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Diagnostic performance of selected commercial HEV IgM and IgG ELISAs for immunocompromised and immunocompetent patients

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ABSTRACT

Background: Hepatitis E virus (HEV) genotype 3 is recognised as an emerging pathogen in industrialised countries. The currently commercially available HEV-specific enzyme linked immunosorbent assays (ELISAs) are primarily designed for the detection of antibodies against genotypes 1 (Burma) and 2 (Mexico) and may not sensitively detect HEV genotypes 3 or 4.
Objectives: This study aimed to evaluate the analytical and clinical performances of eight commercially available HEV serum antibody immunoglobulin M (IgM)- and immunoglobulin G (IgG)-specific ELISAs for genotype 1 and 3 HEV infections in a clinical setting and to study the antibody responses against HEV of immunocompromised versus immunocompetent patient groups.
Study design: Analytical performance and diagnostic sensitivity and specificity were assessed using well-defined reference samples and samples from patients with polymerase chain reaction (PCR)-confirmed HEV infection ($n = 88$) and a specificity panel ($n = 98$).
Results: Limiting dilutions indicated that the highest analytical sensitivity in head-to-head comparison was measured for the Mikrogen_new IgG assay. Taking the serum working dilutions of each assay into account, the Wantai IgG assay was the most sensitive assay. Receiver operator curve (ROC) analysis showed area under the curve (AUC) values of 0.943, 0.964, 0.969, 0.971, 0.974 and 0.994 for the DSI, Mikrogen_old, MP Diagnostics, Mikrogen_new, Wantai and DiaPro anti-HEV IgM assays, respectively. The highest specificity of currently available assays was found for the IgM Wantai assay (>99%). If anti-HEV IgM and IgG results from each supplier were combined, DSI and Wantai assays were able to detect the highest number of (passed) HEV infections.
Conclusions: Our study showed that current commercial HEV ELISAs could be used to diagnose HEV genotype 3 infection adequately in a clinical setting.

1. Background

Hepatitis E virus (HEV) is a positive-sense, single-stranded RNA virus that causes sub-clinical, acute and chronic infections, characterised by hepatitis, though extra-hepatic manifestations have been described. Four genotypes are known to infect humans (genotype 1–4), the epidemiology and geographical distribution of which differs between genotypes 1–2 and 3–4. HEV genotype 3 and, to lesser extent, genotype 4 is recognised as an emerging pathogen in industrialised countries [1,2] and it can cause chronic hepatitis in immunocompromised individuals leading to rapid fibrosis of the liver [3].

HEV infection is diagnosed by laboratory testing since its clinical presentation does not differ from other pathogens causing hepatitis. Till this date, the virus cannot efficiently be cultured for diagnostic purposes; thus, detection of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies and virus RNA are the modes to confirm HEV infection [4].

In immunocompromised patients (e.g., solid organ transplant recipients and haematological patients) antibody production is often delayed [5], and detection of HEV RNA is suggested to diagnose HEV infection in this patient group [6]. In immunocompetent individuals, the narrow window in which HEV RNA can be detected in serum or faeces is confined to the acute phase of the disease

Abbreviations: HEV, hepatitis E virus; ELISA, enzyme linked immunosorbent assay; RT-PCR, reverse transcriptase polymerase chain reaction; RNA, ribonucleic acid; ORF, open reading frame; WHO, World Health Organization; LIMS, laboratory information management system; EBV, Epstein–Barr virus; CMV, cytomegalovirus; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; OD, optical density; S/N, signal to noise; ROC, receiver operator curve; AUC, area under the curve.

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Table 1
Sample distribution of diagnostic sensitivity panel.

Time of drawl (*t* =)	Samples (*n* =)	Patients (*n* =)	Samples per immune status[a] (*n* =)			Samples per genotype (*n* =)		
			IC	ICT	Unkn	geno1	geno3	Unkn
Prior to infection	12	12	12	0	0	0	9	3
<6 wks	34[b]	31	16	14	4[b]	7	18[b]	9
>6 wks <6 mos	22	19	15	4	3	0	16	6
>6 mos	20	16	16	3	1	1	17	2

[a] ICT Immunocompetent, IC = immunocompromised, Unkn = unknown.
[b] One sample was excluded in the Mikrogen_new assay due to sample volume.

(mean 28, range 17–48 days) [1]. Therefore, serology is needed to diagnose HEV infection in patients who present themselves after a viraemic period.

Although HEV has one serotype, the role of currently commercially available serological assays is questioned in genotype 3 and 4-endemic countries. The current commercial HEV-specific enzyme linked immunosorbent assays (ELISAs) have recombinant open reading frame 2/3 (ORF 2/3) antigens coated, which are primarily designed for the detection of antibodies against genotypes 1 (Burma) and 2 (Mexico) and they may not sensitively detect HEV genotype 3 or 4 [6–9]. The sole ELISA assay using genotype 1 and 3 antigens (Mikrogen) was recently introduced in the market.

A few in-house assays have been described [10,11], but since continuity and robustness of routinely used assays are essential in clinical settings, most laboratories prefer (CE-marked/Food and Drug Administration (FDA)-approved) commercial assays. The HEV-specific immunoblot (Recomblot, Mikrogen) [8,12] uses the same antigens as the Mikrogen IgM/IgG ELISA and is labour intensive. Knowledge on the performance of current commercially available IgM and IgG ELISAs is limited, specifically if IgM and IgG of the same commercial company are preferably combined in routine work-up.

Available assays differ in the accuracy of detection of an acute or past HEV infection. In addition, previous publications have assessed the performance of either HEV-specific IgG [7,13–16] or IgM [10,12] solely.

2. Objectives

We first aimed to evaluate the analytical and diagnostic performance of selected commercially available IgM and IgG ELISAs for the detection of both genotype 1 and 3 HEV infections using a well-defined serum panel of polymerase chain reaction (PCR)-confirmed HEV infected patients. Second, we targeted investigation of the HEV antibody responses in immunocompetent and immunocompromised patients.

3. Study design

3.1. Sample collection

The samples used in our retrospective study had been collected in the time period 2003–2011 during hospitalisation and routine visits by patients to our outpatient clinic for clinical assessments. Serum/ethylenediaminetetraacetic acid (EDTA)–plasma samples have been stored at −20 °C and −80 °C, respectively.

3.2. Sensitivity panel

In order to assess the analytical sensitivity we performed a twofold end-point titration of a genotype 1 and 3 HEV IgM and IgG antibody-positive serum, starting from 1/125 and 1/25, respectively. Presence of HEV antibodies of these secondary standards was confirmed by the – previous – routinely used MP Diagnostics ELISA and HEV RNA was detected by HEV reverse transcriptase polymerase chain reaction (RT-PCR). Additionally, for IgG a twofold end-point titration (starting from 1/25) was performed using the World Health Organization (WHO) reference reagent for HEV antibody, (human serum NIBSC code: 95/584) of which the antigenic trait is unknown (WHO IS) [17]. Lower limit of detection (LLOD) was calculated for each anti-HEV IgG ELISA using the world health organisation international standard or reference reagent (WHO IS), taking the working dilution of each assay into account, as described before [7].

In order to select samples for the diagnostic sensitivity panel, a laboratory information management system (LIMS) database search was performed for HEV RNA positivity, HEV genotype, immune status, sequential sample availability and clinical information. A total of 88 samples were selected from 17 immunocompromised, 15 immunocompetent and four patients with an unknown immune status (total 36), whose HEV infections were confirmed by real-time RT-PCR. The time frame of infection was determined with reference to clinical symptoms and retrospective HEV PCR testing (Table 1).

3.3. Specificity panel

In order to assess the assay's specificity, a serum/EDTA–plasma sample panel was constituted of acute Epstein–Barr virus (EBV) infection (*n* = 10), human cytomegalovirus (CMV) infection (*n* = 10), B19 virus infection (*n* = 10), hepatitis A virus (HAV) (*n* = 10), hepatitis B virus (HBV) (*n* = 10), hepatitis C virus (HCV) infections (*n* = 10) and healthy blood donors (*n* = 28). Additionally 10 samples were selected from 10 HEV-infected transplant recipients prior to their HEV infection.

3.4. Selection of immunoassays for HEV antibody detection

For the detection of both anti-HEV IgM and IgG in serum or EDTA-plasma samples, eight commercially available HEV ELISAs were selected on the basis of prior publications [7,10], common use in Dutch laboratories and availability. IgM and IgG ELISAs were selected from MP Diagnostics, (IgM v3.0; MP Biomedicals, Singapore, former Genelabs (GL)), Dia.Pro (Milan, Italy), DRG (Marburg, Germany), DSI [10] (RPC Diagnostic Systems, Nizhniy, Novgorod, Russia), MP Products (Diacheck; The Netherlands), Wantai Biological Pharmacy (PE2-assay; Beijing, China) and Mikrogen (recomWell; Neuried, Germany). From Mikrogen, two versions of recomWell anti-HEV IgM assays were selected, one available in The Netherlands up until February 2011 and the 'new' version available from November 2012 in The Netherlands.

All HEV IgM and IgG ELISAs indirectly detected HEV antibodies using synthetic ORF2 and 3 peptides coated on a polystyrene plate, except for the HEV-IgM PE2 ELISA from Wantai, which was a μ-chain-capture ELISA [18]. In all ELISAs, genotype 1 and 2 antigens were used, except for the new version recomWell HEV-IgM assay (Mikrogen), which is coated with genotype 1, 2 and 3 ORF3 antigens.

Table 2
Performance of IgM and IgG specific ELISAs.

	Analytical sensitivity (titres)						Clinical performance IgM		
	IgM (titres)		IgG (titres)			LLOD[a] IgG (IU/ml)	ROC[a] analysis	Sens.	Spec.
	geno1	geno3	geno1	geno3	WHO[a]	WHO[a]	AUC (95%CI)		
Mikrogen_old	4000	250	6400	800	1600	6.31	0.964 (0.940–0.989)	52%	>99%
Mikrogen_new	32,000	16,000	>12,800	3200	3200	3.16	0.971 (0.938–1.000)	74%	99%
MP Diagnostics	>64,000	4000	3200	100	800	2.63	0.969 (0.948–0.991)	74%	84%
DSI	8000	4000	3200	800	800	1.25	0.943 (0.900–0.986)	71%	90%
Dia.Pro	32,000	32,000	6400	100	800	2.63	0.994 (0.982–1.000)	81%	98%
Wantai	>64,000	>64,000	>12,800	1600	1600	0.69	0.974 (0.941–1.000)	75%	>99%
DRG	32,000	32,000	6400	100	800	2.63	Excluded		
Diacheck	1000	125	3200	100	400	5.25			

[a] World Health Organization reference reagent (WHO), lower limit of detection (LLOD) taking the serum work dilution of each assay into account, receiver operator curve (ROC), area under the curve value (AUC), confidence interval (CI), sensitivity (Sens.), specificity (Spec.).

ELISA procedures were used according to the manufacturer's instructions; positive and blank controls were taken for quality assurance. Signal to cut-off (s/co) ratios were interpreted according to the manufacturer's instructions. Subsequent statistical analysis was performed using IBM SPSS statistics software v.20.

3.5. HEV-RNA detection and sequence analysis

The internally controlled quantitative real-time RT-PCR amplified a conserved ORF3 region of 77 bp as described before [3,6]. For phylogenetic analyses, ORF1 RdRp (nt 4254–4560) sequences of 321 bp were generated on an ABI3130XL using previously described methods [6].

4. Results

4.1. Analytical sensitivity

IgM limiting dilutions indicated that the highest analytical sensitivity among the IgM ELISAs for genotype 1 was achieved by MP Diagnostics and Wantai assays. For genotype 3 IgM antibody titration, the Wantai assay was the most sensitive assay (Table 2). Remarkably, a wide variety of s/co ratios was observed, among which the IgM and IgG Wantai assay stood out (s/co ratios of 18 as upper limit of detection). IgG limiting dilutions indicated that the highest analytical sensitivity in head-to-head comparison (titration curves) was measured for the Mikrogen_new assay, though the Wantai assay had an equal sensitivity for genotype 1 (Table 2). Remarkably, if the LLOD was calculated taking the serum working dilutions of each assay into account, the Wantai anti-HEV IgG assay was the most sensitive assay with 0.69 IU ml^{-1} (Table 2). For all samples, the IgM and IgG Diacheck assays had the lowest sensitivity and were, therefore, excluded from further validation. Additionally, both Dia.Pro and DRG proved to be exactly the same assay, though from two different manufacturers and we, therefore, excluded the more expensive assay (DRG) from further validation.

4.2. Specificity of anti-HEV IgM ELISAs

The specificity panel was assessed only for IgM, since no gold standard for IgG is available. From the specificity panel (n = 98), 16 samples were reactive in the IgM MP Diagnostics assay, 10 samples in the IgM DSI assay, two samples in the IgM Dia.Pro assay, one sample in the IgM Mikrogen_new assay and none in the IgM Mikrogen_old and Wantai assays. HEV RNA could not be detected in these reactive sera by sensitive real-time RT-PCR assay. The boxplot (Fig. 1) shows a high variety of aspecifc reactions among the different IgM assays in specific subpanels of acute infections. Two IgM assays (DSI and MP Diagnostics) showed aspecific reactions with sera from both acute CMV (n = 6 and 5, respectively) and HAV infections (n = 2 and 3, respectively). Remarkably, the DSI IgM ELISA had a median s/co ratio above the cut-off of the assay in case of acute CMV infections. Noteworthy are the low s/co ratios in all groups of the specificity panel of the Wantai assay, since this assay had the highest s/co ratios in HEV-IgM/IgG-positive sera. The overall specificity was calculated (Table 2) resulting in the highest score for IgM Mikrogen_old (>99%) and IgM Wantai assays (>99%).

4.3. Diagnostic sensitivity of the anti-HEV IgM ELISAs

In order to assess the clinical accuracy of the IgM ELISAs, the diagnostic sensitivity panel was processed using six IgM ELISAs. Cohen's kappa (κ) concordance was calculated, with 'intermediate' results (s/co ratios interpreted according to the manufacturer's instructions) both considered as reactive or non-reactive, which yielded higher or equal concordance levels for the assays using 'intermediate' results assigned as non-reactive (Table 3). The κ-levels ranged between 0.562 and 0.948 and the highest κ-levels among the IgM ELISAs were obtained for the Dia.PRO/Wantai IgM assays (κ = 0.900) and the Mikrogen_new/Wantai IgM assays (κ = 0.948).

Furthermore, receiver operator curve (ROC) analysis resulted in highest area under the curve (AUC) of 0.971, 0.974 and 0.994 for the Mikrogen_new, Wantai and DiaPro HEV IgM assays, respectively (Table 2).

The overall diagnostic sensitivity ranged from 52% for the Mikrogen_old IgM assay to 81% for the IgM Dia.PRO assay (Table 2). If only acute samples (<6 weeks after infection, n = 34) were taken into account, the diagnostic sensitivity was 65% for Mikrogen_old, 73% for Mikrogen new, 74% for MP Diagnostics, DSI and Wantai and 79% for Dia.Pro assays.

4.4. Performance of combined anti-HEV IgM and IgG ELISAs

As in most clinical laboratories IgM and IgG ELISAs of one commercial provider are combined in routine work-up, we assessed the combined results of both anti-HEV IgM and IgG ELISAs of one provider to diagnose an HEV infection at different time points in the infection. None of the assays was able to detect anti-HEV IgM or IgG in nine of 78 samples, though HEV RNA could be detected (median viral load 2.35 log 10 IU ml^{-1}, range 1.41–7.09). These nine samples belonged to seven immunocompromised transplantation recipients, of which six samples were drawn <6 weeks after infection and two between 6 weeks and 6 months after infection. Anti-HEV IgM and/or IgG antibodies (IgM/IgG ratio) were detected in 51 (40/40), 61 (57/33), 63 (62/39) and 66 (56/56) samples for Mikrogen_old, MP Diagnostics, Dia.PRO assay and Mikrogen_new assay, respectively, whereas both DSI and Wantai IgM and IgG assays combined could

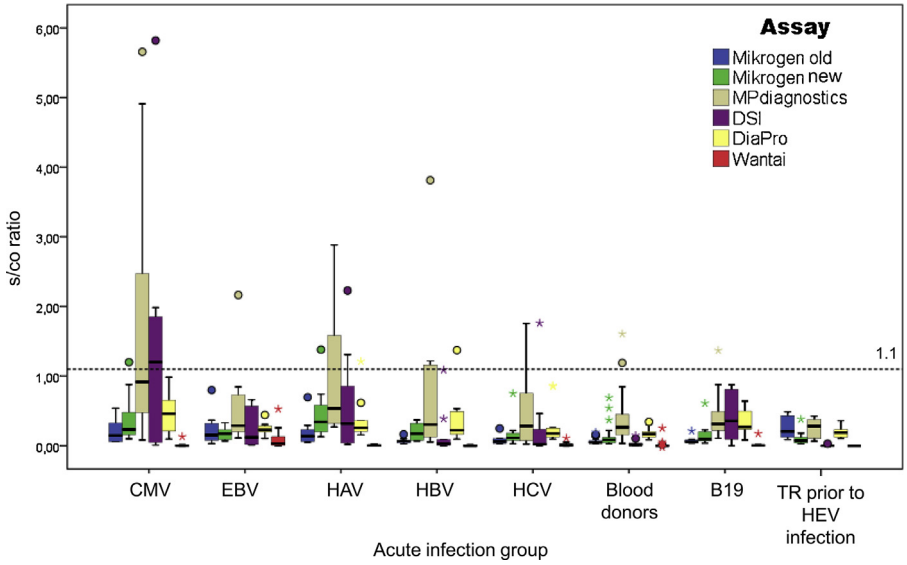

Fig. 1. Specificity of selected anti-HEV IgM ELISAs. Boxplot showing the mean, interquartile ranges, outliers (○) and extremes (*) of the signal to cut-off (s/co) ratio's of six HEV-IgM detecting ELISAs in patients with diagnosed acute infections of CMV ($n = 10$), EBV ($n = 10$), HAV ($n = 10$, $n = 9$ for Mikrogen_new assay), HBV ($n = 10$), HCV ($n = 10$), B19 ($n = 10$), healthy blood donors ($n = 28$) and samples of transplant recipients (TR) before HEV infection ($n = 10$). Colours are indicated in the legend (Mikrogen_old, Mikrogen_new, MP Diagnostics, DSI, DiaPro, Wantai).

Table 3
Cohen's Kappa concordance of anti-HEV IgM and IgG specific ELISAs.

	Anti-HEV IgM					
	Mikrogen old	Mikrogen new	MP Diagnostics	DSI	Dia.Pro	Wantai
Anti-HEV IgG						
Mikrogen_old	–	0.744	0.562	0.642	0.653	0.721
Mikrogen_new	0.739	–	0.743	0.810	0.873	0.948
MP Diagnostics	0.643	0.559	–	0.738	0.726	0.723
DSI	0.783	0.854	0.621	–	0.792	0.763
Dia.Pro	0.678	0.667	0.813	0.653	–	0.900
Wantai	0.730	0.893	0.530	0.892	0.610	–

detect HEV-specific antibodies in 67 of 78 samples. For these two assays, the IgM/IgG nominator of positive samples was 55/50 and 58/57 samples, respectively.

4.5. Antibody kinetics of genotypes 1 and 3 in the immunocompetent and immunocompromised

In order to gain insight into the antibody kinetics in groups with different immune status and detected responses to the two assessed genotypes, the s/co ratios of these different panels were plotted (Figs. 2 and 3). Fig. 2 shows a difference in magnitude of the immune responses in the compared groups. However, anti-HEV IgM could be detected in only in 7/16 immunocompromised patients and in 18/18 immunocompetent patients in the acute phase (<6 weeks) of the infection, indicating a delayed immune response in the immunocompromised group. The immunocompetent group shows a classic pattern of quickly rising IgM levels immediately after infection, which declines after 6 weeks and becomes undetectable after 6 months (Fig. 2, lower left panel). This is in contrast to the immunocompromised group (of which 50% were chronically infected), having low IgM levels immediately after HEV infection, which rise during the course of infection. Furthermore, Fig. 3 shows that genotype 3 IgM antibodies can efficiently be detected in assays which have genotype 1 and 2 antigens coated.

The difference between immunocompromised and immunocompetent groups in IgG antibody kinetics is less apparent than for IgM, both showing seemingly steady IgG antibody levels after 6 weeks of infection (Fig. 2, right panels). However, only two assays (Mikrogen_new and Wantai) were able to detect IgG in all samples of the immunocompetent group ($n = 4$) 6 months after infection. Also for IgG, genotype 3 antibodies were detected using genotype 1 and 2-coated ELISAs.

5. Discussion

Autochthonous HEV infection, caused by genotype 3, is recognised as an emerging infectious disease in industrialised countries. Only limited data are available on the diagnostic performance of commercial IgM and IgG ELISAs or combination of these two, and the sensitivity of ELISAs coated with genotype 1 and 2 antigens is questioned for the detection of genotypes 3 and 4. Our study gives more insight into the diagnostic performance and antibody kinetics of commercial anti-HEV IgM and IgG assays of immunocompromised and immunocompetent patients with genotype 1 and 3 HEV infections.

The results of this study show a variety in analytical sensitivity among the tested assays for both IgM and IgG antibodies and if used on clinical specimens, they also show varying clinical specificity ranging from 84 to more than 99% and sensitivity ranging from 52%

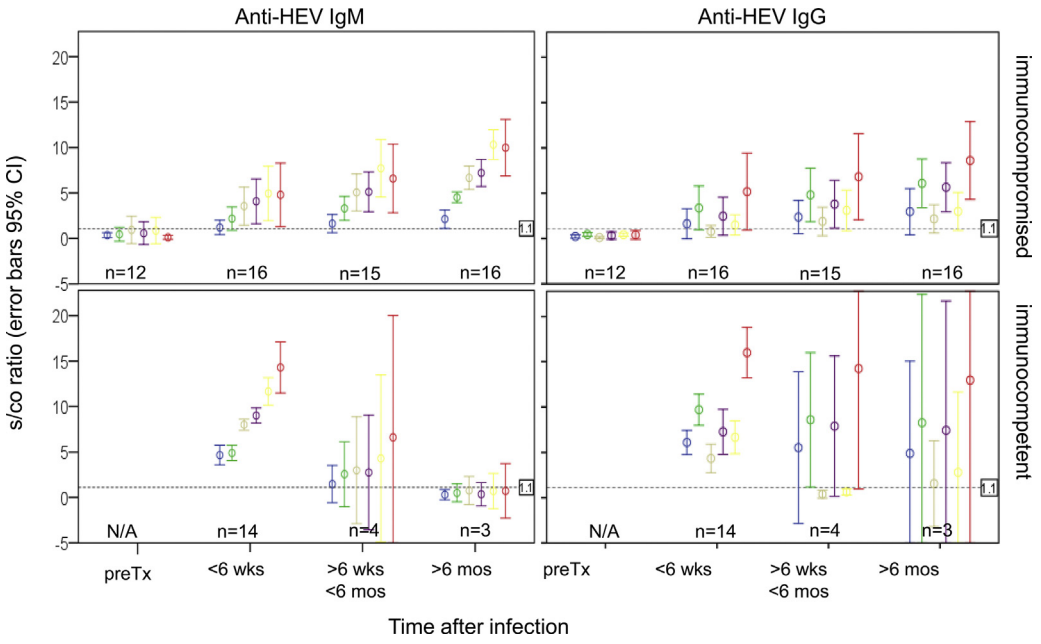

Fig. 2. Anti-HEV IgM and IgG antibody kinetics per assay and immune status. Plotted are the s/co-ratios and 95% confidence intervals (C.I.) per assay (Mikrogen_old, Mikrogen_new, MP Diagnostics, DSI, DiaPro, Wantai) in time (pre = prior to HEV infection). N/A = not applicable.

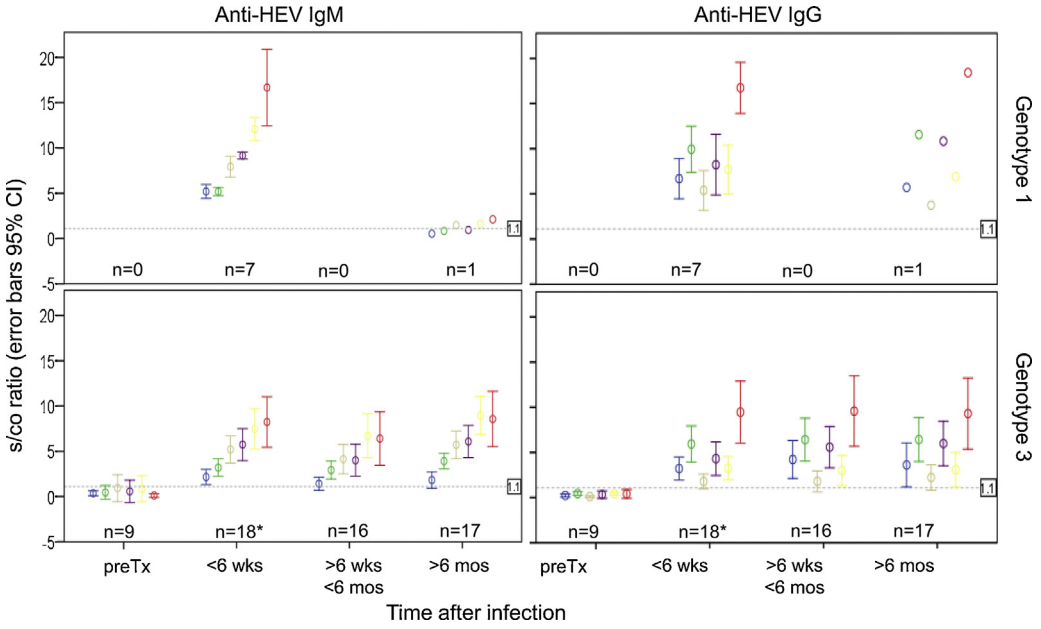

Fig. 3. Anti-HEV IgM and IgG antibody kinetics per assay and genotype. Plotted are the s/co-ratios and 95% confidence intervals (C.I.) per assay (Mikrogen_old, Mikrogen_new, MP Diagnostics, DSI, DiaPro, Wantai) in time (pre = prior to HEV infection). * One sample was excluded in the Mikrogen_new assay due to sample volume.

to 79%. If the IgM and IgG results from each commercial supplier were combined to assess the diagnostic value, only DSI and Wantai assays were able to identify HEV-specific antibodies in 86% of samples. These data should be interpreted for each setting separately, depending on the objective for which the ELISAs is used. In clinical practice, IgM is the most valuable serologic tool to diagnose an acute HEV infection. Comparing the currently available assays, the Wantai assay had the best specificity (>99%), most discriminative s/co ratios and comparable diagnostic sensitivity. For public health studies in genotype 3-endemic areas, it should be taken into account that the Mikrogen_new assay had a better analytical sensitivity for IgG if compared head to head with other assays. However, taking the working dilution of this assay (1:101) and the Wantai assay (1:11) into account, the latter had the lowest LLOD (0.69 IU ml^{-1}). Furthermore, specificity could not be assessed for IgG and we did not include samples with a long (>5 years) follow-up period and can, therefore, not draw any conclusions on performance of the selected assays over longer periods of times.

It is known that antigenicity of the pE2 peptide (aa 394–606), used in the Wantai assay, is superior to other shorter E2 peptides because of the stabilising effect that a 60-amino-acid extension has on the dimer formation of the antigen [19]. The Wantai assay is the only assay which uses an μ-chain-capture strategy in the anti-HEV IgM assay. Generally, μ-chain-capture ELISAs have higher specificity and sensitivity than indirect ELISAs [20,21]. Taken together, this may explain the high s/co ratios seen in the Wantai anti-HEV IgM and IgG assays.

The seemingly moderate diagnostic sensitivity in this study may be explained by the 67.1% of the samples in this sensitivity panel, which were selected from 17 immunocompromised individuals, having HEV RNA in their peripheral blood, though inferior antibody levels than immunocompetent individuals. In 11.5% of the patients, none of the assays could detect any antibody responses.

Furthermore, antibody kinetics directed against genotype 1 and 3 infections in groups with different immune status confirmed the findings of previous studies [12,16], leading us to conclude that there are no sensitivity problems in the detection of genotype 3 using ELISA assays coated with only genotype 1 and 2 antigens. Although the diagnostic sensitivity panels used in our study indicate different antibody kinetics in the immunocompromised group compared to the immunocompetent group, the numbers used were relatively small and need to be assessed eventually in larger panels to gain statistical significance; this was a limitation in our study. A second limitation is the absence of HEV genotype 2 and 4 samples, which were not available for this study.

Collectively, though there is a wide variety among the selected assays, our data show that the HEV ELISAs can be used to diagnose both HEV genotype 1 and 3 infections in a clinical setting. Considering the high concordance and specificity of the best-performing assays and the availability of HEV-RNA assays, there is no longer a need to perform serologically confirmatory testing in diagnostic settings.

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Conflicts of interest

S.D.P received travelling and accommodation expenses from Mikrogen. A.D.M.E.O. is chief science officer of Viroclinics Biosciences BV, a spin-out Erasmus MC contract research organisation that collaborates with pharmaceutical companies. The other authors have no conflicts of interest to disclose.

Ethical approval

This study was approved by the hospital medical ethical committee (MEC-2011-277).

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