Serology versus nucleic acid amplification to diagnose acute hepatitis E, United Kingdom, 2014–18

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\textbf{S U M M A R Y}

Objectives: Diagnosing hepatitis E infection usually involves specific IgM testing, but sensitivity/specificity concerns mean many guidelines and practices include confirmatory tests. We studied whether additional information confirmatory tests provide justifies their use.

Methods: We examined 9131 records of anti-hepatitis E IgM assays, 7615 of IgG assays, and 1726 of RT-PCR assays from our regional laboratory, spanning October 2014–October 2018. We paired 495 IgM assay results with a RT-PCR result. We examined whether IgM results predicted PCR results, reviewed discrepant pairs, and investigated the correlation between IgG and PCR results in patients with strongly reactive IgM assays.

Results: Anti-hepatitis E IgM titres are bimodal. A high cut-off value optimises prediction of RNA detectability. 7/404 low-IgM samples had detectable RNA, 6 from immunosuppressed patients. 26/91 high-IgM samples did not have detectable RNA. In high-IgM samples, RNA detectability was not associated with IgG titre (one-tailed Mann-Whitney U test, \(p = 0.14\)).

Conclusions: In immunocompetent patients, tests beyond IgM seldom add clinically useful information. In patients with immunocompromise, IgM and RNA could contribute information. Additional tests\’ extra costs/intervention delays cannot be justified. IgM assay cut-offs should reflect titres\’ bimodal distribution, with values standardised using international units.

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\textbf{Introduction}

Hepatitis E viruses are small, positive-sense RNA viruses. Four main genotypes are known to infect humans, with genotypes 1–2 associated with faecal-oral transmission in the developing world, and genotypes 3–4 associated with zoonotic transmission from undercooked meat in the western world. There are case reports of human infection by other zoonotic genotypes.\textsuperscript{1,2} Infection is usually asymptomatic. Severe disease can cause fulminant hepatitis and death; extra-hepatic manifestations can be seen. There is a particular association between pregnancy and severe disease.\textsuperscript{3,4} There is a single serotype of hepatitis E virus (HEV) infection, but immunity is not lifelong and reinfection is possible. HEV genotypes 1 and 2 were estimated to have caused approximately 20.1 million infections, 3.4 million symptomatic cases, 70,000 deaths and 3,000 stillbirths worldwide in 2005.\textsuperscript{3} HEV infection is estimated to cause approximately between 60,000 and 100,000 infections in the United Kingdom annually.\textsuperscript{5,6} A vaccine exists but is not widely deployed and there is no specific treatment for acute infection.\textsuperscript{7,8}

Clinical laboratory diagnosis of HEV infection is made either by amplification of HEV RNA or by detection of antibodies against HEV. However, anti-HEV IgM may persist for many months,\textsuperscript{9} whereas HEV RNA may be undetectable in blood by the time specialist tests are undertaken, even in those with symptoms.\textsuperscript{10} Possible cross-reactivity leading to reactive anti-HEV IgM plus anticytomegalovirus (CMV) IgM and/or anti-Epstein-Barr virus (EBV) IgM assays, when it is not clear that more than one active infection is present, has been reported.\textsuperscript{11,12}

In 2018, the National Infection Service, Public Health England (since succeeded by the UK Health Security Agency) introduced a new set of Standards for Microbiology Investigations for HEV infection.\textsuperscript{13} These standards offer a variety of laboratory pathways for testing for acute HEV infection in the immunocompetent, which amount to testing for HEV IgM assay reactivity, and then using IgG assay reactivity or RNA amplification in blood to exclude a non-specific IgM reactivity, with the presence of either IgG or RNA
sufficient to diagnose acute infection and the presence of neither leading to a conclusion of non-specific IgM assay reactivity. This strategy assumes that, in most infections, either there will be viraemia or IgG will have become detectable. In the immunocompromised, HEV RNA in blood alone is used to diagnose or exclude infection.

Also in 2018, the European Association for the Study of the Liver introduced a set of Clinical Practice Guidelines for HEV infection, which advocated testing HEV IgM, IgG and RNA amplification in all patients with symptoms consistent with acute hepatitis, although these Guidelines acknowledged that in some situations not all tests will be undertaken. In 2019, the European Centre for Disease Prevention and Control issued its own testing and surveillance guidelines, which advocate either RNA amplification, or both IgM and IgG assay reactivity, as criteria for diagnosis of HEV infection. The World Health Organization recommends diagnosing HEV infection by IgM alone in high-prevalence regions, and additionally undertaking RNA amplification in blood and stool in low-prevalence regions.

A testing pathway that uses IgG or nucleic acid detection to confirm an initial IgM result contains the implicit assumption that the risk of nongspecific IgM reactivity and consequences of a false positive based on this alone outweigh both the risk of a false negative result from confirmatory tests, and the risk of harm from confirmatory testing leading to delayed reporting of a positive result. We therefore sought to evaluate these assumptions by investigating results from HEV testing in our regional diagnostic laboratory.

Materials and methods

The Cambridge Clinical Microbiology and Public Health Laboratory is a regional clinical laboratory in the East of England. Samples were received within the routine diagnostic service.

Serum was tested respectively for anti-HEV IgM and IgG using the FORTRESS HEV-IgM ELISA and FORTRESS HEV-IgG ELISA (Fortress Diagnostics Limited, Antrim, United Kingdom), with assays performed either manually or using a DYNEX DS2 system (Dynex Technologies, Chantilly, Virginia, United States of America). These assays derive a cut-off value by taking a light absorbance offset from the mean of three negative control values, and reporting test results as a ratio (index value) with respect to this cut-off value. The manufacturer recommendation is that a ratio <0.9 should be reported negative, a ratio >1.1 should be reported positive, and a ratio between 0.9 and 1.1 inclusive should be regarded as borderline; borderline results are retested and the finally reported result is that of the final retest (where the retest result remained borderline, the final report was “indeterminate”). Laboratory standard operating procedure required ratios of borderline and positive, but not of negative, reports to be recorded.

HEV RNA was detected by RT-PCR using a previously described assay. In summary, the assay targets a region of the HEV ORF-2 region that is conserved across genotypes; samples with amplification and a cycle threshold (Ct) ≤35 are reported as RNA detected.

Details of all HEV tests undertaken between October 2014 and October 2018 inclusive were extracted from the laboratory database. Tests for HEV IgM and RNA undertaken on samples drawn from the same patient less than 24 h apart were correlated. Such tests may have been run on the same sample, or on samples drawn closely in time from the same patient.

Receiver operating characteristic analysis of PCR result prediction by IgM assay result was undertaken using R, version 3.6.3, with the PRROC package. Statistical testing of association between IgG index values and PCR positivity was undertaken using built-in functions in Mathematica 12.1.

Following receiver operating characteristic analysis, records were reviewed corresponding to patients with HEV IgM results above the predicted cut-off. Results of CMV and EBV VCA IgM assays (LIAISON platform, Diasorin, Saluggia, Italy; second-line CMV IgM assay VIDAS platform, bioMérieux, Marcy-l’Etoile, France) and PCRs for CMV and EBV DNA (in-house assay; polymerase gene targets) for these patients were collated, for any tests undertaken within two months of the HEV IgM result (two months was chosen as a time window to allow for additional testing for the same acute presentation to have been undertaken at a return visit).

To inform consideration of external validity of the results derived, we collated descriptive statistics regarding age and sex of patients who provided samples tested for HEV IgM but not RNA, for RNA but not IgM, and who provided samples for paired testing. For samples requested from within our local tertiary hospital (where our laboratory is based), we used data on the requesting clinician’s speciality, the speciality of single-speciality clinics from which samples were sent, and, where necessary, review of individual patient notes, to identify the speciality of the clinician who requested HEV testing. Where testing was suggested during a consultation by another speciality, that speciality was listed as having requested testing. A full description of the procedure followed to determine requester speciality may be found in Fig. S1.

This study was confirmed by use of the UK Health Research Authority decision tool and accompanying documentation to be a service evaluation not requiring ethical approval.

Results

We examined 9131 records of HEV IgM assays, 7615 records of IgG assays and 1726 records of RNA RT-PCR assays. In 495 cases we paired an IgM assay result with a RT-PCR result (Fig. 1).

More detailed examination of samples demonstrated a clearly bimodal distribution of quantitative IgM assay results. Examination of paired samples showed that the first mode clearly corresponded primarily to IgM non-reactive samples but with a tail extending into the positive reporting range, and almost no samples had HEV RNA detectable by PCR. The second mode clearly corresponded primarily to most samples where HEV RNA was detected by PCR, but around one quarter of the samples clustering around that mode did not have detectable RNA (Fig. 2).

The presence of a clearly bimodal distribution of IgM index values with well-separated clusters about the modes motivated us to consider whether it would be possible to set an appropriate index value cut-off to discriminate between these clusters. Receiver operating characteristic analysis of the use of IgM index value to predict PCR positivity showed a maximised Youden index for any cut-off values between 3.931 and 4.386 inclusive, with a true positive rate of 0.90 and a false positive rate of 0.06 (Fig. 3).

We then proceeded to investigate samples that yielded a discordance between the prediction of PCR positivity from the IgM index value and the results of PCR testing. Our dataset contained 91 samples with IgM values above the cut-off, and 404 samples with IgM values below the cut-off. Firstly, we considered samples with a low IgM index value (below the cut-off) and RNA detection by PCR. There were seven such samples corresponding to five distinct patients (Table 1). Four of the patients had electronic records from our local hospital, and it was possible to determine that all four of these patients were immunosuppressed. One of the samples was sent by a general practitioner and there were no electronic records from our local hospital. The patient’s general practice lay within our hospital’s catchment area, allowing us to deduce that the absence of records meant it was unlikely that the patient had been heavily immunosuppressed (since the patient had never been reviewed in an outpatient clinic). This patient had a strongly reactive HEV IgG assay, and the C5 of the HEV PCR was 35, with a good sigmoidal curve.

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Secondly, we considered samples with a high IgM index value (above 3.931) and RNA non-detection by PCR. There were 26 samples, corresponding to 22 patients. According to the current United Kingdom Standards for Microbiology Investigations, these samples should be further investigated by IgG assay, so we looked for contemporaneous IgG assay results. 19 samples, corresponding to 16 patients, had an IgG index value above 8. 4 samples, corresponding to 3 patients, had an IgG index value below 1. 3 samples, corresponding to 3 patients, did not have IgG assay results (we note that testing was undertaken prior to publication of the Standards for Microbiology Investigations requiring the assay to be performed). Of these 3 patients, one had a preceding strongly reactive IgG assay, and the other two had falling alanine transaminase levels. All samples with low IgG index values were submitted from external hospitals and no further clinical details were available. Of the samples with high IgG index values, one was from a patient with transformed chronic lymphocytic leukaemia, one was from a patient with compensated cirrhosis secondary to hepatitis C virus, and five samples were follow-up testing samples (with the largest interval between first sample and follow-up testing being 14 months).

The Standards for Microbiology Investigations recommend using at least one of RNA detectability and IgG reactivity to determine exposure to HEV. This recommendation carries the implicit assumption that as RNA becomes undetectable, IgG titres will have increased to a level of detectability. We therefore investigated the association between PCR positivity and level of IgG reactivity in samples with a high IgM index value (above 3.931). There was no apparent association between IgG index value and PCR positivity in these samples (one-tailed Mann-Whitney U test, n = 84, p = 0.14, noting the relatively small sample size; Fig. 4).

Because of the potential for cross-reactivity involving HEV, CMV and EBV assays, we investigated the association between HEV PCR positivity and signals of CMV and EBV detection in samples with a high HEV IgM index value (above 3.931). 59/91 and 58/91 samples had any results available for CMV and EBV, respectively. 4/47 HEV PCR positive and 0/12 PCR negative samples had any suggestion of CMV reactivity (IgM or PCR; where a second-line IgM assay was performed following an indeterminate first-line assay, the second-line result only was considered). 5/46 HEV PCR positive and 1/12 PCR negative samples had any suggestion of EBV reactivity (VCA IgM or PCR). Statistical testing results for these data are not reported owing to confounding from HEV testing being more often requested when no other possible cause of hepatitis had been identified.

Finally, to evaluate external applicability of conclusions drawn, we considered the demographics of samples where IgM and RNA testing could be paired versus the overall datasets. The groups had similar age and sex profiles (Figs. S2 and S3). External requesters were over-represented in IgM testing in comparison with RNA testing and paired testing (Fig. S4). In each category of testing, most samples sent by the local tertiary centre were assigned to one of the hepatology, haematology, or transplant specialties. Few samples were received from other specialties that might see patients presenting with extrahepatic manifestations of HEV infection, or from obstetricians.

**Discussion**

Guidelines requiring confirmation of HEV IgM-reactive samples by assaying a different marker of infection are predicated (implicitly or explicitly) on the idea that there is a clinically significant overlap between non-specific low-level reactivity in an IgM assay and low-level reactivity in the presence of disease. The clearly bimodal reactivity distribution we have demonstrated is inconsistent with this interpretation for most patients. Moreover, we have shown that for most patients, a low HEV IgM (including a low IgM in the manufacturer-defined positive range of the assay evaluated) is a strong predictor of RNA non-detectability by PCR. This means that if there is ongoing clinical suspicion of infection, PCR is unlikely to provide additional information and may even provide false reassurance. The key clinical/public health implication of this finding is that in most situations, decisions should be made based on an IgM result alone, without incurring extra cost and delaying action whilst awaiting further testing. Accordingly, we recommend that guideline writing groups should consider revising in accor-

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**Table 1**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>IgM index value</th>
<th>PCR Ct</th>
<th>IgG index value</th>
<th>Clinical details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>20</td>
<td>3.941</td>
<td>Chronic myeloid leukaemia (progressed soon after to acute myeloid leukaemia)</td>
</tr>
<tr>
<td>2</td>
<td>1.438</td>
<td>23</td>
<td>5.027</td>
<td>Haemopoietic stem cell transplant 5 months prior to episode</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>20</td>
<td>Not done</td>
<td>Methotrexate/ctyramine in preceding 2 weeks for lymphoma</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>24</td>
<td>0.033</td>
<td>General practice sample; no further details</td>
</tr>
<tr>
<td>5</td>
<td>2.255</td>
<td>24</td>
<td>5.868</td>
<td>Chronic myeloid leukaemia (progressed soon after to acute myeloid leukaemia)</td>
</tr>
</tbody>
</table>

* Where serology assays were reported negative, laboratory protocol permitted reporting an index value of 0.
which and PCR proportion distribution sponding Fig. J.P.ues). are not plotted detected. 4. Histograms of IgM assay result value to cut-off value ratios (index values). A: all samples, B: only samples with corresponding PCR results; results corresponding to RNA detection ("PCR positive") and RNA non-detection ("PCR negative") are plotted below and above the x-axis, respectively. In both cases a clearly bimodal distribution can be seen. When PCR results are considered, it becomes clear that the second, higher mode corresponds mainly to samples where RNA was detected, although within that cluster is a substantial minority of samples in which RNA was not detected.

Fig. 3. Receiver operating characteristic curve for prediction of PCR positivity from IgM index value. The cross represents the point with maximal Youden index (equivalent to maximal sum of sensitivity and specificity of IgM for predicting PCR positivity), which occurs for any IgM index value cut-offs between 3.931 and 4.386 inclusive.

dance with this implication the aspects of their guidelines pertaining to which diagnostic assays should be undertaken. (Our work does not recommend any changes to the indications for requesting testing.)

There are three main patient groups in whom this interpretation must be treated with care, and in whom PCR testing is still indicated. The most important group is those with immunosuppression, particularly those in whom a good IgM response cannot be expected. This group accounts for all but one of the low-IgM, PCR-positive samples in our dataset. Our findings are consistent with another study in which all samples with detectable HEV RNA but non-reactive IgM assays came from immunocompromised patients.21 The second group is those with known historical HEV infection, who may not mount a substantial IgM response upon reinfection. Past HEV infection is a plausible explanation for the high IgG, low IgM, late amplifying RNA sample from a patient not

Fig. 4. Histograms of IgG index values for samples with a high IgM index value, grouped by whether HEV RNA was detected by PCR. The histograms are plotted below and above the x-axis, respectively, for detection and non-detection. The histograms have been normalised to show proportions of samples within categories (i.e. the total proportion of samples in each of the PCR positive and PCR negative groups is 1), to allow easier comparison between samples in which RNA was detected and samples in which RNA was not detected. The chart corresponds to 23 PCR negative and 61 PCR positive samples. There is no evidence of association between lower IgG index value and PCR positivity (one-tailed Mann-Whitney U test, p = 0.14).
known to be immunosuppressed in our dataset. (It is also plausible that low-level contamination from positive control material caused a false positive PCR result.) We note that despite the high annual incidence of HEV infection, and likely persistence of elevated risk for zoonotic infection in individual patients, we observed this phenomenon in only one of 404 paired samples with low HEV IgM titres. This suggests that reinfection with low IgM titre is sufficiently rare not to indicate routine PCR testing of all samples. The third group is those with extrahepatic manifestations of HEV infection. It is uncommon for diagnostic laboratories to receive sufficiently detailed clinical histories to determine the presence of possible extrahepatic manifestations; we note that very few samples in our dataset were requested by specialists whose involvement one might expect in evaluating florid extrahepatic signs of infection. Because of this lack of data, the small number of patients involved, and the biologically plausible idea that viraemia might be present more commonly in extrahepatic infection, it would be more appropriate to undertake testing for HEV RNA in those with extrahepatic manifestations of HEV infection.

Whilst it may be possible to obtain yet further granularity in testing and interpretation with an appropriate clinical history regarding timing of symptom onset and hepatic transaminase results, it is important to understand that such histories are seldom provided to diagnostic laboratories alongside testing requests, and even if they were, the high-throughput nature of laboratories renders it impracticable for those booking in requests at the initial, high-volume stage, to apply more than straightforward interpretation of what assays should be undertaken. The general rule we propose of testing IgM only in most cases, with clearly defined exceptions that can be captured on a request form, represents what we believe is the best response to the realities of the challenges faced by the typical clinical diagnostic laboratory.

Varying sensitivities of HEV IgM testing have been reported in the literature. Our results emphasise the point that there is no gold standard test for infection status. Consequently, reports of sensitivity depend upon the gold standard utilised, and so are not directly comparable. Many reported gold standards contain IgM positivity as one of the criteria. It is important to emphasise that the goal of a clinical laboratory is to facilitate clinical management of patients and public health responses. Test accuracy is only one component contributing to this goal. Further tests that give more accurate information must be justifiable by the delay to management they incur and the cost effectiveness of undertaking the extra testing.

Our results did not show a substantial overlap between strongly reactive HEV IgM and markers of infection with CMV or EBV – and where seen, overlap was more common in samples with detectable HEV RNA. However, because during this period HEV markers were not always tested when another infective cause of hepatitis was suggested by prior testing, it is not possible to determine the true extent of overlap in reactive IgM markers. In cases where results raise concern for serological cross-reactivity, and where it remains clinically relevant to determine which virus or viruses explain a patient’s presentation, then follow-up PCR could be considered.

In common with many assays in clinical use, the assay used in our study does not give antibody titres in international units, limiting the applicability of our numerical results to other assays. The use of assays with standardised outputs would allow manufacturer-independent guidelines for cut-offs to be used in HEV IgM titres, and could promote movement towards an international consensus strategy for testing for acute infection. At least one attempt at widespread standardisation of assay implementation has been made, but the key additional step required to allow clinically useful cut-offs to be set is the standardisation of reporting between different assays. Failing this, assays should be validated against a broad collection of serum samples to ensure cut-offs reflect the broad range of non-specific reactivity that can be seen in human populations. Our results do clearly show it is possible to use an IgM assay that dichotomises most samples into being either effectively non-reactive or strongly reactive.

The presence of at least one patient with unequivocal immunosuppression in the antibody-reactive, RNA undetectable cohort, plus approximately one quarter of patients with high IgM being RNA negative, raises the possibility that RNA testing alone for acute HEV infection in immunosuppressed patients may lack sensitivity when compared with combined RNA and IgM testing.

HEV IgM is known to persist for a long time, up to many months following infection. The presence of samples that are strongly reactive for HEV IgM but that pair with RNA-undetectable samples may reflect the passage of time since onset of immune response, and this would fit with there being very few correlated samples where IgM, but neither IgG nor RNA, was detected. However, the lack of significant correlation between IgG titre and RNA positivity in such cases raises an alternative explanation that viraemia may not be reliably present early in infection and/or may persist longer than previously thought in some patients (Fig. 5). If this second scenario were the case, there may be important implications for infection prevention and control and consequently public health guidance. Directly determining the duration of viable HEV shedding in stool is therefore a key open question. We caution that the lack of correlation between IgG titre and RNA positivity was determined with a relatively small sample size (total of 84 samples). We also caution that a reliable estimate of the power of the Mann-Whitney U test undertaken here is impracticable, because standard methods of calculating the power of the Mann-Whitney U test assume an underlying data distribution.

European testing guidelines list a rising HEV IgG titre in serial samples amongst possible diagnostic criteria for infection. Our data do not speak to this strategy, as the receipt of serial samples was too rare for analysis. As a rising IgG titre cannot be determined from a single sample, and as most clinical laboratories would be able to return to stored samples to assay IgG in the small number of cases where treating clinicians deem follow-up testing clinically relevant, the overall most efficient testing strategy for most laboratories would be to only assay IgM first-line, and to consider testing IgG on original and repeat samples in the few cases where follow-up sampling is undertaken.

Because our laboratory used HEV IgM as the screening assay in immunocompetent patients, it is possible that our study overlooked entirely some patients with undetectable IgM who would have had detectable RNA. Viraemia preceding IgM response has been observed in experimental primate infections and has been suggested as the explanation for discordant IgM versus RNA assays in one human study, although that study did not go on to demonstrate subsequent development of IgM response. This would likely only affect clinical management in critically ill patients, in whom RNA could be assayed if another cause of hepatitis were not found. In other patients, convalescent serology could make a diagnosis. In addition to these patient groups, the potential issue of overlooking viraemia preceding IgM response applies to well allogenic haemopoietic stem cell donors, in whom the purpose of testing for acute HEV infection is not to explain clinical syndrome or biochemical markers, but to look for infectious viruses. It is not possible from this study to determine which strategy is optimal for detecting asymptomatic or pre-symptomatic infectious individuals, although it would be logical to presume that detection of RNA in peripheral blood would correlate best with infectiousness from haemopoietic stem cell donation.

Our data do not speak to two further indications for assaying HEV RNA: firstly, determining the status of chronic infection (in immunocompromised individuals); secondly, with a view to geno-
typing for prognostication (although confounding between routes of transmission and geographical risk may limit the value of genotyping).

We have not studied the use of assays on stool for the diagnosis of acute HEV infection. In many outpatient clinical settings, it is less practical to request a stool sample than to perform testing on blood samples sent to test for multiple causes of acute hepatitis. One study has suggested that, overall, RNA detection in stool is a less sensitive test than RNA detection in serum, although it did identify patients with detectable RNA in stool but not in serum. 25

Table 2 summarizes our recommendations for testing arising from this study.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2022.06.017.

References