

Full Length Research Paper

Comparative clinical study on diagnostic detection of hepatitis E virus between nested polymerase chain reaction (PCR) and serological tests

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This study was carried out to evaluate the diagnostic value of nested reverse transcriptase-polymerase chain reaction (RT-PCR) for HEV (hepatitis E virus) RNA detection relative to anti-HEV immunoglobulin G (IgG) and IgM enzyme-linked immunosorbent assays. One hundred and twenty six (126) patients with sporadic acute hepatitis E were included in this study. We focused on the popular genotype I and IV of HEV-positive patients in China, and selected the conserved region located in ORF2 for designing a set of nested RT-PCR primers. HEV RNA was detected in 53.2% (67/126) of patients, while all control subjects were negative for HEV RNA. The total agreement between IgM and nested RT-PCR detection was 80.9%, showing a fine coincidence. The results further suggested that there was a significant difference between nested RT-PCR detection and IgM ELISA: 3 cases with positive results for HEV RNA showed negative anti-HEV IgM at the early phase, and presented positive IgM reaction in succession after the trail of detection. HEV RNA was detected in serum samples from sporadic acute hepatitis patient usually by day 1 to 12 after the onset of symptoms, but showed a decreasing sensitivity with the increasing disease course. From these experiments, we can conclude that HEV RNA detection is of great clinical significance, which has an obvious advantage in diagnosis of early infection of HEV.

Key words: Hepatitis E virus, nested reverse transcriptase-polymerase chain reaction (RT-PCR), RNA, antibody.

INTRODUCTION

Hepatitis E (HE) remains an important public health problem in developing countries, and is diagnosed in some developed countries with sporadic cases (Emerson and Purcell, 2003). The HEV (hepatitis E virus) target population is young to middle aged adults, 15 to 40 years of age. The clinical symptoms are typical of acute viral hepatitis that includes jaundice, malaise, anorexia, nausea, abdominal pain, fever and hepatomegaly. The disease is self-limiting and generally no chronic sequelae has been reported (Emerson and Purcell, 2003). Increased morbidity and mortality is observed in chronic

liver disease patients superinfected with HEV. A unique clinical feature is its increased incidence and severity in pregnant women, with mortality rates of 15 to 20% (Kumar et al., 2004). Therefore, research on the discovery and validation of biomarkers of hepatitis E patients and their association with disease severity would be important.

Hepatitis E virus (HEV) is a non-enveloped RNA virus and its genome is a single-stranded, positive-sense RNA of 7.2 kb. It contains a short 5'-untranslated region (5'-UTR) followed by three ORFs (ORF1, ORF2 and ORF3)

and then a short 3'-UTR with a poly(A) tail. The ORF2 of HEV encodes its capsid protein (pORF2) of 660 amino acids and is proposed to encapsidate the viral RNA genome (Chandra et al., 2008). Based on nucleotide sequence analysis, mammalian HEV has been divided into four genotypes, namely genotypes 1 to 4. Genotype 1 is responsible for the majority of HEV infections in developing countries; genotype 2 consists of strains not only in Mexico but also in African countries including Chad, Namibia and Nigeria; genotype 3 is widely distributed throughout the world except in Africa; and genotype 4 is distributed exclusively in Asian countries. In China, genotypes 1 and 4 have become the dominant cause of HE (Zhu et al., 2011; Tai et al., 2009). Recently, detection of serum IgM specific for HEV by enzyme-linked immunosorbent assay (ELISA) is the most popular method used for the diagnosis of hepatitis E, but with lower sensitivity and missing cases with HEV infection (Favorov et al., 1994). Since various kinds of factors could influence the stability of the detection, including HEV window phase, patient autoimmune status and the specificity of tests, it is necessary to develop another assay with higher diagnostic value for HEV detection. The aim of the present study was to detect HEV RNA in serum using nested reverse transcriptase (RT)-PCR and to evaluate this detection for clinical diagnosis of hepatitis E in comparison with the well-adopted ELISA assay.

MATERIALS AND METHODS

Sample collection

A total of 126 serum samples were randomly collected from 34 patients with sporadic acute hepatitis E in Guangzhou, Guangdong, China. Twenty four of them were sampled for 4 times during the treatment, and the other 10 of them were sampled for three times during the treatment. Besides, 20 serum samples from patients with hepatitis A, 30 from patients with hepatitis B and 30 hepatitis C serum samples were collected for comparison. Thirty healthy volunteers were included in the study as controls. After centrifugation at 3500 rpm for 10 min, sera was separated and stored immediately in a -80°C freezer until use.

Detection of serum HEV RNA by nested RT-PCR

Since only genotypes 1 and 4 of HEV were causing pandemic in China, we focused on genomic sequences of genotypes I (EMBL ID: D11092, L08816, L25547, M94177) and IV (EMBL ID: AB108537, AJ 272108) and selected the conserved region located in ORF2 for designing a set of nested RT-PCR primers. RT-PCR was performed using a QIAGEN One-Step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. cDNA was produced in a reaction tube containing 10 µL of the template RNA, 4 µL of the 5×QIAGEN One-Step RT-PCR buffer, 0.2 µL primer (50 mmol L⁻¹), 2 µL of the dNTP mix (containing 10 mM of each dNTP), 20 U of the RNase Out RNA inhibitor, 12 U of AMV reverse transcription enzyme mix, 2.3 µL of the RNase-free water. The thermal cycling conditions included one step of reverse transcription for 1 h at 42°C. The primers for nested PCR were as follows: the external forward primer M1[5'-AATTATGCCGAGTAACCGGGTT-3'] and reverse primer M2 [5'-CCCTTATCCTGCTGAGCATTCCC-3']. The

reaction system in a volume of 50 µL contained 5 µL of 10×PCR buffer, 1 µL of each primer (M1, M2, M3 and M4) (50mmol L⁻¹), 1 µL of the dNTP mix (10 mM of each dNTP), 0.5 µL of Taq enzyme (5 U µL⁻¹), 5 µL of the RT-PCR product. The nested forward primer M3[5'-GTTATGTTTTGCATAGAAATGGC-3']], products from first round were used as the templates for the second round of nested PCR. The nested reverse primer M4 [5'-AGCCGACGAAATCAATTCTGTC-3']; house keeping gene β-actin: [sense-primer 5'-GTCGTACCACTGGCATTGTG-3', anti-sense 5'-CCATCTCTTGCTCGAAGTCC-3] served as replication control. Both thermal cycling conditions for nested PCR were the same: 35 cycles of denaturation for 45 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C, and a final incubation for 10 min at 72°C. The amplified PCR products were examined by 1.5% agarose gel electrophoresis.

IgM and IgG anti-HEV ELISA

All serum samples were thawed at room temperature and tested with IgM and IgG anti-HEV ELISA kits (manufactured by Genelabs Diagnostics). The samples with an optical density less than the cutoff value (mean optical density for the three negative controls on each plate plus 0.4 for IgM, plus 0.5 for IgG) were considered as negative. Samples with an optical density greater than or equal to the cutoff value were tentatively considered reactive and then retested to confirm the result. The absorbance was determined at 450 nm. The ELISA was performed according to the protocols provided by the manufacturer.

Statistical analysis

The IgG and IgM anti-HEV tests were compared with the HEV RNA test by RT-PCR for concordance. Fisher's exact test and the chi-square test were used to compare the prevalence of anti-HEV among tested groups. A *p* value of less than 0.05 was considered significant.

RESULTS

The specificity of nested RT-PCR for HEV

Nested RT-PCR for HEV was performed on 20 HAV patients, 30 HBV patients, 30 HCV patients and 30 healthy persons. All the results were negative. Besides, their anti-HEV IgM detection results showed negative as well. Nested RT-PCR for HEV was able to amplify specifically without any cross reaction with other hepatitis virus.

The frequency of IgM and IgG anti-HEV detected by ELISA

One hundred and twenty six (126) serum samples collected from 25 patients with sporadic acute hepatitis E during treatment were detected for IgM and IgG anti-HEV by ELISA. Our study showed that cases with positive results for IgM alone were 6.3% (8/126), IgG alone were 25.4% (32/126), and both for IgM and IgG were 56.3% (71/126). The frequency of HEV-negative cases both for IgM and IgG were 11.9% (15/126). In all, the frequency of HEV-positive for IgM was 62.7% (79/126), IgG 81.7%

Table 1. The nested RT-PCR results of 126 serial serum specimens of HE.

Anti-HEV	Number	Positive cases for nested PCR	Positive rate (%)
IgM+IgG-	8	2	25.00
IgM+IgG+	71	52	73.20
IgM-IgG+	32	10	31.30
IgM-IgG-	15	3	20.00
Total	126	67	53.20

Table 2. The test result of two methods comparing 126 serial serum specimens of HE.

HEV RNA result	anti-HEV IgM		Total
	Positive cases	Negative cases	
Positive cases	61	6	67
Negative cases	18	41	59
Total	79	47	126

$\chi^2 = 49.159$, $P=0.000$; $P=0.023$

(103/126).

Nested RT-PCR detection

HEV RNA was detected in 53.2% (67/126) of patients with sporadic acute hepatitis E, 25.0% (2/8) in anti-HEV-IgM-positive alone samples, 31.3% (10/32) in anti-HEV-IgG-positive alone samples, 73.2% (52/71) in anti-HEV positive both for IgM and IgG samples, and 20.0% (3/15) in anti-HEV negative for IgM and IgG samples (Table 1).

The agreement between IgM and nested RT-PCR for positive results was 77.2% (61/79), and the agreement for negative cases was 87.2% (41/47), with the total coincidence of 80.9% (102/126) (Table 2). Our study showed a concordance of the laboratory results for HEV between nested RT-PCR detection and IgM ELISA ($Kappa = 0.613$, $P=0.000$). Under analysis of *Pearson* χ^2 and *McNemar* χ^2 , though with some complementarity, it was suggested that there was a significant difference between nested RT-PCR detection and IgM ELISA ($\chi^2 = 49.159$, $P=0.000$; $P=0.023$), so that they could not be replaced by each other for HEV detection.

Correlation between course disease and lab results for both assays

Among the 126 serum samples of 25 patients, 3 cases with positive results for HEV RNA showed negative anti-HEV IgM at first, and presented positive IgM in succession after the trail of detection. HEV RNA was detected in serum samples from sporadic acute hepatitis patient usually by day 1 to 12 after the onset of symptoms, but showed a decreasing sensitivity with the progression of disease. While anti-HEV IgM presented a

high positive frequency up to week 2 after the onset of symptoms (Table 3).

DISCUSSION

HEV infection often causes viremia. Patients were found to excrete HEV in stool after there was a high concentration of virus in bile. Hepatitis E viremia can be detected before the onset of liver abnormality which was accompanied by humoral immune reaction. Currently, the exact moment at which HEV caused the antibody reaction was unknown. Besides, since there is no robust system to grow HEV in culture, there are some limitations in the field of HEV study (Chandra et al., 2008). Studies on experimentally infected macaques first defined the clinical and serological course of HEV infection. In those studies, serum anti-HEV immunoglobulin G (IgG) appeared around 3 to 4 weeks post-inoculation at the peak of ALT elevation. A human volunteer study showed anti-HEV IgM to peak in the symptomatic period and then decline to baseline within 3 to 6 months of illness. Serum anti-HEV IgG levels continued to rise during the symptomatic phase and became detectable in the convalescent phase for 2 years (Meng, 2010; Zhu et al., 2008). Therefore, both molecular and serological methods are important for HEV diagnosis.

The diagnosis of hepatitis E is usually made serologically by commercial ELISA kits. However, limitations for serodiagnosis exist due to various envelope antigens in different kits. Since different HEV genotypes determine the differences of their antibody response to corresponding antigens, the detection results from different ELISA kit might present discordance (Chen et al., 2005). Evidences showed that anti-HEV antibodies, developed

Table 3. The result of 126 serial serum specimens detected by two methods of HE.

Course of disease (days)	Number	HEV RNA		anti-HEV IgM	
		Positive cases	(%)	Positive cases	(%)
1-4	24	16	66.7	14	58.3
5-8	45	28	62.2	30	66.7
9-12	21	12	57.1	15	71.4
13-15	17	7	41.2	11	64.7
>15	19	4	21.1	9	47.4
Total	126	67	53.2	79	62.7

in various laboratories using synthetic peptides or recombinant proteins derived from different genotypes and/or subtypes of HEV, showed a wide variation in sensitivity in seroprevalence studies (Zaki et al., 2009; Ma et al., 2011). Furthermore, derived from the same genotype and/or subtype of HEV but expressed in different expression system, the antigenicity and epitope displayed significance difference (Chau et al., 2006). Depending only on serology for hepatitis E can either miss the diagnosis of early cases or over-diagnose cases without true viremia. Thus aetiology will be of great help for HEV diagnosis. Methods for aetiological diagnosis include immune electron microscopy and traditional RT-PCR assay. These methods, with lower sensitivity, were limited to detect latent infection and early acute infection. Recently, nested RT-PCR shows a better way for detection with a higher specificity and sensitivity (Kumar et al., 2011; Mirazo et al., 2011).

HEV RNA was usually detected in both serum and stool in late latent period and early acute infection of patients with HE. In some HE cases with both negative results of anti-HEV IgM and IgG, HEV RNA was positive. In the present study, 3 of 15 serum samples with both negative results of anti-HEV IgM and IgG had detectable positive HEV RNA. It is believed that HEV RNA detection is of great help to make a diagnosis in HEV early infection, covering up the shortage of serological method. Since only genotype I and IV HEV were reported in China, we focused on genomic sequences of genotypes I and IV HEV and selected the conserved region for designing a set of nested RT-PCR primers, of which GC content was equal to AT content. We found that the selected sequence, located in the junction region between N-terminal sequence of ORF2 and ORF3 in HEV, showed a high conservatism, but were rich in GC that was not beneficial to PCR amplification. Sequences located in inner ORF2 was not only relatively conservative, but also had the appropriate GC content for PCR amplification (Ahmad et al., 2011; Johne et al., 2010). We thus selected a set of nested RT-PCR primers for this region and resulted in an expected high degree of specificity. HEV RNA was detected in 53.2% of patients with sporadic acute hepatitis E, which bled in 12 days after the onset of disease. Consistent with other relevant reports, the positive frequency of HEV RNA was lower

than ELISA with 62.7 (anti-HEV IgM) and 81.7% (anti-HEV IgG), respectively. We assumed that it might be due to the short period of viremia, which usually lasted for only 2 weeks, and the presence of anti-HEV IgM often lasted longer than viremia (Cheng et al., 2012; Khudyakov and Kamili, 2011). In our study, the total coincidence of agreement between IgM and nested RT-PCR detection was 80.9% ($Kappa = 0.613$, $P = 0.000$) in 126 patients with sporadic acute hepatitis E. Under analysis of Pearson X^2 and McNemar X^2 , though with complementarity, it was suggested that there was a significant difference between nested RT-PCR detection and IgM ELISA ($X^2=49.159$, $P=0.000$; $P=0.023$), so both molecular and serological methods must be applied for accurate diagnosis.

Noted above, HEV RNA was usually detected in early acute infection of patients with HE, and tended to decline to an undetectable level with the course of disease progressing. On the other hand, since anti-HEV IgM might not reach the detectable level in early infection stage, depending mainly on serological tests could misdiagnose cases with early viremia before seroconversion. Instead, positive result of HEV RNA would be more helpful for early diagnosis. From this study, we could conclude that HEV RNA detection is of great clinical significance, which shows an obvious advantage of making a diagnosis in early infection of HEV.

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