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Short communication

# Use of hepatitis E IgG avidity for diagnosis of hepatitis E infection

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A B S T R A C T

The diagnosis of acute hepatitis E infection is based on the detection of HEV RNA or specific IgM in immunocompetent patients. Viraemia and excretion of HEV RNA in faeces are not observed in all patients and commercial kits vary in their performance for anti-HEV IgM detection. Additional diagnostic tests must therefore be considered. The value of anti-HEV IgG avidity index for differentiating between acute infection and previous exposure to HEV in countries of low endemicity was investigated. 132 specimens were included, with 39 serum samples from patients with known HEV infection, studied retrospectively. IgG avidity index was high (>60%) in patients with previous infection ($n = 16$) or polyclonal activation ($n = 3$) but was low (<40%) in patients with acute infection ($n = 20$). Then, 93 serum samples from patients, checking for acute hepatitis (detection of anti-HEV IgM but not of HEV RNA) were investigated. IgG avidity index was <40% in 77 of these patients, consistent with acute infection. It exceeded 60% in 15 patients, providing evidence of contact with HEV up to six months previously. One patient had an uninterpretable biological profile, with an IgG avidity index between 40% and 60%. IgG mature slowly during HEV infection, over a period of six months. IgG avidity index can therefore be used to exclude primary infection. This method should improve the diagnosis of acute hepatitis E.



Hepatitis E was first characterised in countries with poor sanitary conditions, but sporadic cases of hepatitis E have since been reported in many industrialised countries. Hepatitis E is recognised increasingly as a cause of acute hepatitis in the developed world, and has been found in North America, Europe, Japan and Australia. In France, the number of indigenous cases of hepatitis E infection diagnosed has increased dramatically, from 47 in 2006 to 97 in 2007 (Nicand et al., 2008), together with the number of samples tested for HEV markers ($n = 600$ in 2006 and $n = 1100$ in 2007). The genetic heterogeneity of hepatitis E has been characterised, and four major genotypes have been identified on the basis of sequence comparisons and phylogeny (Lu et al., 2006). Viruses of genotypes 1 and 2 are isolated most frequently in countries in which the virus is endemic and infect only humans. Genotypes 3 and 4 have been isolated from sporadic cases in endemic conditions and indigenous cases in industrialised countries. The sources of contamination remain unclear, but there seems to be an animal reservoir and similarities between human and porcine isolates have suggested that viruses of these two genotypes may be transmitted from animals to humans (Meng et al., 1997).

The diagnosis of hepatitis E infection is based principally on the detection of HEV RNA by RT-PCR or the detection of anti-HEV IgM in immunocompetent patients. Routine serological tests make use of enzyme immunoassays involving antigens derived from genotypes 1 and 2 viruses. However, commercial kits vary in performance for anti-HEV IgM detection. A Dutch study evaluated recently the performance of serological assays for diagnosing infections with genotype 3 hepatitis E viruses (Herremans et al., 2007a). This study concluded that the antibody levels detected following infection with genotype 3 were lower than those following infection with genotype 1. Factors complicating the diagnosis of acute HEV infection include a lack of sequential sampling in most cases, together with a high frequency of false-positive results due to the poor specificity of IgM ELISA. Increases in the number of sporadic indigenous cases to levels higher than the anticipated seroprevalence in regions in which the virus is not endemic complicate the interpretation of serological profiles. The IgG avidity index is widely used in the diagnosis of infections with other viruses, including rubella, cytomegalovirus, hepatitis A and West Nile virus (Hofmann and Liebert, 2005; Baccard-Longere et al., 2001; Roque-Afonso et al., 2004; Fox et al., 2006). The potential utility of IgG avidity index for diagnosing HEV infections was investigated. The diagnostic value of IgG avidity index for assessing changes in the antibody response was evaluated with a total of 132 sera in which anti-HEV IgG was detectable.

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**Table 1**
IgG avidity index as a function of patient group: group 1: past HEV infection; group 2: acute HEV infection; group 3: polyclonal activation (retrospective study) and group 4: prospective study.

| Patient groups | Group 1 | Group 2 | Group 3 | Group 4 | | |
|---|---|---|---|---|---|---|
| Sub-group (according IgG avidity index) | | | | <40 | 40–60 | >60 |
| *n* (total = 132) | 16 | 20 | 3 | 77 | 1 | 15 |
| IgG anti-HEV | + | + | + | + | + | + |
| IgM anti-HEV | – | + | + | + | + | + |
| HEV RNA | – | + | – | – | – | – |
| IgG avidity index (%) | 50–100 | 1.8–96.8 | 61.1–87 | 2–40 | 46.1 | 64–100 |
| Average of IgG avidity index (%) | 74.4 | 18.4 | 70.7 | 15.7 | 46.1 | 78.5 |
| Profile | Past infection | Acute infection | Polyclonal reactivation | Acute infection | Indeterminated | Polyclonal reactivation/non-specific reaction/reinfection |

Samples were collected from January 2006 to June 2008 at the National Reference Laboratory for Hepatitis E and were stored at −80 °C until use. Each serum sample was tested for anti-HEV IgG, IgM and HEV RNA. Several different profiles were included in the study. Past HEV infection was defined as the detection of anti-HEV IgG in asymptomatic individuals. Acute HEV infection was identified on the basis of positive detection of both anti-HEV IgM and HEV RNA. All the strains detected were of genotype 3. An additional profile was included in which immune polyclonal activation during Epstein-Barr virus infection was observed (positive anti-HEV IgM, anti-CMV IgM and negative HEV RNA). Anti-HEV IgG avidity index was first determined retrospectively for 39 specimens assigned to three groups on the basis of past and current HEV infection: group 1: 16 patients with past HEV infection; group 2: 20 patients with acute HEV infection and group 3: 3 patients with polyclonal immune activation (Table 1). For those patients, recent EBV infection was diagnosed by detection of anti-VCA IgM and IgG in a first serum sample and EBNA seroconversion in a second serum sample one month later. Sequential serum samples were tested for five patients from group 2. These samples were collected monthly over a period of four months ($n = 3$ patients) and after one year ($n = 2$ patients). Then, 93 serum samples collected from patients investigated for acute hepatitis E infection (group 4), with anti-HEV IgM detection but no HEV RNA detection (Table 1) were tested.

Serological tests for anti-HEV IgG and IgM with the EIAgen HEV IgG® and EIAgen HEV IgM® kits (Adaltis, Bologna, Italy), respectively, were performed according to the manufacturer's instructions. These indirect enzyme immunoassays tests are based on synthetic immunodominant determinants encoded by ORF2 (amino acids 619-660) and ORF3 (amino acids 101-123), derived from Burma virus (genotype 1) and the Mexican prototype strain (genotype 2). The IgM kit contained anti-IgG immunoglubulins to neutralise interference due to rheumatoid factor. For each serological marker (anti-HEV IgG, anti-HEV IgM), ratios of optical density (OD) to cut-off (CO) exceeding 1 were considered to indicate a positive result. The presence of anti-HEV IgM was confirmed with a second test (Assure® HEV IgM, MP Diagnostics, Illkirch, France) for the rapid detection of IgM antibodies. This assay is a solid-phase immunochromatographic IgM capture assay using a recombinant conformational epitope (amino acids 394-660). IgG avidity index was determined with the EIAgen HEV IgG® kit and a slightly modified version of the manufacturer's protocol. Briefly, two wells of a 96-well ELISA plate were used for each serum sample. The wells were treated as described in the manufacturer's instructions. After incubation of the serum in an antigen-coated plate for 45 min at 37 °C, three steps of washing using buffer in the first well and washing using buffer supplemented with 6 M urea in the second well were added. Plates were incubated for 5 min at 37 °C at each step. All wells were then washed six times with washing buffer. Negative and positive controls were included on each plate, together with an internal control, consisting of serum samples from two patients, one with previous and the other with current (acute) infection. A 10% difference in the median for five assays with these serum samples was considered acceptable for validation of the plates. IgG avidity index was calculated as a percentage, as follows: IgG avidity index (%) = (OD of well with urea/OD of well without urea) × 100. Values below 40% were considered to be low, and those above 60% were considered to be high. IgG avidity index values of 40–60% were considered equivocal.

For the molecular detection of HEV, total RNA was extracted from 200 μl of serum with the MagNA Pure LC RNA isolation kit (MagNA Pure LC instrument, Roche Diagnostics, Meylan, France). The HEV genome was detected in samples by amplifying the internal part of the ORF2 corresponding of the capsid protein (Cooper et al., 2005). A first fragment of 730 nt (5711–6441 nt) was obtained by RT-PCR and a fragment of 358 nt (5996–6343 nt) was amplified by nested PCR with specific primers. The 348 bp PCR product was visualised by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Genotype was determined by sequencing both strands of the DNA fragment with gene-specific primers, the DTCS sequencing kit and an automated DNA sequencer (CEQ8000, Beckman Coulter, Villepinte, France) and comparing the sequence obtained with HEV sequences available in GenBank.

The results of the retrospective study are presented in Table 1. Samples from patients with previous infections (group 1) had IgG avidity index values of over 50%. Most ($n = 18/20$) of the samples collected from patients with acute hepatitis E infection (group 2), had a low avidity, between 1.8% and 38.4%. Unexpectedly, two serum samples taken from patients with HEV viraemia displayed strong avidity (96% and 96.8%), confirmed in two independent assays. Serum samples collected from three patients with polyclonal activation of the immune system (group 3) had a high IgG avidity index (mean: 70.7%). Student's *t*-tests comparing avidity index values showed that the differences between groups 1 and 2 and between groups 2 and 3 were significant ($p < 0.05$) whereas the difference between groups 1 and 3 was not ($p = 0.7$; Fig. 1).

IgG avidity index for a pair of sequential samples (one taken during acute infection and the second taken nine months later) for two patients were compared. This comparison showed that IgG avidity index increased over this period, from 1.7% to 73% in one patient (patient a, Fig. 2) and from 23% to 75% in the other (patient b, Fig. 2). Lower avidity was recorded for the serum samples of three patients followed up over a period of four months, the final sample having an IgG avidity index of less than 40%, with no significant difference in avidity between the first and last samples (patients c, d, e, Fig. 2).

For patients studied for hepatitis E diagnosis (group 4), 77 specimens had an IgG avidity index value below 40%, consistent with acute HEV infection, whereas 15 patients had a high IgG avidity index value, exceeding 60% (Fig. 1 and Table 1). The biological features of these 15 patients are summarised in Table 2. Immunochromatographic test did not confirm IgM detection in

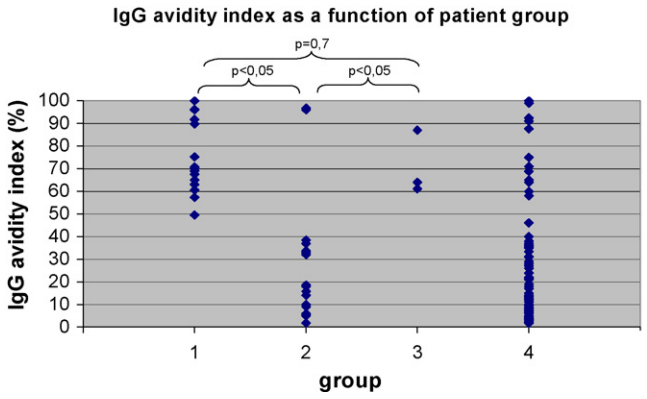


**Fig. 1.** IgG anti-HEV avidity index, as a function of clinical data.

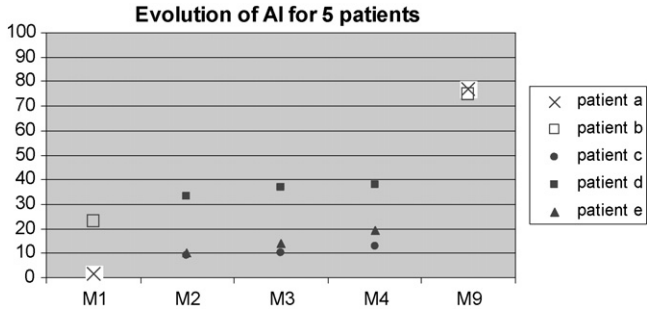


**Fig. 2.** Change in IgG avidity index for 5 patients (patients a and b from month 1 (M1) to month 9 (M9) and patients c, d and e from month 2 (M2) to month 4 (M4)) (sequential serum sample).

11 of these patients (1–11), whereas detection was confirmed in the other patients of group 4. Eight patients had a low IgM index (<2) in the IgM ELISA and four had an IgM index greater than 3. Patient 12 was known to have rheumatoid factor, but immunochromatographic test results were nonetheless positive for this patient. Polyclonal activation was excluded for this patient by the absence of detection of anti-VCA IgM and anti-CMV IgM. Polyclonal activation was demonstrated for patient 13. Patients 14 and 15 had a high IgM ratio in the ELISA that was confirmed by immunochromatographic test, with no polyclonal reactivation or rheumatoid factor. No detailed clinical history was available for these last two cases. Finally, one patient from group 4, with a low IgM ratio in the ELISA, had an IgG avidity index of 40–60%.

In countries in which HEV infection is non-endemic, genotype 3 is the genotype isolated most frequently, often from sporadic cases in elderly patients with no history of travel in zones in which the virus is endemic. The diagnosis of acute HEV infection is based on HEV RNA detection and specific IgM detection. Viraemia and excretion of the virus in faeces may last from a few days to a few weeks and are limited to the acute phase of the illness (Lin et al., 2000; Aggarwal et al., 2000). Viral load varies between patients, with values of $2.0 \times 10^3$ to $1.7 \times 10^7$ copies/ml reported (Takahashi et al., 2007). Furthermore, the detection of viral RNA may be intermittent (Zhang et al., 2002). As HEV RNA is not always detectable, diagnosis may require the detection of serological markers (Lin et al., 2000). IgM is detectable from the onset of symptoms, but careful interpretation of data concerning its presence is required for the diagnosis of acute HEV infection. IgM may be detected more than six months after the acute phase of infection (Favorov et al., 1992) and is also detected in cases of polyclonal activation of the immune system due to a virus with the same clinical presentation. In addition, most of the commercial tests available are based on an indirect ELISA that can generate false-positive results for samples containing high levels of rheumatoid factor not effectively neutralised by the neutralising reagent in the kit. Frozen samples containing fibrin particles or aggregates after thawing have also been shown to generate some false results. Additional methods of testing should therefore be considered, to overcome the problems of misdiagnosis associated with these discrepancies. The testing of sequential serum samples can be used to monitor increases in IgG titre, but such monitoring is not always possible, as most patients recover rapidly. A combination of immunoblotting and ELISA has been proposed (Herremans et al., 2007b) for the diagnosis of HEV infection in countries in which this virus is weakly endemic. The determination of IgG avidity index is an alternative method that is simple to perform, based on an ELISA, and has already been used for the diagnosis of many acute viral infections, including infections due to hepatitis A virus (Roque-Afonso et al., 2004). The immune system's initial response to any infection is the formation of low-avidity antibodies. As the infection proceeds, antigen-adapted IgG is produced, and avidity increases. IgG avidity index determination can therefore be used to distinguish between primary and past infection. This method has been shown to be relevant for distinguishing between recent infection with low IgG avidity index and past infection with high IgG avidity index in studies of hepatitis A virus and West Nile virus (Roque-Afonso et al., 2004; Levett et al., 2005). Avidity index values between 40% and 60% were considered to be in a grey area that is difficult to interpret.

All serum samples from patients with past infection tested in this study contained high-avidity IgG antibodies. IgG avidity index

**Table 2**
Serological profile of patients (group 4) with high IgG avidity index.

| Patient | Anti-HEV IgG OD/CO | Anti-HEV IgM OD/CO | IgG avidity index (%) | IgM capture assay | HEV RNA | Interpretation |
|---|---|---|---|---|---|---|
| 1 | 8.6 | 1.2 | 68.8 | Neg | Neg | Contact over six months |
| 2 | 7.6 | 1.5 | 59.9 | Neg | Neg | Contact over six months |
| 3 | 8.4 | 1.2 | 87.6 | Neg | Neg | Contact over six months |
| 4 | 9.4 | 2 | 91 | Neg | Neg | Contact over six months |
| 5 | 8 | 2.1 | 99 | Neg | Neg | Contact over six months |
| 6 | 1.3 | 1.3 | 65 | Neg | Neg | Contact over six months |
| 7 | 1.3 | 2.1 | 64 | Neg | Neg | Contact over six months |
| 8 | 4.4 | 1.2 | 75 | Neg | Neg | Contact over six months |
| 9 | 3 | 3.4 | 58 | Neg | Neg | Contact over six months |
| 10 | 4.7 | 4.5 | 71 | Neg | Neg | Contact over six months |
| 11 | 8.8 | 8 | 100 | Neg | Neg | Contact over six months |
| 12 | 8.7 | 11 | 75 | Pos | Neg | Rheumatoid factor or contact over six months or reinfection |
| 13 | 2.9 | 10.62 | 77 | Pos | Neg | Polyclonal reactivity |
| 14 | 8.5 | 7.14 | 92.4 | Pos | Neg | Contact over six months or reinfection |
| 15 | 8.4 | 9.65 | 92.5 | Pos | Neg | Contact over six months or reinfection |

was low in most cases of acute infection, as reported previously (Bendall et al., 2008; Zhang et al., 2002). IgG matures slowly in HEV infections and those results suggested that 15 of the cases had been in contact with the virus over six months, with two patients followed up over nine months having an avidity index greater than 60%.

Patients presenting documented polyclonal activation had high IgG avidity index values similar to those obtained for patients with past infection. Unexpectedly, two immunocompetent patients with recent HEV infection had high IgG avidity index values despite having concomitant viraemia. In group 4, patients number 14 and 15 had very high IgM in combination with very high IgG avidity. Reinfection is the most likely explanation and more patients should be studied in the future. Several studies have suggested that reinfection with a different type of HEV may occur. Bendall et al. (2008) also described one patient with acute HEV infection and high IgG avidity index. However, no earlier serum sample was available to determine whether this patient displayed reinfection. Seriwatana et al. (2002) described a typical memory response, characterised by low levels of IgM and extremely high levels of IgG, suggesting possible reinfection. Ke et al. (1996) reported a failure to develop IgG after acute infection in some patients. The risk of reinfection is unclear for such patients. The duration of natural immunity is also not well established. Serological investigations in primates have suggested that anti-HEV IgG may be detected for many years and that low antibody titre can protect against reinfection (Arankalle et al., 1999). Previous infection in monkeys may give cross-genotype and cross-host species protection, although viral replication has been detected in rechallenged monkeys without hepatic cytolysis (Huang et al., 2008).

No gold standard method could be identified, given the wide range of performances observed for commercial serological kits. The EIAgen HEV IgM® kit appeared to be less specific than the immunochromatographic test for detecting IgM, suggesting that cross-reactivity may occur, as reported for flavivirus infections (Koraka et al., 2002). Previous serological studies have evaluated the performance of both tests and showed a possible persistent response (over six months) with ELISA. By contrast, the anti-HEV IgM were detected on a shorter period (three months) with a rapid test (Legrand-Abravanel et al., 2009). In 11 cases of patient with high IgG avidity index, IgM detection by indirect ELISA was not confirmed with a different method (rapid test), suggesting contact over six months. In this sub-group, discrepancy between the two IgM anti-HEV tests can be explained by the difference in antigen targets.

The presence of rheumatoid factor and polyclonal activation may lead to false-positive IgM detection using indirect ELISA. However, IgG avidity index determination cannot differentiate past infection from recent infection when the IgG avidity index value obtained is between 40% and 60%, corresponding to the defined grey zone.

Given the limited performance of serological markers, IgG avidity index is a useful tool that is simple to use for the diagnosis of HEV infection. As IgG matures slowly in HEV infection, over a period of six months, IgG avidity index can be use to exclude recent infection in cases of polyclonal activation of the immune system or non-specificity of the IgM ELISA (Bendall et al., 2008). Given the problems encountered for the detection of viral RNA or anti-HEV IgM, combinations of markers are required for reliable diagnosis. IgG avidity index may therefore help to improve the diagnosis of acute hepatitis E infection.

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