A Comparative Study of Detection of Hepatitis E Virus RNA by RT-PCR and Digoxin Probe Techniques

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Abstract

Techniques for detecting hepatitis E virus nucleic acid by RT-PCR and digoxin probe spot hybridization tests were developed. The examination by RT-PCR test of 250 stored stool specimens from patients with acute hepatitis E gave a positive rate of 40.9% and was positive in one stool specimen collected 28 days after the patient had contracted the illness. Serologic examination yielded a positive rate of 66.7%, and also utilizing the DIG probe labelled with PCR product it was 66.6%. RT-PCR with the digoxin probe proved to be suitable for clinical diagnosis and basic research on hepatitis E.

Introduction

Detection of serum antibodies against hepatitis E virus (HEV) cannot replace viral diagnosis, although it has been used in the clinical diagnosis of HEV as a result of rapid advances in diagnostic methods for hepatitis E (HE). Currently the most commonly used laboratory methods with high specificity and sensitivity for detection of viral nucleic acid are polymerase chain reaction (PCR) and nucleic acid probe. To our knowledge, there have been no reports on the detection of HEV nucleic acid (HEV RNA) using a digoxin probe (DIG probe). The use of reverse transcription-“nested” polymerase chain reaction (RT-PCR) for detection of HEV RNA is currently in progress. Our laboratory has developed RT-PCR and DIG probe techniques for detection of HEV RNA in stool specimens and sera of patients with hepatitis E seen during the 1986–1990 period.

Material and Methods

Subjects:
Sera and stools of patients with confirmed HE seen in Hetian in 1986–1987, in Turfan in 1990 and Kashi in 1992 were collected 7–30 days after the onset of the illness as confirmed by immune electron microscopy (IEM). The stool samples were stored at −35°C. The control samples consisted of HBV sera, HCV sera, and HAV stools from patients, treated in the Department of Infectious Diseases of our hospital, who had been diagnosed previously. Pararotavirus strains A (RV-A) and KB63 (RV-KB63) were kindly supplied by the Unit of Microbiology, the August First Agricultural College of Xinjiang, and cytomegalovirus (CMV) strain by the Institute of Pediatrics of our hospital.

Reagents:

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1. The principal reagents used to extract viral nucleic acid were: PEG 6000, 2-mercaptoethanol, RNAsin, guanidine isothiocyanate and diethyl pyrocarbonate. The latter two reagents were purchased from Sigma Co., USA.

2. HEV Primers\(^1\): the following primers were synthesized by the Biotechnology Center of the Chinese Academy of Zootecnics and Veterinary Science.

   F1 5'-GCTATTATGGAGGAGTGTGG-3' (4459-4478)
   R1 5'-CAGGGCCCAATTCTTCTTCT-3' (4859-4876)
   F2 5'-GCGTGGATCTTGCAGGCC-3' (4522-4539)
   R2 5'-TTCAACTTCAAGCCACAGCCC-3' (4741-4760)

3. The principal reagents for RT-PCR: Reverse transcriptase and Taq enzyme were purchased from Promega Co., USA and 4 × 10 mM dNTP from Boehringer Mannheim Co., Germany.

4. DIG probe: The DIG-labelled kits for detection were obtained from Boehringer Mannheim Co., Germany.

The main instruments were:

1) PCR detector: Auglone-BELGLUM, Rue August Joivet 8-4031.
2) Table Type Ultracentrifuge: Tomy Mc-120, Japan, 12000 rpm.
3) TG-16G Ultracentrifuge: made in Beijing, China, 15000 rpm.
4) Model TR-A Ultraviolet Detector: made in Shanghai, China.

Methods:

1. Virus concentration for extraction of nucleic acid: one-step extraction with guanidine isothiocyanate was used.

2. RT-“nested” PCR\(^2\):

   (1) The first PCR proceeded synchronously with reverse transcription: 5 μl of 10× PRC buffer solution, 3.5 μl of 25 mM MgCl₂, 4 μl of 4 × 10 mM dNTP, 3 μl of 10 μmol R₁, 3 μl of 10 μmol F₁, 2.5 μl of Taq enzyme and 4 μl of avian myeloblastosis virus (AMV) reverse transcriptase. The above virak extracts were treated with diethyl pyrocarbonate (DEPC) containing RNAsin and were dissolved in a 31 μl 1 quantity of water. Liquid paraffin embedding and mounting was performed. Reverse transcription proceeded at 42°C, and PCR was carried out 20 min later.

   Circulation conditions: 94°C 1 min, 37°C 1 min and 72°C 2 min, with 30 circulations and extension at 72°C for 5 min.

   (2) The second PCR was carried out by taking the product of the first PCR as the template and adding R₂ and F₂ in quantities equal to those in the first PCR, under the same operational condition and employing the procedures described above.

   (3) Electrophoretic analysis: electrophoresis was carried out by adding 1 μl of sample solutions to 5 μl of the second amplified product with 1.5% TAE agarose gel (containing 1 μg/ml ethidium bromide) and 1 × TAE as electrophoresis buffer solutions at 80 volts. Under an ultraviolet detector, a strip at 239 bp was judged to be positive.

   3. DIG probe: Detection utilizing the DIG probe was performed in accordance with the requirements described in the detections for the DIG kit. The DIG-labelled probe was the second product (239 bp) of detection of HEV RNA by RT-PCR in this laboratory, and a spot hybridization test was carried out on a nitrocellulose membrane.

Results

The detection by RT-“nested” PCR of HEV RNA in stool specimens from patients with acute HE showed strips at 239 bp. On the other hand, the examinations of stool extracts from normal subjects and patients with rotavirus, pararotavirus and hepatitis A, and hepatiits B and hepatitis C sera which
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had undergone amplification by the same method, yielded negative results, demonstrating the proper selection of primers and the successful application of these methods (Fig. 1).

The positive detection rates from 250 stored stool samples collected 7-30 days after the onset of the illness were as follows: 16.6% in Hetian for the 1986-1987 period, 40.0% in Turfan in 1990, and 66.0% in kashi in 1992; an overall positive rate of 40.9% was obtained. In comparison with ELISA⁴, the RT-"nested" PCR method was more than twice as sensitive. The duration of sample preservation is the main factor influencing positive results; the longer the duration of preservation, the more HEV lysis and the lower the positive rates. One stool sample collected 28 days after the illness onset showed positivity, the longest time lag for detecting HEV RNA in a patient stool specimen³, the longest duration of antigen persistence in a stool specimen⁴ and the longest viral excretion period as demonstrated by IEM⁵ hitherto reported in the literatura. This suggests that the present method is highly specific and sensitive and can detect even very low levels of virus within a sample.

Fig. 1 Detection of HEV by RT-"nested" PCR.
1-3: HAV; 4-6: HBV; 7 and 9: HEV, RNA; 8: Molecular PBR 322/Hae; 10-12: HCV; 13: RV-A; 14: RV-KB63; 15-16: CMV.

Fig. 2 The spot hybridization with DIG probe for detection of HEV RNA.
A1–15: Solution for nucleic acid;
B1–15: 10% solution for nucleic acid;
C1–15: 1% solution for nucleic acid;
D1,2,4: The first PCR products;
D3: The first PCR equivocal products;
D5–7: The second PCR products;
D8: The second PCR equivocal products;
D9: Denatured salmon sperm DNA;
D10: HCV-RNA;
D11: double-distilled water;
D12: 239 bp segment after 4 reclamations;
E1–8: different respective dilutions of 239 bp segment 10µg, 1 µg, 100 pg, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg.

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At the same time, RT-PCR was used for virus detection in sera collected 8 days after the illness onset and yielded a positive rate of 66.7% (8/12), suggesting that viremia was marked in the acute period, and providing precise evidence for the diagnosis of sporadic HE and pathogens of multi-virus mixed infection.

The results of the spot hybridization test with the DIG probe on a nitrocellulose membrane were positive in 66.6% of 15 samples. Results obtained after ten-fold dilution were positive, but those obtained after 100-fold dilution were negative. All positive samples in the first PCR were positive in the second PCR, though equivocal samples in the first PCR were negative in the second PCR. The sensitivity of spot hybridization approached approximately 100 pg (Fig. 2).

Discussion

The PCR technique has been widely used in the diagnosis of viral and bacterial diseases in China over the past two years because of its high sensitivity and specificity. The drawbacks of this technique are the occurrence of false positive reactions and vulnerability to laboratory contamination. A rapid and simple alternative with high specificity and quantitative characteristics is presently an ardently pursued line of research. The primers used in this study were situated between 4459-4876 bp, belonging to the posterior end of open reading frame 1 (ORF1), and clear of the high variability 2011–2325 bp zone of HEV. The synchronization of reverse transcription with the first PCR, with a period of about 5 hours, is capable of meeting the requirements of sensitivity, rapidness and specificity, and is suitable for clinical diagnosis. First of all, successful use of the PCR technique depends on the accurate selection of primers.

Upon using the RT-PCR technique, Tsarev et al.6) studied viral excretion by detecting the HEV gene in bile, stool specimens and sera from monkeys infected with HEV. Schlauder et al.7) showed that HEV RNA from the sera of 4 children suffering from HE 2-3 days after the onset of the illness were positive by RT-PCR. In this study, RT-PCR was used to detect HEV in stool specimens from patients to ascertain viral excretion; a 40.9% positive rate was obtained for HE patient stool specimens, thus providing a reference for the clinical isolation period, specifically a 30 day isolation period after diagnosis of the illness. The serum HEV RNA positive rate 8 days after the onset of the illness was 66.7% (8/12), confirming that viremia existed in most patients with acute HE and providing objective parameters for HE virological diagnosis.

The main contribution of the nucleic acid hybridization technique to viral diagnosis is its rapidness, sensitivity and specificity. This technique can be used in monitoring medicinal effectiveness and viral mutation and in molecular epidemiological surveys. Nucleic acid probes are mainly of two kinds: isotopic and non-isotopic. Non-isotopic probes have gradually come into increasing use in clinical practice since the 1980’s, replacing isotopic probes which have certain difficult to overcome drawbacks. Though photosensitive biotin labelling is simple, less expensive and easy to perform, our laboratory has experienced several labelling failures. According to the BRL data8), it is difficult to label 239 bp segments with photosensitive biotin; even when labelling is successful, the sensitivity is unavoidably very low. Therefore, we selected the DIG probe for labelling because of its safety, convenience, rapidness, low cost and sensitivity. Routinely prepared nucleic acid can be labelled directly. At present, no reports are available on detection with oligonucleotide probes and the random synthesis of segments as probes is done in the dark to a certain extent. Hybridization with a probe labelled with a PCR product for detection of HEV RNA proved to be convenient, reliable and practical. Our study confirmed that equivocal PCR products become positive after hybridization, serving as a complement to PCR.

A comprehensive evaluation of these two methods is presented. The RT-PCR technique is
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sensitive, specific, rapid and easy to perform but has certain burdensome laboratory requirements and is expensive whereas the DIG probe technique is relatively insensitive and has a long circulation period as well as having minimal laboratory requirements and being inexpensive. If cloned cDNA segments could be directly labelled, the DIG probe technique might be more practical and easier.

Studies on HE have gradually taken on greater depth over the past decade, and the establishment of laboratory diagnostic methods lays the foundation for the development of clinical diagnosis and basic research. The establishment of RT-PCR and DIG probe techniques for detecting HEV RNA has both facilitated and promoted studies on HE. The feasibility of both techniques has been experimentally established. Comparison of the two laboratory techniques involved led us to conclude that the RT-PCR technique can be extended to clinical application and that the DIG probe is more advantageous in HEV basic research and the identification of false PCR positivity.

References


RT-PCR およびジゴキシグニンプローブを用いた E 型肝炎ウィルス RNA 検出の比較検討

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要 旨

RT-PCR およびジゴキシグニンプローブスポットハイブリダイゼーションによる E 型肝炎ウィルス核酸検出法を開発した。急性 E 型肝炎の250の保存便中40.9％がRT-PCRで陽性を示した。また1例では発病後28日で対照中に陽性を示した。血清学的には66.7％が陽性で、ジゴキシグニンプローブを用いた PCR では66.6％陽性であった。ジゴキシグニンを用いた RT-PCR は E 型肝炎の臨床診断および基礎研究に有用であることが示された。

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