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New immunoassays for total, IgA and IgM antibodies against hepatitis E virus: Prevalence in Italian blood donors and patients with chronic liver or kidney diseases

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ABSTRACT

Background: Hepatitis E virus (HEV) is a zoonotic agent that causes acute hepatitis in humans with sporadic infections and outbreaks in developing countries worldwide. The global spread of HEV remains underestimated because of subclinical infections and lack of sensitive diagnostic assays.
Aims: To study the prevalence of HEV antibodies (anti-HEV) in sera of blood-donors and patients with chronic-liver-disease and chronic-renal-disease, using newly developed anti-HEV assays.
Methods: 396 sera from 199 blood-donors, 109 chronic-liver-disease patients and 88 chronic-renal-disease patients and three standard reference serum panels were tested in parallel with a sensitive reference anti-HEV assay and newly developed assays for IgA, IgM and total anti-HEV based on HEV-like-particles produced by recombinant baculo-viruses.
Results: Overall, total anti-HEV was detected in 12.9% (7.0% blood-donors, 9.2% and 30.7% chronic-liver-disease patients and chronic-renal-disease patients, respectively). We observed a higher anti-HEV prevalence in older subjects and in chronic-renal-disease patients in relation with degree on immune-depression ($p < 0.001$). Results from reference serum panels showed an optimal and slightly better performance of the new assay over the commercially available assay.
Conclusions: Newly developed anti-HEV assays using recombinant HEV-like-particles showed optimal diagnostic performances assessing that HEV-infection is endemic in Italy with seroprevalence ranging from 7% to 30% in blood donors and immune-compromised hosts, respectively.

1. Introduction

Hepatitis E virus (HEV) is a non-enveloped, single-stranded RNA virus. It causes zoonosis and acute hepatitis E in humans by fecо-oral transmission [1]. HEV infection associates with two distinct epidemiological patterns; acute epidemics in developing countries and sporadic infection in developed countries [2]. There are at least 5 genotypes and 4 of them are shown to cause acute hepatitis in humans [3]. Genotype 1 and 2 infections are endemic in Africa and Asia where they account for several outbreaks of severe hepatitis in developing countries [4]. Genotype 3 and 4 are zoonotic, affecting humans and a large variety of animal species, including boars, deer, pigs, rabbits and rats [5]. These types are primarily responsible for the sporadic cases of acute hepatitis E in western Europe by up-takings of contaminated, undercooked meat [6]. Nowadays, in endemic countries, HEV accounts for 25% of all non-A, non-B

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acute hepatitis and the prevalence among healthy blood donors exceeds 40% [7]. On the contrary, in developed countries, earlier epidemiological reports indicated that the prevalence of HEV infection was rather low ranging from 1% to 4% [8]. However, other studies have shown a higher prevalence of anti-HEV antibodies with considerable inter-region differences [9,10]. Nevertheless, the spread of HEV infection remains underestimated because of the subclinical and asymptomatic course of most infections and the variability in sensitivity and specificity of diagnostic assays [11]. Thus an improvement of existing anti-HEV assays or the development of new assays with better performance is needed.

Here, we describe the usefulness of a new anti-HEV (total, IgM and IgA) antibody assay using as reference HEV antigens a mixture of HEV-Like Particles (HEV-LPs) produced in recombinant baculoviruses containing of N-terminal 111-amino acid (aa)-deleted DNA fragments of genotype 1, 3 and 4 HEV-ORF2 [12–14]. Using this assay, we studied the epidemiological impact of HEV infection in Italy assessing its prevalence in the sera of three different groups of subjects; healthy blood donors, patients with chronic-liver-disease (CLD) and chronic-renal-disease (CRD).

2. Subjects and methods

2.1. Subjects

Serum samples were obtained from 396 consecutive subjects, 240 males (60.6%) and 156 females (39.4%), median age of 50 years (range 18–90) and were divided into three groups as follows: (i) 199 (50.2%) healthy blood donors, (ii) 109 (27.5%) patients with CLDs, and (iii) 88 (22.3%) patients with CRDs undergoing haemodialysis (14 of them after previous kidney transplant failure). All sera were stored at −30 °C till used and tested all together in a single run for IgA, IgM and total anti-HEV antibodies using serological new assays (Dia.Pro, Milan, Italy) based on HEV-like particles (HEV-LPs) and for IgG anti-HEV by commercial assay (Wantai HEV-IgG ELISA, Wantai Biologic Pharmacy Enterprise, Beijing, People's Republic of China) according to manufacturer's instruction. Samples positive for HEV serological markers were also tested for HEV-RNA by RT-PCR.

2.2. Methods

2.2.1. Production and purification of genotype 1, 3 and 4 HEV-LPs

To produce HEV-LPs, recombinant baculo-viruses containing of N-terminal 111-amino acid (aa)-deleted DNA fragments of genotype 1 (DQ079624), genotype 3 (DQ079627) and genotype 4 (DQ079631) HEV ORF2 were infected to *Trichoplusia ni* BTL-Tn 5B1-4 (Tn5) [15] cells (Invitrogen, San Diego, CA), respectively. The recombinant baculovirus-infected Tn5 cells were harvested on day 7 post-infection. The intact cells, cell debris, and progeny baculoviruses were removed by centrifugation at 10,000 × *g* for 60 min. The supernatant was then spun at 32,000 rpm for 3 h in a Beckman SW32Ti rotor. The resulting pellet was re-suspended in EX-CELLTM 405 medium and kept at 4 °C overnight. For the CsCl gradient centrifugation, 4.5 ml of the samples were mixed with 2.1 g of CsCl, and then centrifuged at 35,000 rpm for 24 h at 10 °C in a Beckman SW55Ti rotor. The fraction which contain HEV-LPs was collected and diluted with EX-CELLTM 405 medium and centrifuged for 2 h at 50,000 rpm in a Beckman TLA55 rotor to sediment HEV-LPs [12–14].

2.2.2. Total anti-HEV assay

Polystirene micro-plates (Biomat, Italy) were coated with purified HEV-LPs dissolved in PBS and incubated overnight at room temperature. After washing, micro-plates were post-coated with 1% BSA in PBS and then dried. HEV-LPs were labelled with HRP with the Nakane's method [16] and the conjugate was purified by gel filtration on Sephadex G75 in PBS.

For the assay, 100 μl samples were incubated a +37 °C for 45 min. After washing, the micro-plate wells were filled in with 100 μl HRP-labelled HEV-LPs in PBS + 1% Bovine serum albumin (BSA) and incubated again at +37 °C for 45 min.

The conjugate was washed out, and 100 μl of TMB/H_2O_2 in phosphate/citrate buffer were added for 15 min at room temperature, then the enzymatic reaction was stopped with 0.3 M sulphuric acid and the micro-plate read at 450 nm (OD450 nm). A cut-off value is determined on the mean OD450 nm value of the negative control (NC) by the formula: Cut-off = NC + 0.250. Test results are interpreted as a ratio of the sample OD450 nm and the cut-off value (S/Co). Values of S/Co <0.9 indicate a negative sample and values between 0.9 and 1.1 were considered borderline. A positive sample is obtained for S/Co values > 1.1.

2.2.3. Anti HEV-IgM and HEV IgA assays

Polystirene micro-plates coated with purified HEV-LPs are prepared as indicated above. For both the assays the procedure was: 50 μl of neutralizing reagent and 100 μl of 1:100 diluted samples were incubated a 37 °C for 1 h. Sample diluent was Na-citrate buffer with 0.1% Tween 20 and 2% casein, while goat anti-human-IgG was used as neutralizing reagent. Neutralization of IgG anti-HEV, carried out directly in the well, was performed to block IgG interference in determining IgM or IgA. The conjugate for HEV IgM and IgA were goat polyclonal anti-human-IgM and anti-human-IgA, respectively, labelled with HRP using SATA/sulfo-SMCC method, conjugated and purified by FPLC on Sephacryl S-300. After washing, micro-plate wells were filled in with 100 μl of goat anti-human-IgM-HRP or anti-human-IgA-HRP in Tris buffer +5% BSA and incubated again at +37 °C for 1 h. The conjugate was washed out and 100 μl of TMB/H2O2 in phosphate/citrate buffer were added for 20 min at room temperature. The enzymatic reaction was stopped with 0.3 M sulphuric acid and the micro-plates were read at 450 nm. A cut-off value was calculated as mentioned above. For the IgA assay, a negative and positive sample is determined by S/Co values <1.0 and >1.1, respectively, with borderline results between 1.0 and 1.1. Regarding the IgM assay, S/Co values <1.0 and >1.2 indicate a negative and positive sample, respectively, while, values between 1.0 and 1.2 were considered borderline.

2.2.4. HEV-RNA detection

Briefly, after extraction of viral RNA from 200 μl of serum, HEV RNA was amplified by nested RT PCRs in two different regions of HEV genome, ORF1 and ORF2, as previously reported [17].

2.2.5. Reference panels

We used three standard references panels: (a) Cat. No. K-ZMC003, Hepatitis E Mixed Titre Panel (Zeptometrix Corporation, Buffalo, NY, USA), (b) Cat. No. SCP-HEV-001b HEV Seroconversion Panel (Biomex GmbH, Heidelberg, Germany) and (c) Cat. No. SCP-HEV-006b HEV Seroconversion Panel (Biomex GmbH, Heidelberg, Germany).

In the first panel, mixed titre HEV-positive samples were tested with both Wantai and Dia.Pro assays. The analytical sensitivity was assessed on four additional samples that turned out to be positive at a pre-screening with a commercial available assay (Wantai HEV-IgG ELISA, Wantai Biologic Pharmacy Enterprise, Beijing, People's Republic of China). We performed a twofold endpoint titration in two samples and a tenfold endpoint titration in the remaining two. A twofold endpoint titration was also performed using the first WHO reference reagent for HEV antibody, NIBSC code 95/584. In the seroconversion panels, sera from two HEV genotype 3 infected patients were used to test total, IgA and IgM Dia.Pro assay in comparison with IgG Wantai assay.