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Confirmation of specificity of reactivity in a solid phase ELISA for the detection of hepatitis E viral antigen improves utility of the assay

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ABSTRACT

Genotype 3 hepatitis E virus (HEV) can lead to persistent infections in immunocompromised hosts. A recently available commercial assay for the detection of HEV antigen (HEV-Ag ELISA, Wantai diagnostics) may enable the study of HEV-Ag dynamics in such persistent infections, however currently there is no confirmatory test available. We generated a putative neutralising reagent from a pool of four convalescent blood donor samples and explored neutralising activity against HEV antigens from clinical samples, HEV tissue-culture and virus-like particles. Using this neutralisation method we were able to differentiate true reactivity from non-specific reactivity in plasma, stool and urine samples. This could also facilitate the introduction of HEV-Ag detection as a screening assay or the study of HEV-Ag in different body fluids.

1. Introduction

Systemic viral infections are often associated with circulating non-virion associated viral antigens in plasma, such as p24 antigen in HIV infection, p22 antigen in HCV infection and hepatitis B surface antigen (HBsAg) subviral particles in HBV infection (McHugh et al., 1988; Rydell et al., 2017). The detection of circulating antigenaemia typically is less sensitive than the detection of nucleic acid by PCR particularly in acute infections, however commonly more cost-effective than genome-based assays (Behrendt et al., 2016; Fiebig et al., 2003; Tremeaux et al., 2016). In some circumstances the detection of viral antigens can provide enhanced diagnostic information, for example loss of core antigen can predict viral clearance during therapy for HCV and HBsAg titres during interferon therapy for HBV inform stopping rules by predicting when a virological response is unlikely (Moucari and Marcellin, 2011; Tedder et al., 2013). In the context of hepatitis E virus (HEV) infection, the detection of the viral ORF2 antigen (HEV-Ag) using a solid-phase enzyme linked immunoassay (ELISA) has recently been shown to be sensitive for the detection of chronic HEV infections in immunocompromised patients (Behrendt et al., 2016). This study also demonstrated ongoing detection of plasma HEV-Ag following clearance of plasma HEV RNA in a subset of treated patients, however the clinical relevance of this is unknown nor is the detection of HEV-Ag in non-plasma analytes such as urine or stool (Behrendt et al., 2016; Geng et al., 2016). To study HEV-Ag detection in such analytes or in the context of screening for infection, it is vital that any reactivity in an ELISA is confirmed for specificity (Cameron and Briggs, 1980). Here we describe strategies for confirming the specificity of HEV-Ag in plasma, stool and urine samples reactive in the recently released commercial assay produced by Wantai diagnostics (HEV-Ag ELISA, Fortress Diagnostics, Antrim, Northern Ireland, UK), including the selection of reagents for this purpose.

Abbreviations: ALT, alanine aminotransferase; BSA, bovine serum albumin; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; G3, genotype 3; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; HEV-Ag, hepatitis E virus antigen; IR, initial reactive; IU, international units; IQR, interquartile range; NHP, normal human plasma; NHSBT, National Health Service Blood and Transplant; OD, optical density; ORF2, open reading frame 2; PCR, polymerase chain reaction; PHE, Public Health England; PPV, positive predictive value; RNA, ribonucleic acid; RR, repeat reactive; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; S/CO, sample over cut-off ratio; TDM, therapeutic drug monitoring; VLP, virus-like particle

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2. Materials and methods

2.1. Patient and blood donor samples

Residual HEV-Ag reactive samples and follow up HEV RNA-positive stool samples were obtained from a screening audit of HEV viraemia in transplant recipients undergoing therapeutic drug monitoring (Ankcorn M et al., manuscript in submission). A urine sample was collected from a patient undergoing treatment for persistent HEV infection with ribavirin. Anonymised blood donor and convalescent blood donor samples from a previous HEV donor-transmission study were donated by NHS Blood and Transplant (NHSBT) (Hewitt et al., 2014). Faecal suspensions were made using a 10 µl disposable loop half-filled with raw faeces resuspended in 1000 µl of stool transport and recovery buffer (S.T.A.R. buffer, Roche Diagnostics) which was vortexed and centrifuged for 5 min at 13,000 rpm for 5 min. A further 1:10 dilution was made in the same buffer.

Pooled normal human plasma (NHP) used for controls and dilutions were generated from citrate plasma packs from NHSBT treated with preservative (Bronidox) which had been screened for markers including HEV RNA, anti-HEV IgG and IgM and filtered prior to storage at −30° C.

2.2. Detection of HEV antigen

HEV-Ag testing was performed using a commercial sandwich ELISA (HEV-Ag ELISA, Fortress Diagnostics, Antrim, Northern Ireland, UK) according to the manufacturer's recommendations. The assay is a sandwich ELISA with polyclonal antibodies directed against the ORF2 product on the solid phase with enzyme-linked monoclonal antibodies in the detection system. Briefly, 50 µl of sample was added to the ELISA plate and incubated for 1 h at 37 °C. Horseradish peroxidase–conjugated monoclonal anti-HEV ORF2 antibody was added followed by a further incubation for 30 min at 37 °C. The wells were washed five times using the supplied buffer and then chromogen added. Following a third incubation in the dark for 15 min at 37 °C the reaction was stopped using Stop solution. Optical densities (OD) were measured immediately with an ELISA plate reader (EL808™ Absorbance Microplate Reader, BioTek; $OD_{450/630}$). The assay cut-off value (CO), the OD threshold above which determines the positive status of a sample (S), was calculated using the mean OD absorbance value of the three negative controls plus 0.16, as per the manufacturer's instructions. We considered any sample with a S/CO ratio > 1.0 on initial testing as reactive (IR) and on repeat testing as repeat reactive (RR) with S/CO > 1.0.

2.3. Measurement of neutralising activity

Neutralising activity was determined as a percentage of the reduction in binding in the HEV-Ag assay of the test incubation mixture (antigen sample plus neutralising reagent) when compared to a non-neutralising control mixture (antigen sample plus NHP) using the following formula:

$$\%\ \text{neutralisation} = 100 - \frac{(OD_{\text{sample+neutralising reagent}} - OD_{NHP})}{(OD_{\text{sample+NHP}} - OD_{NHP})} \times 100$$

For stool samples the OD of STAR buffer was subtracted from the test OD rather than the OD of NHP. Test wells exhibiting greater than 50% reduction in binding were considered neutralised.

2.4. Production of a putative HEV antigen-containing pool

Plasma samples from five patients with HEV viraemia persisting for more than two months with a high HEV-Ag binding ratio (S/CO > 18.0) were pooled to generate a standard antigen. This pooled plasma was titrated in half $\log_{10}$ dilutions in NHP and tested in the HEV-Ag assay.

2.5. Production of a putative antigen neutralising reagent

Ten plasma samples from convalescent blood donors previously HEV viraemic with high IgG binding ratios (Anti-HEV IgG S/CO > 20.0) and HEV-Ag non-reactive (S/CO < 1.0) were selected and screened for neutralising activity. Four of the ten convalescent donor plasma samples which demonstrated potent neutralising activity were used to make an equivolumetric pool of neutralising reagent.

2.6. Generation of tissue culture-derived antigen and virus-like particles

HEV from a genotype 3 faecal sample was propagated in a HepG2/C3a cell line using a previously published method (Tanaka et al., 2007). Cell culture supernatant was harvested on day 45 post–inoculation (HEV RNA quantification 1.11×10^7 IU/ml) and diluted 2 $\log_{10}$, 2.5 $\log_{10}$ and 3 $\log_{10}$ in NHP.

Virus-like particles (VLP) were generated using recombinant bacmids encoding amino acids 112-608 of either G1 or G3 HEV ORF2 created with the Bac-to-Bac® baculovirus expression system (Life Technologies), according to the manufacturer's protocol. Sf9 cells were cultured, harvested after five days and then lysed. The harvest, containing supernatant and lysed cells, was clarified, fractionated on Optiprep (Sigma) and fractions containing VLPs identified by SDS-PAGE and electron microscopy (Fig. 1a/b).

3. Results

3.1. Defining the optimum antigen dilution to give a neutralisable signal

HEV-Ag from the antigen pool derived from five viraemic patients was detectable in the ELISA up to a dilution of 3.5 $\log_{10}$ (S/CO 1.81, Fig. 2). The dilutions of 2.5 $\log_{10}$ and 3 $\log_{10}$ which gave S/CO values of 13.08 and 5.94 respectively were chosen for further work to ensure the antigen was sufficiently dilute to prevent saturation of the putative neutralising reagent. It was notable that attempts to neutralise HEV-Ag reactivity on undiluted samples which were on the plateau were unsuccessful. For example, HEV-Ag pool at a 1 $\log_{10}$ dilution (S/CO 17.10) could only be neutralised by 5% (Table 1).

3.2. Demonstration of neutralising activity in various incubation conditions

In order to identify potent convalescent samples to make a putative neutralising reagent ten plasma samples from convalescent donors were screened for neutralising activity by pre-incubation of each sample with the two dilutions of the antigen pool at a 1:1 ratio (30 µl of each). Incubation was allowed to proceed for either one hour at room temperature or overnight at 4 °C in a round-bottomed microtitre plate prior to 50 µl of the incubation mixture being assayed for HEV-Ag under normal conditions.

All ten donor samples demonstrated significant neutralising activity when incubated undiluted with the diluted antigen pool at 2.5 $\log_{10}$ and 3 $\log_{10}$ dilutions. There was no demonstrable difference between the two incubation conditions (Fig. 3). Six of the ten samples were able to neutralise the antigen pool to below the assay cut-off when used at a pre-dilution of 1 $\log_{10}$ in NHP. Significant neutralising activity was not seen in any samples at dilutions of 2 $\log_{10}$ and 3 $\log_{10}$ (Fig. 4). The four samples demonstrating highest neutralising activity at a 1 $\log_{10}$ dilution were selected to make an equivolumetric pool of putative neutralising reagent.

3.3. Dilution effect reduced by using low volume neutralising reagent

In an attempt to reduce the dilution effect of adding a non-neutralising NHP reagent (non-neutralising control) to the antigen pool