PCR) using the primers for MTase included in ORF1 region (La Rosa *et al*., 2011). Both DNA strands were sequenced using nested primers (1681, 1682) in an automated 3100 genetic analyzer (Applied Biosystems Foster CA, USA), according to manufacturer's instructions. Sequences were aligned and edited separately using CLUSTALX v2.01 (Larkin *et al*., 2007) and Bioedit (Hall *et al*.,1999)

For HEV subtyping, HEV-RNA was tested with primers derived from a well-conserved region of ORF2, according to the previously described method for genotyping (Garbuglia *et al*., 2013; Inoue *et al*., 2006) ORF2 reference sequences were obtained from Smith (Smith *et al*., 2016).

Quantitative real time-PCR. Quantitative HEV-RNA detection was carried out with the hepatitis Ceeram@Tool Kit (Ceeram; S.A.S., La Chapelle sur Erdre, France) according to the manufacturer's instructions using Qiagen RotorGene Q MDX. This assay could detect as low as 300 cp/ml in our condition assay with genotype 3f, linear range 300-2.5x10^5 cp/ml.

Statistical analysis. Statistical analyses were performed using R 3.3.2 software. Descriptive statistics, mean and standard deviation or frequencies and percentage were reported. The association between gender and HEV-Ag results was assessed with Chi Square test.

The relations between HEV-RNA (Log cp/ml) and HEV-Ag (S/Co) levels were analyzed using non-parametric Spearman bivariate correlations, and Bland-Altman plot (Bland and Altman, 1986). The degree of agreement between HEV-RNA (positive/negative) and HEV-Ag (positive/negative) was assessed with Cohen's kappa.

RESULTS

To assess the HEV-Ag sensitivity to other acute hepatitis E-related markers (anti-HEV IgM positivity, HEV-RNA positivity), we tested the presence of HEV-Ag in an anti-HEV IgM-positive sample and samples 15/94 (16%) resulted HEV-Ag positive. Furthermore, among 30 HEV-RNA positive and anti-HEV IgM-positive samples, ten samples (10/30=33.3%) were HEV-Ag positive and two (2/30=6.6%) showed a borderline cut-off *(Table 1)*. Five anti-HEV IgM positive and HEV-RNA negative samples were positive to HEV-Ag (5/64=7.8%) (*Table 2*).

Overall, the concordance of HEV-RNA and HEV antigen was minimal, since Cohen's Kappa ranged from 0.29 (considering two HEV-Ag borderline results HEV-RNA negative) to 0.36 (considering two HEV-Ag borderline results HEV-RNA positive) (*Table 1*); 38 samples collected from immune depressed patients (i.e., HIV-positive) resulted HEV-Ag negative, according to HEV- RNA, IgG and IgM negativity.

The Bland-Altman plot also revealed a low agreement between HEV-RNA (cp/ml) and HEV-Ag (S/Co), (Bland Altman bias=-1.138, data not shown), confirmed by a not significant Spearman's correlation coefficient (rho=0.137, p>0.05, *Figure 1*). No correlation was found between an-

ti-HEV IgG level and HEV Ag positivity (data no shown).
The HEV-Ag assay was negative for all anti-HEV IgM negative samples tested (n=73), and in the 34 sera with markers of active replication of HBV/HAV/HCV, indicating 100% specificity. These results suggest that there is no interference between HEV-Ag, HCV-Ag, HBsAg, and HAV-viral capsid.
To assess if HEV-Ag sensitivity was influenced by HEV genotype or subtype we compared the HEV-Ag positivity to HEV genotype and HEV-RNA viral load. As shown in *Table 3*, HEV-RNA levels of <300 cp/ml led to negative results. In GT 3f samples with a viral load >800 cp/ml, HEV-Ag was positive in four out of five cases (80%), whereas all patients harboring genotype 3e were HEV-Ag negative irrespective of HEV-RNA viral load (HEV-RNA range 310-5000 cp/ml).
The two samples (patient 11, patient 3) in which HEV-Ag had a borderline value showed a low viremia level below 1000cp/ml, they harbored different genotypes 3f and 1. Among genotype 1 (samples n=11), HEV-Ag positivity (S/CO ≥1.1) was observed only in three (27.3%) patients, and in these samples the viremia was >2000 cp/ml.
Overall, for 20/30 patients with HEV-RNA positive results serial ALT values were available, and as shown in *Table 3* the ALT values at T0 were higher in HEV-Ag-positive patients (range, 865 to 2560) than in patients with negative HEV-Ag (range, 38 to 842) (median 1543 *vs* 171, p<0.05). In some patients (*Table 3*) we collected serial ALT values and T0 showed the highest ALT values, suggesting the early phase of acute viral hepatitis (AVH), while the low values observed in patients with negative HEV-Ag suggested that measurement of antigen was carried out in the late phase of acute symptoms.

Table 1 - *The Relationship between HEV Antigen detection and HEV-RNA positivity in the serum samples anti-HEV IgM+/IgG+.*

HEV-Ag	*HEV-RNA pos*	*HEV-RNA neg*	*Tot*
Positive	10	5	15
Negative	18	59	77
Borderline	2	0	2
Tot	30	64	94

Table 2 - *Results of HEV-Ag (Wantai) and HEV-RNA negative and ALT at T0 in anti-IgM+/IgG+ patients.*

Patient code	*HEV-RNA*	*HEV -Ag (S/CO)*	*ALT (d/m/y): ALT value*
1	negative	*7.0* (27/03/12)	*T0* 27/03/12: 361 04/04/12: 72
2	negative	*3.6*	na
3	negative	*5.4* (07/03/16)	*T0* 07/03/16:826 09/03/16:400 11/03/16: 229
4	negative	*1.5* (02/02/16)	na
5	negative	*2.1* (02/02/16)	na

HEV-Ag is positive with (S/CO) >=1, HEV-Ag is negative with (S/CO) <1 and borderline with values between 0.9-1.1; na, not available.

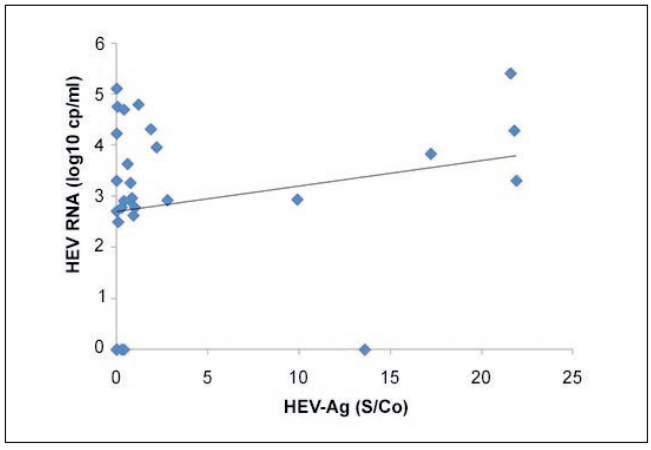

Figure 1 - *Correlation between HEV-RNA concentration and HEV-Ag in 30 patients.*

DISCUSSION

The choice of a sensitive and rapid execution method remains a prominent goal in the correct diagnosis of acute hepatitis E infection. As shown in the literature data both direct and indirect methods for diagnosing acute hepatitis are not yet optimal (Nan & Zhang, 2016). Studies concerning HEV antigen, detected both with homemade (Wen *et al.*, 2015) and commercial methods (Gupta *et al.*, 2013; Zhao *et al.*, 2015; Mishra *et al.*, 2016) gave discordant conclusions on its utility in clinical practice. In fact, in some works HEV-Ag showed a high sensitivity comparable to that of HEV-RNA (Trémeaux *et al.*, 2016), whereas other authors observed a lower sensitivity in in acute infections compared to IgM positivity or RNA detection (Mishra *et al.*, 2016; Behrendt *et al.*, 2016).
In our samples the concordance between anti-HEV IgM, HEV-RNA and HEV-Ag positivity was very weak. In fact, among 94 IgM positive samples only 16% (15/94) resulted HEV-Ag positive. This average is much lower than that found by Trémeuax (88%), and Zhao 59.2% (Zhao *et al.*, 2015), and 55.9% among Indian patients (Gupta et al., 2013). This dissimilarity could be explained by the time of sampling and HEV genotype present in the samples. In our study all analyzed samples belonged to anti-HEV IgM and IgG-positive patients, thus, most probably the patients were in the late phase of acute infection when HEV-Ag declines in peripheral blood (Majumda *et al.*, 2013). Furthermore, in the cited studies HEV genotypes were not indicated, so no conclusion could be drawn on the influence of HEV subtype in HEV-Ag measurement (Zhao *et al.*, 2015).
Overall, 33.3% of HEV-RNA positive samples were positive for HEV-Ag. Notably five samples (7.8%) were positive for HEV-Ag and negative for HEV-RNA (*Table 2*). This discrepancy could be due to the low level of HEV-RNA or viral acid nucleic degradation. It had been demonstrated that HEV-Ag is stable at room temperature over a period of ten days and it can also be included in "empty particles", that are produced after HEV-RNA clearance (Behrendt *et al.*, 2016). Furthermore, our findings underscore the influence of HEV GT subtypes in HEV sensitivity determination. In agreement with Trémeaux (Trémeaux *et al.*, 2016), among GT3f samples the HEV-Ag was positive in samples with a viremia >800 cp/ml, whereas none of

Table 3 - *Results of HEV-Ag (Wantai), quantitative HEV-RNA, HEV genotype or subtype and ALT in anti-HEV IgM+/IgG+ patients.*

Patient Code	HEV-RNA cp/ml	HEV genotype	HEV-Ag (S/CO)	ALT (d/m/y): ALT value
1	<300	1	0.3 (24/04/15)	na
2	2000	1	21.9 (06/06/15)	T0 6/6/15: 2560 9/6/15: 1934 15/6/15: 474 19/6/15: 280
3	<300	1	0.4	na
4	6720	3f	17.2 (26/01/16)	T0 26/01/16: 1250 04/02/16: 28 15/06/16: 62 27/10/16: 11
5	<300	1	0.4	na
6	19080	3	21.8 (02/05/16)	T0 02/05/16: 2320 12/05/16: 125
7	840	3i	2.8 (13/01/16)	T0 13/01/16: 1191 16/01/16: 489 19/01/16: 222 22/01/16: 107
8	50000	3e	0.4 (06/11/13)	06/11/13: 38
9	800	3e	0.4 (02/02/16)	26/01/16: 4752 T0 02/02/16: 358 16/02/16: 218 01/03/16: 93
10	<300	3f	13.6 (03/02/16)	T0 03/02/16: 865 27/02/16: 300 11/03/16: 197 21/03/16: 72
11	420	3f	0.92 (27/01/16)	T0 27/01/16: 34 14/05/16: 28 08/09/16: 33
12	310	3e	0.4 (16/02/16)	26/01/16: 4752 02/02/16: 358 T0 16/02/16: 218 01/03/16: 93
13	600	1	0.99 (02/05/16)	T0 02/05/16: 1836 06/05/16: 685
14	840	3i	0.75 (05/12/11)	17/11/11: 894 25/11/11: 612 T0 05/12/11: 120
15	90000	3f	2.2	na
16	504	3f	0.01 (24/02/12)	T0 24/02/12: 842 27/02/12: 354 03/03/12: 102
17	20600	1	1.9 (13/02/12)	na
18	4240	1	0.6 (17/05/12)	17/05/12: 120
19	1850	3	0.77 (15/01/13)	05/01/13: 124 T0 15/01/13: 85
20	924	1	0.84 (26/02/13)	T0 26/02/13: 469 04/03/13: 214
21	2030	3f	0.01	na
22	<300	3	0.01 (05/10/13)	T0 05/10/14: 531 10/10/13: 143
23	62000	1	1.2 (04/03/13)	T0 04/03/13: 902 11/03/13: 508 18/03/13: 218
24	251000	3f	21.6 (24/09/14)	T0 24/09/14: 1872 01/10/14: 671
25	<300	3	0.01 (23/05/14)	06/05/14: 1155 08/05/14: 715 10/05/14: 526 T0 23/05/14: 84
26	850	3f	9.9 (18/07/16)	na
27	56000	3	0.05 (14/04/16)	na
28	126000	1	0.01 (02/05/13)	na
29	600	1	0.3 (17/08/12)	na
30	17000	3	0.01 (31/10/14)	T0 01/11/14: 232 02/12/14: 168 29/12/14: 31

HEV-Ag is positive with (S/CO) >=1.1, HEV-Ag is negative with (S/CO) <1 and borderline with values between 0.9-1.0. All HEV-Ag positive values are indicated in bold. No subtypes was indicated in genotype 1 patients, since only one sample gave positive result (1a) with Inoue primer set. Alanine aminotransferase, ALT; na, not available; To, represents the time point when HEV-Ag was determined.

the GT3e samples resulted positive for HEV-Ag (range of viral load HEV RNA: 310-5000 cp/ml). Nevertheless, the ALT values were very low (i.e Patient 8, ALT=38), suggesting that the HEV-Ag determination was done in the late phase of acute hepatitis, thus this HEV-Ag negative value could be correlated to T0 and not to HEV subtype. The low sensitivity observed in our samples is apparently in contradiction with those described by Zhao, where a HEV-Ag analytical sensitivity corresponded to 50 IU/ml HEV-RNA. In this case, the WHO standard includes genotypes 3a and 3b, which was been observed among our samples, so the lower sensitivity should be related to GT3 subtypes analyzed. Other conflicting data have been reported on HEV-Ag sensitivity and genotypes. An Indian study indicated that acute genotype 1 infection displayed prolonged HEV-Ag detection over time despite the absence of RNA: HEV-Ag remained detectable in 88% of patients, while HEV-RNA was detected in only 54% of cases (Majumdar *et al.,* 2013).

Conversely, HEV-Ag became undetectable four weeks earlier than HEV-RNA during acute infection with HEV genotype 4 (Zhang *et al*., 2006). Further investigations are needed to assess how HEV genotype and/or subtypes could influence the sensitivity of HEV-Ag detection, similarly to that described in anti-HEV immunoglobulin detection (Ma *et al*., 2009).

This information can be particularly useful since the HEV-Ag could replace molecular tests, which are scarcely available in low income countries and the knowledge of the analytical sensitivity towards all the HEV genotypes gains

fundamental importance to assess the reliability of the test in HEV acute infection diagnosis.

The specificity of the test was instead found to be 100% both in the positive anti-HBIgM /HbsAg-positive samples as well as those HCV-Ag-positive. Furthermore, in contrast to what was reported by other authors (Behrendt *et al*., 2016), we did not observe any cross-reactivity with HAV viral capsid and HEV-Ag detection in positive IgM anti-HAV and HAV-RNA-positive samples. Moreover, this study has a limitation: Ag assay was not performed in samples with an ALT peak, and this aspect could influence the perfomance of assay, but unfortunately no serial samples were available for each patient to check HEV-Ag levels at different time points of acute infection.

Overall, these data suggest that anti-HEV IgM positivity represents the main biological marker of HEV acute infection in the clinical real life setting of developed countries, where patients with acute hepatitis symptoms were tested for HEV positivity long after the onset of symptoms due to lack of awareness among physicians of the presence of HEV in their geographical area. Therefore the employment of an assay with low sensitivity (i.e. HEV-Ag) could erroneously fail to confirm anti-HEV IgM results and which could cause an underestimation of acute HEV infection cases.

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Conflicts of Interest

The authors declare no conflict of interest.

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