



## Research Article

## Hepatitis E Virus Superinfection and Clinical Progression in Hepatitis B Patients



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## ABSTRACT

Hepatitis E virus (HEV) infection may cause acute hepatitis and lead to hepatic failure in developing and developed countries. We studied HEV seroprevalences in patients with hepatitis B virus (HBV) infection to understand the consequences of HEV superinfection in a Vietnamese population. This cross-sectional study was conducted from 2012 to 2013 and included 1318 Vietnamese patients with HBV-related liver diseases and 340 healthy controls. The case group included patients with acute ( $n = 26$ ) and chronic hepatitis B ( $n = 744$ ), liver cirrhosis ( $n = 160$ ), hepatocellular carcinoma ( $n = 166$ ) and patients with both liver cirrhosis and hepatocellular carcinoma ( $n = 222$ ). Anti-HEV IgG and IgM antibodies were assessed in patients and controls by ELISA. HEV-RNA was identified by PCR assays and sequencing. Seroprevalences of anti-HEV IgG among hepatitis B patients and controls were 45% and 31%, respectively (adjusted  $P = 0.034$ ). Anti-HEV IgM seroprevalences were 11.6% and 4.7% in patients and controls, respectively (adjusted  $P = 0.005$ ). Seroprevalences were higher among the elder individuals. When stratifying for patient groups, those with liver cirrhosis had the highest anti-HEV IgG (52%) and anti-HEV IgM (19%) seroprevalences. Hepatitis B patients with current HEV infection had abnormal liver function tests compared to patients with past or without HEV infection. One HEV isolate was retrieved from a patient with both liver cirrhosis and hepatocellular carcinoma and identified as HEV genotype 3. This study indicates high prevalences of HEV infection in Vietnamese HBV patients and among healthy individuals and shows that HEV superinfection may influence the outcome and progression of HBV-related liver disease.

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## 1. Introduction

Hepatitis E virus (HEV) may cause acute hepatitis. Pregnant women are vulnerable to fulminant courses (Boccia et al., 2006; Hamid et al., 2002). Chronic HEV infection may occur mainly in immunocompromised patients with HIV, organ transplants and during cancer chemotherapy

**Abbreviations:** HEV, hepatitis E virus; HBV, hepatitis B virus; AHB, acute hepatitis B; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IgG, immunoglobulin G; IgM, immunoglobulin M; ORF, open reading frame; WBC, white blood cells; RBC, red blood cells; PLT, platelets; AST, aspartate amino transferase; ALT, alanine amino transferase; AFP, alpha-feto protein.

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(Dalton et al., 2009; Kamar et al., 2008). Large hepatitis E outbreaks with 120,000 cases occurred in China in 1986–1988 (Dalton et al., 2013), followed by >30,000 cases in India in 1956 (Shukla et al., 2011). Global annual incidences are 20 million asymptomatic infections and 3.4 million acute cases with 70,000 deaths (Rein et al., 2012). Seroprevalences of 16 to 52% and incidences between 0.2 to 1.7% are reported from regions with poor sanitation (Dalton et al., 2013). More than 60% of infections and 65% of deaths occur in Asia, where seroprevalences exceed 25% in certain age groups (WHO, 2014). The first vaccine for HEV was licensed in China in 2011 and very recently a recombinant HEV vaccine (Hecolin) sustained protection against HEV for up to 4.5 years after first vaccination tested in a Chinese population (Zhang et al., 2015).

HEV is a hepevirus of 27–34 nm in size (Kamar et al., 2012). Its genome consists of ssRNA of 7.2 kb, containing three open reading frames

(ORF1, 2, 3) (Kamar et al., 2012). ORF1 encodes enzymes for RNA replication while ORF2 and ORF3 encode the capsid and a multifunctional protein, respectively. However, viral replication in hepatocytes is not fully understood (Cao and Meng, 2012). Four HEV genotypes have been identified. Genotypes 1 and 2 (HEV1, HEV2) only infect humans, and HEV genotypes 3 and 4 (HEV3, HEV4) can cause human and animal disease (Kamar et al., 2012, 2014). HEV1 is widely distributed in Asia and HEV2 predominates in Africa and Mexico. HEV3 and HEV4 are distributed globally. HEV1, HEV2 and HEV4 are transmitted fecal–orally, while HEV3 infections occur also by consumption of undercooked meat (Kamar et al., 2012).

Hepatitis B virus (HBV) infections are frequent in sub-Saharan Africa and Asia with infection rates between 8% and 20% (WHO, 2013). In Vietnam, approximately 10 million (10%) individuals live with chronic hepatitis B (Dunford et al., 2012; Nguyen, 2012). HEV superinfection in patients with chronic HBV or HCV infections and autoimmune hepatitis has been found associated with clinical outcomes in several geographical settings (Atiq et al., 2009; Bayram et al., 2007; Cheng et al., 2013; Marion-Audibert et al., 2010; Monga et al., 2004; Pischke et al., 2014). This study aims to assess prevalences and consequences of HEV infection in patients with HBV-related liver diseases and to compare it to uninfected Vietnamese controls.

## 2. Materials and Methods

### 2.1. Study Design and Sample Collection

A cross-sectional study was implemented between June 2012 and December 2013. A total of 1318 HBV patients and 340 healthy controls were recruited (108 Military Central Hospital and 103 Hospital of the Vietnam Military Medical University, Hanoi). Based on clinical manifestations and laboratory parameters, patients were assigned to the different clinical subgroups as previously described (Song et al., 2003). Briefly, the acute hepatitis B (AHB,  $n = 26$ ) are patients who presented with the prodromal symptoms preceded the onset of jaundice (e.g. fever, anorexia, nausea, vomiting, and fatigue), an onset of clinical jaundice, the constitutional prodromal symptoms were diminished, hepatomegaly, jaundice, hyperbilirubinemia, serum ALT, and AST at least 5-fold higher than normal range, HBsAg positive, anti-HBc IgM positive, anti-HBc IgG negative. The chronic hepatitis B (CHB,  $n = 744$ ) were characterized based upon clinical syndromes such as fatigue, anorexia, jaundice, hepatomegaly, hard density of the liver, splenomegaly, hyperbilirubinemia, elevated levels of AST and ALT, HBsAg positive for longer than 6 months. The HBV-related liver cirrhosis (LC,  $n = 160$ ) were characterized as patients infected with HBV (HBsAg positive) showing the clinical manifestations such as anorexia, nausea, vomiting, malaise, weight loss, abdominal distress, jaundice, edema, cutaneous arterial “Spider” angiomas, palma erythema, ascites, shrunken liver, splenomegaly, hyperbilirubinemia, elevated levels of AST and ALT, prolonged serum prothrombin time, and decreased serum albumin. The HBV-related hepatocellular carcinoma (HCC,  $n = 166$ ) were characterized as patients infected with chronically HBV (HBsAg positive), abdominal pain, an abdominal mass in the right upper quadrant, blood-tinged ascites, weight loss, anorexia, fatigue, jaundice, prolonged serum prothrombin time, hyperbilirubinemia, elevated levels of AST, ALT and serum  $\alpha$ -fetoprotein (AFP), ultrasound showed tumor, liver biopsy and histopathology showing tumor cells. The patients with liver cirrhosis and hepatocellular carcinoma (LC + HCC,  $n = 222$ ) were characterized if the patients showed clinical manifestations and laboratory tests of both LC and HCC. In addition, the patients with LC were also categorized as Child-A, Child-B and Child-C based on Child–Pugh scores (Cholongitas et al., 2005). We also included 340 healthy individuals without any history of alcohol or drug use as healthy controls (HC). Biochemical and serological tests were performed for all participants. HBV viral loads were measured by quantitative real-time PCR as described previously (Song et al.,

2003). Five milliliters of venous blood was collected from all participants. Serum or plasma was used for biochemical and laboratory assays.

### 2.2. Ethics Statement

Informed consent was obtained at sampling from all participants or from parents if subjects were <18 years. The study was approved by the institutional review board of the 108 Military Central Hospital and the 103 Military Hospital, Hanoi, Vietnam.

### 2.3. Serology Testing for HEV Infection

Anti-HEV IgG and IgM levels were determined in serum from patients and healthy controls through ELISA kits (MP Biomedicals, Santa Ana, California, USA). The MP HEV IgG and IgM EIA test system was chosen for our analysis on the basis of own round robin test and due to recently reported comparative analysis of commercial available HEV IgG/IgM EIA assays showing no inconsistent performances of sensitivity and specificity among different assays including the MP EIA (Avellon et al., 2015; Wu et al., 2013).

### 2.4. HEV-RNA Detection

Viral RNA was isolated from serum obtained from patients and controls (QIAamp Viral RNA Mini Kit; Qiagen GmbH, Hilden, Germany). HEV-RNA was reverse transcribed into cDNA (QuantiTect Reverse Transcription Kit; Qiagen GmbH, Hilden, Germany). Presence of HEV-RNA was examined in all the patients and controls using a nested PCR assay. Primers were designed based on conserved regions of the overlapping HEV ORF1 region. Outer primer pairs were HEV-38 (sense) 5'-GAGGCYATGGTSGAGAARG-3' and HEV-39 (antisense) 5'-GCCATGTTCCAGACRGTRTTCC-3'; inner primers were HEV-37 (sense) 5'-GGTCCGYGCTATTGARAARG-3' and HEV-27 (antisense) 5'-TCRCCAGAGTGYTTCTTCC-3'.

PCR amplification was carried out in 25  $\mu$ l volumes [5 ng viral cDNA, 1  $\times$  PCR buffer (20 mM Tris–HCl, 50 mM KCl, 2 mM  $MgCl_2$ ), 0.2 mM dNTPs, 0.4 mM  $MgCl_2$ , 0.6  $\mu$ M specific primer pairs, 1 unit Taq polymerase (Qiagen GmbH, Hilden, Germany)]. Cycling parameters were denaturation (94 °C, 5 min), 35 cycles of 30 s at 94 °C denaturation, 30 s at 54 °C annealing, 30 s at 72 °C extension, followed by final extension of 10 min (72 °C). Parameters for nested PCR were: denaturation (94 °C 5 min), followed by 40 cycles of 30 s at 94 °C denaturation, 30 s at 56 °C annealing temperature, 30 s at 72 °C extension, followed by 10 min extension (72 °C). A plasmid containing HEV cDNA served as positive control. Amplicons (306 bp) were visualized on 1.5% agarose gels stained with SYBR green.

In addition, the HBV patients were confirmed for the presence of HEV-RNA by applying the same PCR conditions by, however, using other set of primers which amplified a 497 bp ORF2 fragment. Outer primers were HEV-34 (sense) 5'-CCGACGTCYGTGAYATGAA-3' and HEV-36 (antisense) 5'-TTRTCTGCTGAGCRTTCTC-3'; inner primers were HEV-35 (sense) 5'-AAGTGAGCGCCTACAYTAYCG-3' and HEV-29 (antisense) 5'-CTCGCCATTGGCTGAGAC-3'.

### 2.5. HEV Genotyping

PCR products were purified (Exo-SAP-IT kit; USB, Affymetrix, USA) and used as sequencing templates (BigDye terminator v.1.1 sequencing kit; Applied Biosystems, USA, ABI 3130XL sequencer). HEV genotyping was performed by phylogenetic analyses based on the amplified ORF2 sequences using the MEGA 5 software ([www.megasoftware.net](http://www.megasoftware.net)). HEV reference sequences were obtained from the NCBI GenBank database.

## 2.6. Statistical Analysis

Analyses were performed using the SPSS software (SPSS Statistics, IBM, Armonk, NY) and Intercooled Stata (Stata Corporation, College Station, TX, USA). Prevalences and quantitative variables are given as percentages and medians with ranges. Categorical variables were compared applying Chi square or Fisher's exact tests. Kruskal–Wallis or Mann–Whitney U tests were used to compare quantitative variables. A logistic regression model was used to compare HEV seroprevalences between groups and to analyze associations of HEV seroprevalences with disease progression. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. The level of significance was  $P < 0.05$ .

## 3. Results

### 3.1. Demographic and Clinical Characteristics of Hepatitis B Patients and Controls

The baseline characteristics of the 1318 Vietnamese hepatitis B patients and 340 healthy controls are provided in Table 1. The progression of liver disease increased according to median age of patients. Most patients were male (82%). Differences of means were observed for several clinical parameters, including white and red blood cell counts, platelets, AST/ALT, total and direct bilirubin, albumin, prothrombin, AFP and HBV viral loads. Platelet counts were lower in patients with LC compared to non-LC patients ( $P < 0.001$ ). Levels of AST, ALT and total and direct bilirubin were higher in AHB patients compared to other subgroups ( $P < 0.001$ ). Albumin and prothrombin levels were lower in patients with LC and/or with HCC ( $P < 0.001$ ). As expected, AFP levels were higher in HCC compared to non-HCC patients ( $P < 0.001$ ). HBV viral loads were higher in chronic hepatitis B patients without LC and/or HCC than in chronic hepatitis B patients with LC and/or HCC (Table 1).

### 3.2. Seroprevalence of HEV Infection in Patients With HBV Infection and in Healthy Population

Seroprevalence rates of anti-HEV IgG were higher in patients (45%) compared to controls (31%) ( $P = 0.034$ ). When stratified for the clinical subgroups of HBV infection, the anti-HEV IgG seroprevalence was 42% (11/26) in AHB, 41% (305/744) in CHB, 52% (83/160) in LC, 48% (79/166) in HCC and 49% (108/222) in patients with LC and HCC

(Fig. 1A, Supplementary Table 1). Anti-HEV IgM was observed in 19% (30/160) of LC patients, followed by 15% (4/26) of AHB patients, 15% (33/222) of patients with both LC and HCC, 12% (20/166) of HCC patients, 9% (65/744) of CHB patients and 5% (16/340) of controls. The seroprevalence rate of anti-HEV IgM was also higher among patients with HBV infection (11.6%) compared to the controls (4.7%; adjusted  $P = 0.005$ ) (Fig. 1A, Supplementary Table 1). In the HBV group positive for anti-HEV IgG, we observed a higher positivity of anti-HEV IgM in the AHB (36%) and LC (36%) subgroups, followed by patients with both LC and HCC (29%), HCC (20%) and CHB (20%) (Fig. 1B).

### 3.3. Association of Higher HEV Seroprevalence With Increasing Age

Seroprevalences of anti-HEV IgG and IgM were higher in elder compared to younger subgroups of HBV patients and controls. In the HBV group, the mean age of patients positive for anti-HEV antibodies was higher than that in HBV patients negative for anti-HEV antibodies ( $P < 0.001$  and  $P = 0.002$  for anti-HEV IgG and IgM). A similar trend applied to the controls ( $P < 0.001$  and  $P = 0.022$  for anti-HEV IgG and IgM). A prevalence of 51% and 46% of anti-HEV IgG was observed in HBV patients and controls >40 years of age, respectively. The anti-HEV IgG prevalence in HBV patients between 30 to 40 years was higher compared to that in controls of the same age group ( $P = 0.03$ ). No difference was seen between HBV patients and controls <30 years of age (Fig. 1C and Supplementary Table 2). The prevalence of anti-HEV IgM in HBV patients and in controls >40 years of age was 13.7% and 9.2%, respectively. When stratified for age groups and sex, no differences of anti-HEV IgM prevalence was observed between HBV patients and controls (Fig. 1D and Supplementary Table 2).

### 3.4. HEV Seroprevalence and Progression of HBV-Related Liver Diseases

We found higher anti-HEV IgM prevalences in LC patients (16.8%) compared to patients without LC (9.5%) (OR = 1.64; 95% CI = 1.1–2.4; adjusted  $P = 0.01$ ), indicating that anti-HEV IgM was associated with LC in chronic hepatitis B patients (Table 2). Significance did not apply to anti-HEV IgG seroprevalences. A similar trend was found when comparing anti-HEV IgG and IgM prevalences between HCC and non-HCC patients (Supplementary Table 3).

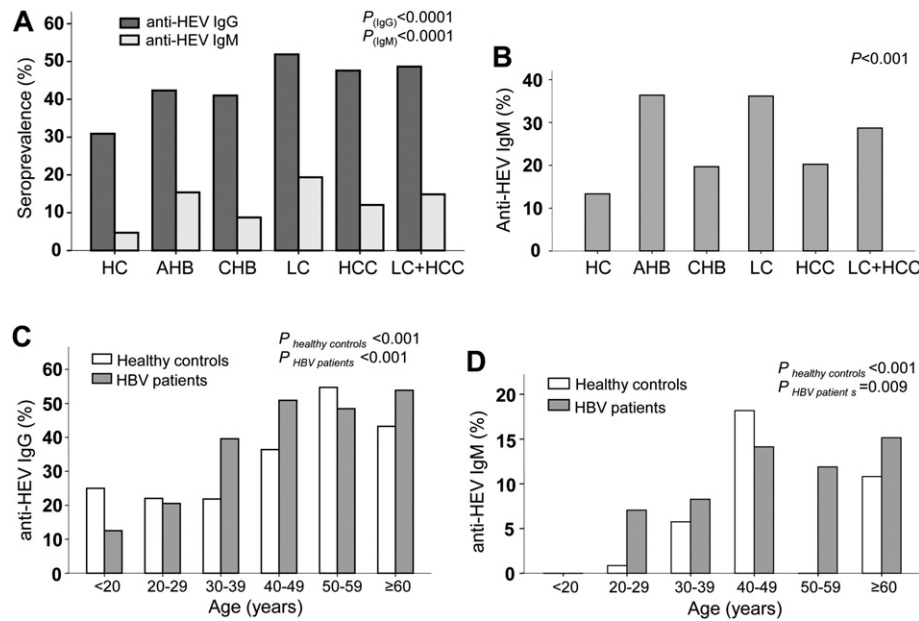
To analyze associations of HEV seroprevalences with underlying LC and its prognosis, we categorized LC patients into three subgroups based on Child–Pugh scores. Anti-HEV IgG prevalences in Child–B and

**Table 1**  
Demographic and clinical characteristics of the studied HBV patients and controls.

Characteristics	AHB (n = 26)	CHB (n = 744)	LC (n = 160)	HCC (n = 166)	LC and HCC (n = 222)	Healthy controls (n = 340)
Age (years)	34 [20–49]	41 [9–84]	57 [15–84]	55 [15–81]	59 [26–81]	33 [15–69]
Gender (M/F)	21/5	557/187	135/25	159/7	210/12	223/117
WBC* ( $10^3$ /ml)	NA	6.5 [4–17]	5.6 [1.7–20.5]	6.3 [3–16]	6 [2.5–17]	Normal
RBC* ( $10^6$ /ml)	NA	4.9 [3–6.8]	4.2 [1.9–9.2]	4.7 [2.1–6.8]	4.3 [2.2–6.2]	Normal
PLT* ( $10^3$ /ml)	NA	223 [19–401]	90 [3.7–441]	208 [68.6–389]	122 [34–361]	Normal
AST* (IU/l)	1064.5 [316–4425]	44 [14–1600]	76.5 [15–1221]	48.5 [17–2158]	72 [14–670]	<30
ALT* (IU/l)	1125.5 [309–3328]	48 [8–2924]	58.5 [8–1426]	40.5 [10–832]	54.5 [10–805]	<30
Total bilirubin* ( $\mu$ mol/l)	184.8 [21.8–558]	15 [4.9–452]	31 [4.1–690]	14 [5–160]	22 [7–419]	<17
Direct bilirubin* ( $\mu$ mol/l)	137.9 [15–353]	5 [1–298]	12 [0.4–440]	4.9 [1–80]	8.2 [0.4–214]	<5
Albumin* (g/l)	36 [27–42]	41.8 [26–48]	34 [20–47]	39 [27–49]	37 [24–47]	>35
Prothrombin* (% of standard)	70 [58–127]	93 [45–215]	56.5 [13–101]	88 [43–172]	75 [19.6–158]	>70
HBV viral load* (copies/ml)	$8.8 \times 10^3$ [1062– $3.7 \times 10^4$ ]	$1.1 \times 10^6$ [189– $4.3 \times 10^{12}$ ]	$6.8 \times 10^4$ [180– $4.7 \times 10^9$ ]	$8.56 \times 10^5$ [450– $1.44 \times 10^9$ ]	$5.85 \times 10^4$ [190– $3.04 \times 10^{10}$ ]	NA
Alpha-feto protein (AFP)* (mg/l)	NA	4.3 [1–200]	7.3 [1.18–300]	196 [1.1–305]	170 [1.6–880]	NA

AHB: acute hepatitis B; CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; WBC: white blood cells; RBC: red blood cells; PLT: platelets; AST and ALT: aspartate and alanine amino transferase; IU: international unit; NA: not available. Values given are medians and range.

\*  $P < 0.001$  for comparison with all other groups.



**Fig. 1.** Seroprevalence of HEV infection in patients with HBV infection and in healthy population. (A): Anti-HEV IgG and anti-HEV IgM in healthy controls (HC) and in HBV patients including acute hepatitis B (AHB), chronic hepatitis B (CHB), patients with only liver cirrhosis (LC), patients with only hepatocellular carcinoma (HCC) and patients with both LC and HCC; (B): Anti-HEV IgM positivity in individuals positive for anti-HEV IgG; (C): Anti-HEV IgG seroprevalence increasing with age; (D): Anti-HEV IgM prevalence in different age groups. *P* values were calculated by Chi square or Fisher's exact tests for comparisons of the seroprevalence among different groups.

Child-C LC patients were higher compared to those in Child-A patients (adjusted  $P < 0.001$  and  $P = 0.002$ , respectively). A similar result was observed for anti-HEV IgM (adjusted  $P = 0.007$  and  $P < 0.001$ , respectively) (Table 2), indicating that higher seroprevalences of anti-HEV IgG and IgM are associated with an increase of LC severity.

### 3.5. Significance of HEV Coinfection in the Outcome of HBV Patients

To analyze the significance of HEV superinfection in HBV patients, we categorized HBV patients into three subgroups based on HEV serology results. Patients negative for anti-HEV IgG and IgM were categorized as “no HEV infection”. Patients who were positive for anti-HEV IgG and negative for IgM were categorized as “past HEV infection”. “Current HEV infection” was defined as HBV patients positive for anti-HEV IgM (anti-HEV IgG positive or negative). Levels of AST, total and direct bilirubin and HBV-DNA viral loads were elevated in the “current HEV infection” group compared to the “no HEV infection” and “past HEV infection” groups ( $P = 0.023$  for AST,  $P = 0.01$  for total bilirubin,  $P = 0.005$  for direct bilirubin,  $P = 0.026$  for HBV-DNA viral loads) (Fig. 2). In contrast, levels of albumin, prothrombin and platelet counts in the “current HEV infection” group were lower compared to the “no HEV

infection” and “past HEV infection” groups ( $P < 0.001$  for albumin and prothrombin,  $P = 0.018$  for platelets) (Fig. 2).

We formed three subgroups of patients with “no HEV infection”, “past HEV infection” and “current HEV infection” for each patient group including AHB, CHB, LC, HCC and patients with both LC and HCC. We observed that AST levels were increased, while prothrombin levels were decreased in the “current HEV infection” patients in CHB group ( $P = 0.01$ ) and for other clinical parameters no differences were observed (Fig. 3).

### 3.6. Prevalence of HEV-RNA and Sequencing Analysis of HEV Isolates

HEV-RNA was detected in only one serum sample from a patient with both LC and HCC. The phylogenetic analysis revealed that the HEV isolate was close to a HEV genotype 3 (NCBI #ssID 1825526) (Supplementary Fig. 1). The patient was negative for both anti-HEV IgG and IgM, indicating that he was in the early phase of HEV infection.

## 4. Discussion

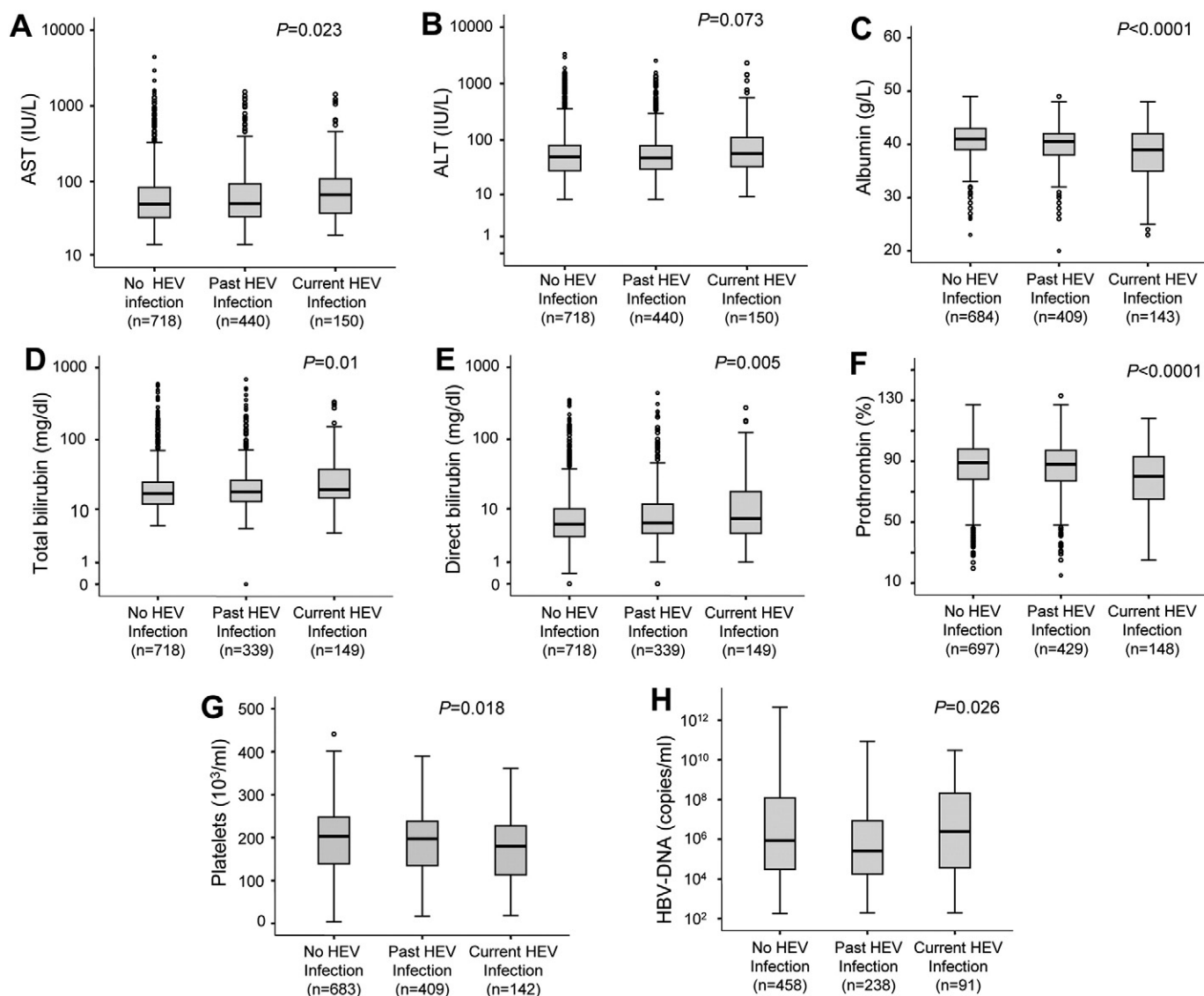
HEV superinfection in HIV-infected patients, but also in patients with chronic HBV and HCV infections may aggravate the course of underlying conditions (Dalton, 2012; Pischke et al., 2014). We describe

**Table 2**  
Association of HEV seroprevalence with liver cirrhosis (LC) and prognosis of LC in HBV patients.

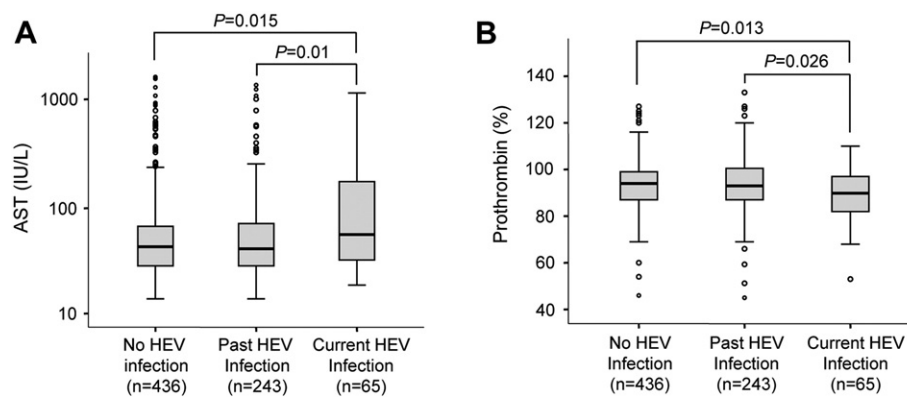
Patient group	Anti-HEV IgG				Anti-HEV IgM			
	Total n	Positive n (%)	OR (95% CI)	<i>P</i> value	Total n	Positive n (%)	OR (95% CI)	<i>P</i> value
<i>Cirrhosis status</i>								
Non-cirrhosis	936	395 (42.2)	1	Reference	936	98 (9.5)	1	Reference
Cirrhosis	382	191 (50)	0.94 (0.7–1.22)	0.65	382	64 (16.8)	1.64 (1.1–2.4)	0.01
<i>Child–Pugh</i>								
Child A	235	91 (38.7)	1	Reference	235	25 (10.6)	1	Reference
Child B	117	79 (67.5)	3.3 (2.05–5.2)	<0.0001	117	31 (21.4)	2.3 (1.3–4.3)	0.007
Child C	30	9 (70)	1.9 (1.3–2.9)	0.002	30	14 (46.4)	2.7 (1.8–4.2)	<0.0001

Non-cirrhosis: HBV patients without liver cirrhosis; Cirrhosis: hepatitis B patients with liver cirrhosis (with or without hepatocellular carcinoma); odds ratio (OR) and *P* values were calculated by using logistic regression model and adjusted for age and gender.





**Fig. 2.** Clinical outcomes of HEV superinfection in HBV patients. Based on the positivity of anti-HEV IgG and anti-HEV IgM, HBV patients were categorized into three different groups as “no HEV infection”, “past HEV infection” and “current HEV infection”. Different clinical and biochemical parameters including AST (A), ALT (B), albumin (C), total bilirubin (D), direct bilirubin (E), prothrombin (F), platelets (G) and HBV-DNA viral loads (H) were compared. Box-plots illustrate medians with 25 and 75 percentiles and  $P$  values were calculated by using Kruskal–Wallis test. The number in parenthesis indicates number of samples analyzed and those numbers in the respective groups vary for the clinical parameters because some patients were not tested for all the clinical parameters.



**Fig. 3.** Clinical outcomes of HEV superinfection in patients with chronic HBV. HBV patients were categorized into three different groups as “no HEV infection”, “past HEV infection” and “current HEV infection” based on the positivity of anti-HEV IgG and anti-HEV IgM. The levels of aspartate aminotransferase (AST) (A) and prothrombin (B) were compared. Box-plots illustrated medians with 25 and 75 percentiles and  $P$  values were calculated by using Kruskal–Wallis test. The number in parenthesis indicates number of analyzed subject samples.

higher HEV seroprevalences in patients with HBV-related liver disease compared to healthy individuals. The frequency of HEV superinfection in HBV patients was differentially distributed among progressive stages of HBV infection. HEV infection was independently associated with the prognosis of HBV-related LC and the clinical outcome of HBV infection.

HEV seroprevalences have previously been determined in general populations and blood donors from various geographical settings (Boutrouille et al., 2007; Cleland et al., 2013; Gallian et al., 2014; Guo et al., 2010; Hau et al., 1999; Pittaras et al., 2014; Ren et al., 2014). Our study shows prevalences of 31% and 5% of anti-HEV IgG and IgM, respectively, in healthy Vietnamese individuals. Anti-HEV IgG seropositivity was considerably higher than previously reported in rural areas of Vietnam (Hau et al., 1999). This difference might result from the sensitivity and specificity of ELISA tests applied (Abravanel et al., 2014; Mansuy et al., 2011). Comparative analysis of the sensitivity and specificity of commercial test systems have been described in recent reports showing comparable performance in terms of negative predictive value and slightly variable performance in relation to sensitivity and specificity (Avellon et al., 2015; Pas et al., 2013; Wenzel et al., 2013; Wu et al., 2014). In our study, the used MP HEV EIA showed minor limitation with regard to sensitivity (IgM and IgG, approx. 80% and approx. 75%, respectively) in agreement to recent reports. However, the MP HEV EIA assay seemed to be robust and suitable for our analysis generating a feasible and true number of positive HEV IgM and IgG samples. Social and demographic characteristics may also cause differences of seroprevalences (Hau et al., 1999; Mansuy et al., 2011). Our results support studies in Chinese blood donors, where anti-HEV IgG and IgM prevalences were approximately 30% and 1%, respectively (Guo et al., 2010; Ren et al., 2014). Somewhat lower anti-HEV IgG prevalences of 3% to 17% occur among most European populations (Beale et al., 2011; Boutrouille et al., 2007; Cleland et al., 2013; Gallian et al., 2014; Juhl et al., 2014; Pittaras et al., 2014; Ren et al., 2014). HEV seroprevalences were, however, exceptionally high in southern France (53%) and Denmark (21%) (Christensen et al., 2008; Mansuy et al., 2011).

This is the first study on HEV superinfection in Vietnamese HBV patients. Prevalences of anti-HEV IgG and IgM in patients were 45% and 12%, respectively. The HEV seroprevalence in our patient group differed from other studies on HBV, HCV and HIV infections (Atiq et al., 2009; Bayram et al., 2007; Feldt et al., 2013; Hamid et al., 2002). Anti-HEV IgG prevalences in chronic HBV Turkish and American patients were 14% and 8% (Atiq et al., 2009; Bayram et al., 2007). In agreement with other findings (Atiq et al., 2009), our results indicate that the HEV seroprevalence was higher in patients with HBV-related liver diseases compared to healthy individuals, suggesting patients with HBV-related liver diseases might have a higher risk for HEV infection (Hamid et al., 2002). Nevertheless, similar findings were not observed in other studies (Bayram et al., 2007; Hamid et al., 2002). A probable explanation could be the sample size utilized in these studies and the study area. These both factors may largely contribute for such dissimilarities in seroprevalence rates. In Europe, HEV infection was observed to be higher in autoimmune hepatitis patients and not in patients with either chronic hepatitis B or C (Pischke et al., 2014), whereas in endemic areas like Vietnam, individuals usually acquire the HBV early in their life and HEV infection subsequently through the fecal–oral transmission later. A plausible explanation is that the patients primarily infected with HBV may show an altered immune response and thus likely to be more susceptible for HEV as a secondary infection. In addition, HEV superinfection in HIV patients and development of chronic hepatitis E has also been described (Dalton et al., 2009). Recently, a study has found a seroprevalence of anti-HEV IgG in Ghana (45%) and Cameroon (14%) (Feldt et al., 2013). However, no contribution of HEV infection to liver pathology was observed. The HEV seroprevalence in our study group was significantly associated with age, both in HBV patients and controls, indicating cumulative exposure to HEV. Aging of the immune system might also favor acquisition of HEV infection.

Although most HEV-infected individuals are asymptomatic, the outcome of HEV superinfection in HBV patients appears more severe (Cheng et al., 2013; Marion-Audibert et al., 2010; Monga et al., 2004). Our results show increased levels of liver enzymes and total and direct bilirubin and decreased levels of albumin, prothrombin and platelet counts in HBV patients with concomitant HEV infection. In contrast to an earlier study suggesting that chronic HBV infections may be inactive during HEV–HBV coinfections (Cheng et al., 2013), we found higher HBV-DNA loads in coinfections. Biochemical and serological tests suggest that HEV superinfection contributes to inflammation and liver failure. HBV-DNA loads were lower in HBV patients with past HEV infection compared to those with no or patent HEV infection, suggesting that host immune responses contribute to control HBV replication. HBsAg positive individuals had a poorer prognosis after HEV superinfection (Chow et al., 2014; Wu et al., 2013).

Although associations of HEV infection with development and progression of LC were reported (Gerolami et al., 2008; Kumar et al., 2007; Marion-Audibert et al., 2010), the mechanisms of LC induction are unclear. According to previous studies, we show that HEV infections were independently associated with underlying LC and progression in chronic hepatitis B. Hepatocyte damage and immune responses during HEV superinfection that leads to increased liver inflammation can progress to LC. The severity of LC is classified based on the Child–Pugh score, determined by a number of clinical and laboratory parameters such as bilirubin, albumin, and prothrombin levels. The association of HEV superinfection with increased abnormalities of bilirubin, albumin, and prothrombin levels supports that HEV superinfection contributes to severity of HBV infection. Although the difference was not significant, the prevalence of anti-HEV antibodies was increased among patients with HBV-related HCC. Therefore, tumorigenesis of HEV cannot be excluded.

In this study, we detected the presence of HEV-RNA in only one serum sample from patient with both LC and HCC (negative for both anti-HEV IgG and IgM) showing that this patient was in the early stage of HEV infection. However, we could not follow up longitudinally the patient confirmed positive for HEV-RNA to verify the chronicity of HEV infection. HEV-RNA can persist longer in the stool than in the blood (Kamar et al., 2012), therefore another limitation of the study is that the nested PCR for detection of HEV-RNA from stool of the HBV patients and controls had not been performed due to the unavailability of stool samples.

In conclusion, this study indicates high HEV seroprevalences in Vietnamese patients with HBV-related liver diseases and in healthy individuals. HEV infection may aggravate the clinical outcome of HBV infection, especially in liver cirrhosis.

#### Author Contributions

NXH, HVT, NH, BTS, and PM contributed to performing of the experiments. LHS, NLT, PGK, CTB and TPV contributed materials and reagents. CTB, PGK and TPV designed the study. NXH, LHS and NLT recruited patients and collected samples. NXH and HVT performed statistical analyses. HVT, NXH, CTB and TPV wrote the manuscript. CGM and JK corrected the manuscript and contributed to the study design. NXH and HVT contributed equally to this work. All authors agreed with the results and conclusions. All authors agreed with the results and conclusions.

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data collection and analysis, decision to publish or preparation of the manuscript.

## Conflict of Interest

All authors have no conflicts of interest to declare.

## Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2015.11.020>.

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