Detection of hepatitis D virus RNA in serum by a reverse transcription, polymerase chain reaction-based assay

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Summary. We designed a reverse transcription, polymerase chain reaction-based assay for serum hepatitis D virus RNA. Amplified hepatitis D virus cDNA was revealed by ethidium bromide staining, followed by blotting onto a nylon membrane and hybridization with a 32phosphorus-labelled oligonucleotide, or by a DNA enzyme immunoassay (DEIA) using a double stranded DNA-specific monoclonal antibody. The absolute sensitivity was expressed as number of hepatitis D virus RNA molecules, using a serum of known viral RNA concentration. Three sets of primers were used, encompassing the base positions 66-686 (variable rod-stabilizing region), 701-962 (conserved, viroid-like domain) and 886-1,333 (portion of the open reading frame 5 encoding for the carboxyterminus of the hepatitis D antigen) of the viral genome. The lower detection limits, after amplification of the three RNA portions, as assessed by ethidium bromide staining, were 7.5 x 10^6, 7.5 x 10^4 and 7.5 x 10^2 molecules of hepatitis D virus RNA per assay, respectively. The region encompassing bases 886-1,333 was chosen for blotting and hybridization to a radiolabelled oligonucleotide probe or for a capture-based DNA enzyme immunosassay, where the microplate was coated with this same probe. The two procedures showed comparable sensitivity, i.e., about 10 molecules of viral RNA per assay. The specificity of the assay was further on a panel of both anti-hepatitis D-positive and -negative sera. Amplification of serum hepatitis D virus RNA by reverse transcription/polymerase chain reaction followed by detection of the amplified cDNA by DNA enzyme immunosassay is a promising and feasible routine assay for detecting low amounts of circulating virions.

Key words: Serum hepatitis D virus RNA – Reverse transcription – Polymerase chain reaction – DNA enzyme immunoassays

Introduction

The hepatitis D virus (HDV) genome is an unusually small RNA molecule (only 1,676–1,683 nucleotides in size) bearing some structural similarity with the plant viroids and virusoids genomes [1]. HDV RNA is a single-stranded circle, with a high degree of self-complementarity and G plus C content, that causes the circle to collapse in a rod-like structure under non-denaturing conditions [2, 3]. Furthermore, a significant sequence heterogeneity (as high as 39%) has been found among the several different HDV isolates sequenced to date [2-13], and a classification into genotypes has been tentatively proposed [13]. The cloning and sequencing of the HDV genome has allowed the preparation of nucleic acid probes for detecting HDV RNA in infected fluids by molecular hybridization-based assays [14-17]. More recently, a higher level of sensitivity has been reached with assays based on reverse transcription (RT) and polymerase chain reaction (PCR) amplification of HDV RNA [18-23]. However, the extensive sequence heterogeneity of the different viral isolates has made the choice of suitable primers for the amplification of HDV RNA difficult, since only a few highly conserved regions exist within the HDV genome. We present here the optimization of a RT-PCR-based assay for detecting HDV RNA in serum using three sets of primers encompassing different regions of the HDV genome. The sensitivity limits of the three amplification reactions were then compared in order to define the best set of primers for use in a routine assay.

Materials and methods

Sera. We used serial tenfold dilutions (in normal human serum) of a standard reference serum of known HDV RNA concentration to assess the sensitivity of the RT-PCR assays [24]. This serum was taken at the time of the acute delta hepatitis that followed the experimental challenge with HDV of chimpanzee 57, as part of a serial HDV transmission study performed at the National Insti-
tute of Health (USA), and shown to contain 0.95 μg of HDV RNA/ml, corresponding to 1 x 10^12 HDV RNA molecules/ml [24]. All dilutions were stored at -70°C until use. The sensitivity of the RT-PCR assay was expressed as the number of HDV RNA molecules corresponding to the lowest amount of amplicons producing a detectable signal by each of the three detection methods, i.e., ethidium bromide staining, DNA enzyme immunooassay hybridization and DNA enzyme immunooassay (DEIA).

Sera from 17 anti-HD positive individuals were also studied; 13 had histologically confirmed chronic delta hepatitis, with IgM anti-HD and high serum titers (> 10^5) of total anti-HD: circulating HDV RNA could be detected by slot-blot hybridization [13] in 7. Four additional sera were from patients with chronic delta hepatitis who had been treated with α-interferon and had a sustained remission of liver damage 0.6–6 years after the end of therapy (i.e., persistently normal, monthly transaminase levels throughout follow-up). At the time of sampling, all these 4 sera were still anti-HD positive, but HDV RNA negative by slot-blot hybridization [15]. Sera from 7 additional patients with chronic hepatitis B, but lacking markers of HDV infection, served as negative controls. All sera were stored at -20°C until use.

RT-PCR. Total RNA was extracted from 150 μl of each serum according to the guanidinium isothiocyanate procedure [25]. After ethanol precipitation, the RNA pellet was rinsed twice with 70% ethanol and resuspended in 20 μl of DEPC-treated water. For RT and subsequent PCR amplification three couples of primers were used, encompassing base positions 66–686 (region A), 701–962 (region B) and 886–1,333 (region C) of the HDV genomic RNA sequence reported by Wang et al. [2] (Table 1). The RT-PCR amplification reactions had been optimized previously [18, 21]. Since optimal conditions were largely overlapping, a procedure was chosen for all three sets of primers, and this was the only variable to be optimized.

Briefly, for cDNA synthesis 10 μl of each of the RNAs resuspended in water were heated at 90°C for 5 min and quickly chilled on ice. After adding 100 pmol of the antigenomic primer, 1 mM each of the four dNTPs, 40 U of ribonuclease inhibitor (RNasin, Promega Biotech, Madison, Wis., USA), 2.5 mM magnesium chloride (MgCl2) and 15 U of avian myeloblastosis reverse transcriptase (Promega), the mixture (20 μl) was incubated for 1 h at 42°C. The PCR amplification mixture contained 10 mM TRIS-HCl, pH 8.3, 50 mM potassium chloride, 1.5 mM MgCl2, 200 μM dNTPs, 100 pmol each of the genomic and the antigenomic primer and 2.5 U of Taq polymerase (Perkin Elmer Cetus), which were directly added to the RT reaction tube. The amplification was run for 35 cycles (1 min at 94°C, 1 min at 45°C and 1 min 30 s at 72°C). One-tenth of the amplified DNA was run on a 1.2% agarose gel, directly stained with ethidium bromide and transferred [26] to a nylon membrane. After hybridization for 4 h at 42°C to a 32 phosphorus (P)-end-labelled oligonucleotide probe (D529) (Table 1), the filter was washed for 10 min at room temperature and then for 20 min at 55°C in 6x SSC containing 0.1% sodium dodecyl sulphate and finally exposed overnight to a Kodak XAR film at -70°C.

Amplified HDV cDNA was also detected by a DEIA using a double stranded DNA-specific monoclonal antibody (Sorin Biomedica, Saluggia, Italy) [22, 27]. Streptavidin-coated microtiter plates were incubated with probe D529, to which a biotin molecule had been attached [22]; 20 μl of the PCR amplification mixture were heat denatured (15 min at 100°C), cooled on ice, diluted with the hybridization buffer (1x SSC, 2x Denhardt’s solution, 10 mM TRIS-HCl, pH 7.5, 1 mM EDTA) and added to the wells, according to the manufacturer’s specifications (Sorin Biomedica). Hybridization to the probe bound to the solid phase was carried out for 2 h at 30°C. After five washes with 0.3 ml of the washing buffer [phosphate-buffered saline (PBS) containing 0.004% 2-ethylmercuricthio-5- benzalcarboxylic acid, sodium salt and 0.1% Tween 20], each well was incubated for 1 h at room temperature with 100 μl of the anti-double stranded DNA antibody diluted 1:1,000 in PBS/10% fetal calf serum. After five washes with the washing solution, the bound antibody was detected with 100 μl of horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody, diluted 1:20,000 in PBS/10% fetal calf serum (1 h at room temperature). After further washes, 100 μl of the chromogen/substrate solution (0.1 M citrate buffer, pH 5, containing o-phenylendiamine hydrochloride and 10 μl hydrogen peroxide) were added and plates were incubated for 30 min at room temperature in the dark. The reaction was stopped with 200 μl of 1 N sulfuric acid and the optical density (OD) read at 450 nm. A sample was considered HDV RNA positive when the OD value was above the cut-off level established using the sera from 7 chronic hepatitis B patients lacking markers of HDV infection (negative controls).

**Results**

Serial tenfold dilutions of the chimpanzee 57 reference serum were subjected to RT-PCR amplification of the three regions of HDV RNA. The DNA products were run on an agarose gel and stained with ethidium bromide: the lower detection limits reached after amplification of regions A, B and C were 7.5 x 10^4 (Fig. 1) and 7.5 x 10^2 (Figs. 1, 2) molecules of HDV RNA per assay, respectively.

RT-PCR amplification of the two regions B and C was also performed on sera from 13 anti-HDV-positive patients with chronic delta hepatitis, 4 individuals with interferon-induced remission of chronic delta hepatitis and 7 patients with anti-HDV-negative chronic hepatitis B. An amplicon of the expected size was observed in 8 of 13 and 10 of 13 sera from the first group of patients after RT-PCR amplification of regions B and C, respectively of the HDV genome, but in none of the other cases.

To further analyze the difference in sensitivity in the RT-PCR amplification of regions B and C, we performed a PCR amplification of region B (the highly conserved, viroid-like domain of HDV RNA genome) using cloned HDV cDNA instead of HDV RNA. As DNA substrate we used the recombinant plasmid BMB-4/38, which contains a full-length, cloned HDV cDNA inserted at its unique HindIII site, located at position 1 of the sequence reported by Wang et al. [2] (kind gift of Dr. B. M. Baroudy). Although the sequence of the HDV RNA isolate from which clone BMB-4/38 was obtained is highly divergent from that found in chimpanzee 57, the primers encompassing the region B are perfectly matched with the template

**Table 1. Primers for amplification and probe for detection of hepatitis D virus genomic RNA**

| Amplification of region A (base positions 66–686) | D452 (genomic) TAAAGAGCATTGGAACGGT | D453 (antigenomic) CATCAGTTAAGAAGAT |
| Amplification of region B (base positions 701–962) | D729 (genomic) GCCTCTCGCTGGCGCGGCTTGGGCAACT | D731 (antigenomic) CCCCCAGTGAATTAGGGGTTTCCACT |
| Amplification or region C (base positions 886–1,333) | D455 (genomic) ATGCCATGGCCACCGGAAGAGGAA | D454 (antigenomic) CTCAGGGGAGATTCCGGGAC |
| Probe for region C (base positions 1,266–1,305) | D525 GAGGCGAGGTTACCGACAGGAAGAGGCGCTTGAGAACAA |