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 32P-phosphorus-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 immunoassay, 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 immunoassay 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

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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 amplons producing a detectable signal by each of the three detection methods, i.e., ethidium bromide staining, DNA enzyme immunoassay hybridization and DNA enzyme immunoassay (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 [15] 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 antigenic 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 μl of avian myeloblasosis virus 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 antigenic 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 32P-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 sulfate 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 denaturated (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 to the solid phase was carried out for 2 h at 50°C. After five washes with 0.3 μl of the washing buffer [phosphate-buffered saline (PBS) containing 0.004% 2-ethylmercurio-s-5-benzoxalcarboxylic 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 five further washes, 100 μl of the chromogen/substrate solution (0.1 M citrate buffer, pH 5, containing o-phenylenediamine dihydrochloride 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 10^3 (data not shown), 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 7 chronic hepatitis B patients lacking markers of HDV infection (negative controls).

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

| Amplification of region A (base positions 66–686) | TAAAGAGCATTGGGAAAGT |
| D452 (genomic) | CATCAGCTAAGAAGAT |
| D453 (antigenic) | CCCCCCCAGTGAATAAAGGGGT |
| **Amplification of region B (base positions 701–962)** | GCCTTCCTCGCTGGCCGGCCGTCGGGC-AACAT |
| D729 (genomic) | CCCCCAGTGAATAAAGGGGTTC-C CAT |
| D731 (antigenic) | CCCCCCCAGTGAATAAAGGGGTTC-C CAT |
| **Amplification of region C (base positions 886–1,333)** | ATGCCATGGCAGCAGGAGGAAAGG |
| D455 (genomic) | CCCCCAGTGAATAAAGGGGTTC-C CAT |
| D454 (antigenic) | CCCCCAGTGAATAAAGGGGTTC-C CAT |
| **Probe for region C (base positions 1,266–1,305)** | D525 GAGGCGAGGATTCAGCAGGAGAAGGGCAGAGAGAGG |

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phorus (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 sulfate and finally exposed overnight to a Kodak XAR film at -70°C.
lower detection limits of the reverse transcription-polymerase chain reaction (RT-PCR) amplification of regions B (top panel) and C (middle panel) of the hepatitis D virus (HDV) RNA contained in serial ten-fold dilutions of a reference serum (the numbers on top of the lanes correspond to the logarithms of the starting dilutions) compared with the lower detection limit of the PCR amplification of region B using cloned HDV cDNA as starting material (bottom panel). Amplicons were run on 1% agarose gels and stained with ethidium bromide.

Fig. 2. Comparison of the lower detection limits of three different procedures for revealing the amplicons obtained after RT-PCR amplification of region C of the HDV RNA contained in serial ten-fold dilutions of a reference serum. Amplicons were run on a 1% agarose gels and stained with ethidium bromide (top panel), transferred to a nylon membrane and hybridized to a 32phosphorus-labelled internal oligonucleotide probe (middle panel) or revealed by a specific microtiter DNA enzyme immunoassay (bottom panel). The broken line in the chart denotes the cut-off level of optical density (OD=0.140) above which the original serum dilution is considered as HDV RNA positive.

(B. M. Baroudy, personal communication). The lower detection limit of the PCR amplification using cloned HDV cDNA as starting material was 10⁵ molecules per assay, as evaluated by ethidium bromide staining of the electrophoresed amplicons (Fig. 1), thus suggesting that the limiting step of the RT-PCR amplification of region B of HDV RNA was the RT.

Amplicons obtained after RT-PCR of the region C were thus transferred to the nylon membrane and hybridized with the ³²P-labelled probe D529. After autoradiography, two more tenfold dilutions (up to a 10⁻⁹ dilution) of the reference HDV RNA-positive serum produced a detectable signal (Fig. 2), bringing the lower detection limit of the assay to 7.5 x 10⁶ molecules of HDV RNA. These same amplicons were also detected by the DEIA. For this assay we first calculated the cut-off level by assessing the OD of the reaction products of sera from 7 anti-HDV-negative patients with chronic hepatitis B. Thus, only sera showing OD values above the mean of the controls plus three times the standard deviation were considered as HDV RNA positive. The lower detection limit of the DEIA was reached after RT-PCR amplification of a 10⁻⁹ dilution of the standard reference serum (as in the case of the blot hybridization procedure), here corresponding to 15 x 10⁶ molecules of HDV RNA per assay (Fig. 2).

Similarly, the amplicons corresponding to the region C obtained through RT-PCR amplification of the sera from the 17 anti-HDV-positive individuals and the 7 negative controls were blotted and hybridized. HDV RNA, as suggested by the presence of a specific autoradiographic signal, was found in all the 13 patients with chronic delta hepatitis, whereas the 4 sera obtained from the anti-HDV-positive patients who had recovered after therapy with α-interferon and the 7 control sera were negative.

Discussion

We report the optimization of a RT-PCR-based assay for serum HDV RNA. In previous experiments several parameters were analyzed, including the extraction procedure, the presence of denaturing agents, the concentration of magnesium and the temperature of annealing of the primers [18, 21]. In the present report, we found a different sensitivity when we amplified different regions of
the HDV genome, despite maintaining the same RT-PCR amplification conditions and despite the perfect base-pairing of the primers to the template RNA. Region A is highly variable. Its significance is unknown, besides stabilizing the rod structure. Region B corresponds to the viroid-like domain that is highly conserved. However, this region has a high degree of tertiary structure, contains a ultraviolet light-sensitive element and the catalytic domains. This region has been previously proposed as a candidate for RT-PCR amplification of clinical samples. Region C encompasses the portion of the open reading frame 5, encoding for the carboxyterminus of the HDAg. At its boundaries there are two highly conserved domains, but the portion in between is relatively heterogeneous.

As the amplification of region C gave the highest sensitivity, this was chosen for further work. After hybridization with a 32P-labelled internal oligonucleotide probe the lower detection limit was lowered to less than 10 molecules of HDV RNA. The difference in sensitivity during the amplification of region B starting from HDV RNA or, respectively, known amounts of cloned HDV cDNA, indicate that the limiting step in the amplification of HDV RNA may be the RT. These observations suggest that, to choose a region for RT-PCR amplification, one should consider, rather than the sequence conservation of that region per se, its suitability to RT; tertiary structure constraints may in fact severely limit the first strand synthesis. From our observations and in keeping with previous reports [18–23], one can therefore conclude that the candidate region for RT-PCR amplification of HDV RNA corresponds to the portion of open reading frame 5 encoding for the carboxyterminus of the HDAg. Furthermore, the sequences recognized by the primers used for amplification of this region are indeed among the very conserved throughout all HDV sequences reported so far [2–13].

The high sequence divergence found among different HDV RNA isolates may severely hamper the development of a universal RT-PCR-based assay. Although the assay reported here may work well on HDV RNAs belonging to the genotype I, as are most isolates found in the Western hemisphere [13], detection of highly heterogeneous sequences found in isolates coming from different geographical areas of the world will require newly designed assays. Using the present assay, we were able to assess the eradication of HDV infection in four of four patients in whom hepatitis remission followed the administration of α-interferon 0.6–6 years after the end of therapy. These results are at variance with those recently reported by Farci et al. [28], where long-term responders to treatment may occasionally have rather high circulating levels of HDV RNA. This discrepancy may be explained by sampling bias due to the low number of patients in both studies, or to different criteria in assessing the long-term biochemical response to therapy.

The evaluation of the sustained response to treatment is only one of many possible applications of an RT-PCR-based assay for serum HDV RNA. In previous studies, low amounts of circulating HDV RNA have been found in both acutely and chronically infected patients, where detection of HDV RNA by slot-blot hybridization or HDAg by immunoblot had both failed to identify the virus in anti-HDV-positive patients [19, 20, 22, 23]. However, detection of trace amounts of HDV RNA by RT-PCR may be useful in other clinical settings, such as the follow-up of patients with end-stage liver cirrhosis related to HDV infection who have undergone liver transplantation of the diagnostic evaluation of fulminant hepatitis of unknown etiology. Finally, the availability of a microplate-based DEIA, the sensitivity of which was found comparable to that obtained with a radioactive probe, may facilitate the adoption of this assay by routine diagnostic laboratories.

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