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Rapid Reverse Transcription-PCR Detection of Hepatitis C Virus RNA in Serum by Using the TaqMan Fluorogenic Detection System

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We describe the application of a new fluorogenic probe-based PCR assay (TaqMan; Perkin Elmer Corp./Applied Biosystems, Foster City, Calif.) for the detection of hepatitis C virus RNA in serum and plasma. This assay allows for the direct detection of specific PCR products within minutes of completion of the PCR by monitoring the increase in fluorescence of a dye-labeled oligonucleotide probe. We evaluated this assay by comparing the results obtained by nested PCR with those obtained by TaqMan PCR. Test samples included two separate dilutions series of plasma samples from experimentally infected chimpanzees and a panel of 48 serum specimens from patients with community-acquired hepatitis C virus. The quantity of HCV RNA in each chimpanzee plasma sample was determined by using branched DNA (bDNA) signal amplification assay (Quantiplex HCV RNA assay; Chiron Corp., Emeryville, Calif.). Both PCR assays demonstrated similar levels of detection and could reliably detect 13 bDNA genome equivalents per sample. We found an overall concordance of 88% between results of the two PCR assays with the community-acquired panel, which resolved to 100% when discrepant samples were retested by nested PCR. TaqMan compared favorably with nested PCR with key advantages of speed, increased throughput, and decreased opportunity for false-positive results because of elimination of second-round amplification.

Hepatitis C virus (HCV) is the major causative agent of parenterally transmitted non-A, non-B hepatitis worldwide (1, 2, 7). Methods that permit the direct detection of virus in serum can supplement information from antibody testing and in some cases provide the only means for diagnosis of HCV infection. While no immunologic assay for the direct detection of HCV antigen in serum is currently available, methods for the direct detection of HCV RNA in serum have been developed and include signal amplification by the branched DNA (bDNA) assay (9) and amplification of viral cDNA by reverse transcription (RT) PCR (8, 11, 16). Detection of HCV RNA in serum is helpful in diagnosing infection in patients with chronic non-A, non-B hepatitis who are serologically negative or of indeterminate status and also in monitoring the efficacy of alpha interferon therapy.

The bDNA assay is a quantitative assay that uses a series of specific hybridization reactions coupled with chemiluminescent detection of the hybridized probes in a microtiter plate. This method allows for the direct detection and quantitation of HCV RNA in serum; however, this assay has a detection threshold of 2.0×10^5 genome equivalents per ml, which may limit its use in the diagnosis and management of patients with HCV infection. RT-PCR employs a conversion of viral RNA into cDNA, followed by an exponential amplification of the cDNA. Detection of specific PCR products is achieved by either a second round of PCR or hybridization of products to a signal-enhancing probe. Although RT-PCR can achieve sensitivity levels of 10 to 100 genome equivalents per ml and is the method of choice for detection of samples with low HCV titers, it is a very labor-intensive procedure, taking up to 3 days to achieve final results if a radioactive probe is used.

We report here the application of a fluorogenic probe-based PCR assay (TaqMan; Perkin Elmer Corp./Applied Biosystems, Foster City, Calif. [4, 14]) for the rapid detection of HCV in serum. TaqMan PCR takes advantage of the 5′-3′ endonuclease activity of *Taq* DNA polymerase to digest a probe labeled with a fluorescent reporter and quencher dye. The probe, typically a 20- to 30-bp oligonucleotide, is added directly to the PCR cocktail and is designed to hybridize to a region internal to the flanking PCR primers (Fig. 1). The fluorescent reporter dye FAM (6-carboxy-fluorescein), TET (tetrachloro-6-carboxy-fluorescein), or HEX (hexachloro-6-carboxy-fluorescein) is covalently attached to the 5′ end of the probe. The quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) is attached 6 or more bases downstream from the reporter dye. The quencher dye reduces the emission intensity of the reporter when the probe is intact and phosphorylation of the 3′ end of the probe prevents extension during amplification. During PCR, the probe hybridizes to the template and is digested as *Taq* DNA polymerase extends the PCR primer (Fig. 1). Digestion of the probe releases the reporter from the activity of the quencher, and successive PCR cycles result in exponential amplification of PCR product and fluorescence intensity. PCR products are detected within minutes of completion of the PCR reaction by monitoring the increase in fluorescence of the dye-labeled probe.

In these investigations we compared TaqMan PCR with an in-house nested PCR assay for the detection of HCV RNA in serum and plasma. Test samples included two separate dilution series of experimental chimpanzee plasma and a panel of 48 serum specimens from anti-HCV-positive patients with community-acquired HCV. The quantity of HCV RNA in each chimpanzee plasma was determined by the bDNA assay

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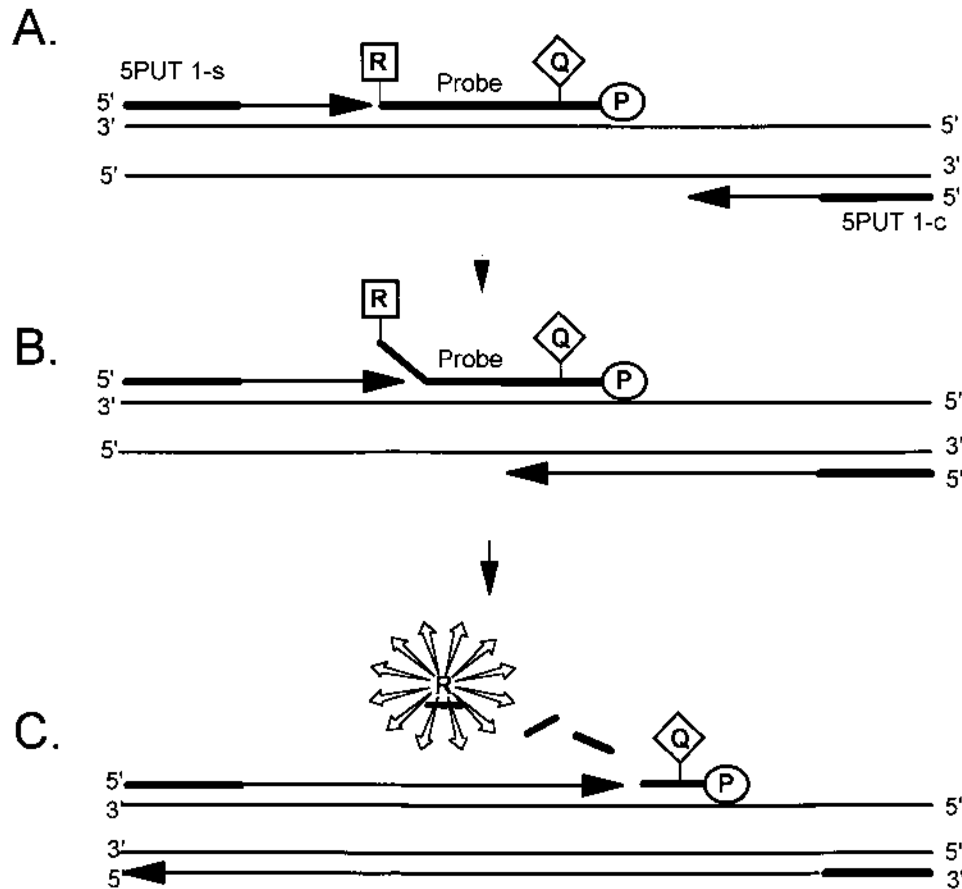

FIG. 1. Diagram of TaqMan 5′ nuclease assay. (A) PCR primers and a TaqMan probe labeled with FAM reporter dye (R) and TAMARA quencher dye (Q) anneal to the DNA template. Extension of the TaqMan probe is blocked by the 3′ phosphate group (P). (B) *Taq* polymerase extends the primer and displaces the TaqMan probe. (C) The displaced probe is endonucleolytically cleaved by *Taq* polymerase resulting in an increase in fluorescence of the reporter.

(Quantiplex HCV RNA assay; Chiron Corporation; Emeryville, Calif.).

MATERIALS AND METHODS

Experimental primate samples. Tenfold dilution series of plasma samples from two chimpanzees (CH 771 and CH 910) experimentally infected with HCV in 1978 (5, 6) were prepared in phosphate-buffered saline and used to assess the limits of detection of TaqMan PCR as compared to nested PCR. HCV RNA in these samples was quantified by bDNA (Quantiplex HCV assay, Chrion Corp.), according to the manufacturer's instruction. The viral titer had been determined previously by chimpanzee infectivity studies (5, 6).

Clinical samples. A blinded panel of anti-HCV positive samples was prepared by using serum specimens obtained from patients identified as part of a population-based study of community-acquired hepatitis C conducted in four sentinel counties in the United States (3). PCR testing was performed only on samples collected independently of those used for serologic testing and were separated within 4 h of collection and stored in aliquots at −20°C or colder.

Sample preparation. Total RNA was isolated from 100 μl of clinical serum or plasma specimens by using a guanidinium isothiocyanate-acid phenol procedure (Tripure; Boehringer Mannheim Biochemicals; Indianapolis, Ind.; or Trizol; GIBCO Laboratories, Gaithersburg, Md.), according to the manufacturer's instructions.

Oligonucleotide primers and probe. Nested PCR was performed by using primer pairs located in the highly conserved 5′ untranslated region (UTR) of HCV (3). TaqMan PCR was performed with a single pair of PCR primers located in the 5′ UTR (15), consisting of forward primer 5PUT1-s (nucleotides [nt] 53 to 76): 5′AACTACTGTCTTCACGCAGAAAGC3′; and reverse primer 5PUTc-1a (nt 268 to 250): 5′CCCAACACTACTCGGCTAG3′. The TaqMan probe 5PUT2FAM (nt 86 to 110): 5′TGGCGTTAGTATGAGTGTCGTGCAG3′ was synthesized in the Biotechnology Core Facility, at the Centers for Disease Control and Prevention. The reporter dye (FAM) was covalently attached to the 5′ end, and the quencher dye (TAMRA) was incorporated via a linker arm thymidine (Amino-Modifier C6 dT, Glen Research, Sterling, Va.) at nt 21 in the probe sequence. Synthesis employed standard DNA phosphoramidites 6-carboxy-fluorescein phosphoramidite and 6-carboxy-tetramethyl-rhodamine succinamidyl ester. Extension from the 3′ end was blocked by attaching a 3′ phosphate group by using 3′ Phosphate Control Pore Glass (Glen Research).

RT-PCR. For both methods described below, 100% of total RNA extracted from 100 μl of serum or plasma was used in the PCR reaction. Nested RT-PCR was performed as previously described (3). TaqMan RT-PCR was performed according to the method described by Nolte et al. (15) with the following modifications. To minimize primer-primer and primer-probe duplex formation and to increase yield, *Taq* polymerase was preincubated with TaqStart Antibody (Clonetech, Palo Alto, Calif.) according to the manufacturer's instructions and 4 pmol of TaqMan probe 5PUT2FAM was included in the 100-μl PCR reaction which contained 60 mM Tris/HCl, 15 mM $(NH_2)SO_4$, and 4.5 mM $MgCl_2$ (Buffer D, Invitrogen, San Diego, Calif.). PCR amplification began with a 3-min denaturation at 95°C followed by 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 62°C for 45 s followed by a final cycle at 72°C for 7 min in a Perkin Elmer 9600 thermocycler. Products were either analyzed immediately or stored at −20°C.

Appropriate precautions were taken to avoid sample-to-sample carryover and contamination by cloned DNA or PCR products (13). Negative control plasmas from anti-HCV-negative blood donors were tested along with the clinical samples, and controls containing no nucleic acid were tested in triplicate.

TaqMan data analysis. Following amplification, data acquisition and analysis were performed by using a Perkin-Elmer LS50 Luminescence Spectrometer equipped with a plate reader linked to the PC-based Fluorescence Data Manager Software (Perkin-Elmer, Norwalk, Conn.). A 50-μl aliquot of each reaction was added to a MicroFLUOR "W" white 96-well U-bottom microtiter plate (Dynatech Industries, Inc., McLean, Va.), and the plate was scanned at 518 nm (FAM) and 582 nm (TAMRA) with an excitation wavelength of 488 nm in the LS50. Data were reduced by the Fluorescence Data Manager Software and exported into a Microsoft Excel worksheet. An increase in emission intensity at 518 nm was seen when the template was present because of the release of the reporter dye (FAM) as the probe was hydrolyzed; the emission at 582 nm

TABLE 1. Comparison of the analytical sensitivities of Nested RT-PCR and TaqMan PCR.

Sample and dilution[a]	Genome equivalents[b]	Result	
		Nested RT-PCR	TaqMan PCR[c]
CH 771			
10^2	3,800	+, +	yes, yes
10^3	380	+, +	yes, yes
10^4	38	+, +	yes, yes
10^5	3.8	−, −	yes, no
10^6	0.38	−, −	no, no
CH 910			
10^2	13,000	+, +	yes, yes
10^3	1,300	+, +	yes, yes
10^4	130	+, +	yes, yes
10^5	13	+, +	yes, yes
10^6	1.3	−, −	no, no

[a] Tenfold dilution series of chimpanzee plasma samples were prepared in phosphate-buffered saline.

[b] Genome equivalents were determined for the undiluted samples by using bDNA (Quantiplex HCV assay).

[c] A TaqMan PCR yes score is assigned when the ΔRQ is greater than the threshold calculated at the 99% confidence level for the standard deviation of the three no-template controls.

remained constant since the intensity from the quencher dye (TAMRA) was not affected.

Analysis and quantitation of the signal were accomplished by applying three calculations. The emission intensity of the reporter was divided by the emission intensity of the quencher to give a ratio defined as RQ^+ for experimental samples and RQ^- for no template controls. In this calculation, the quencher functions as an internal standard, allowing fluctuations in fluorescent intensity due to nonspecific effects to be normalized. In the second calculation, the average RQ^- value for the three no template controls was subtracted from the RQ^+ value of the experimental samples to give a value defined as ΔRQ. Any fluctuation that was not due to PCR-related nuclease digestion was normalized in the ΔRQ calculation. Moreover, the ΔRQ value represented the magnitude of signal generated during PCR. In addition, a third calculation, defined as the threshold ΔRQ, was performed to obtain a numerical cutoff value above which a given ΔRQ should represent a positive result. This value was calculated by multiplying the standard deviation of the three no-template controls by a T-table value. The software will assign a yes value to those samples over the threshold and a no to those below the threshold.

To confirm the specificity of the TaqMan PCR results, 10 μl of PCR products was electrophoresed through a 2% agarose gel, Southern blotted, and hybridized as previously described (15) with probe 5PUT-p1s labeled with [α-^{32}P]dCTP by using terminal transferase (Boehringer Mannheim Biochemicals).

RESULTS

The analytical sensitivity of the TaqMan PCR assay was similar to that achieved by nested PCR. Two separate dilutions series of CH 910 (containing 1.3×10^7 bDNA genome equivalents per ml) and CH 771 (containing 3.8×10^6 genome equivalents per ml) were prepared and HCV RNA was tested in duplicate by nested PCR and by TaqMan PCR. Nested PCR products were evaluated by agarose gel electrophoresis (data not shown). TaqMan products were detected by monitoring fluorescence, and the specificity of the products was confirmed by Southern hybridization. The last dilution (10^{-4}) of CH 771 plasma found to be positive in duplicate by both assays contained approximately 38 genome equivalents (Table 1). When a 10-fold higher dilution containing approximately 3.8 genome equivalents was tested, only one replica was positive by TaqMan PCR and neither replica was positive by nested PCR. The last dilution (10^{-5}) of CH 910 plasma found to be positive in duplicate by both assays contained approximately 13 genome equivalents (Table 1). In all cases Southern hybridization confirmed the TaqMan yes and no assignments (data not shown).

To assess the reproducibility and sensitivity of the assay when testing low-titered samples, 10^{-4} and 10^{-5} dilutions of CH 771 were tested in duplicate by using TaqMan PCR on 10 separate occasions. Both duplicates of 10^{-4} dilutions (38 genome equivalents) were positive 70% of the time, and at least one duplicate was positive in all cases. Both duplicates of 10^{-5} dilutions (3.8 genome equivalents) were positive only 33% of the time; however, at least one sample was positive 78% of the time. These results underscore the need for testing samples in duplicate, particularly when serum specimens may be of low titer regardless of the PCR methodology used.

A blinded panel of samples obtained from patients with community-acquired hepatitis C was also tested by TaqMan PCR. This panel consisted of 48 serum samples obtained from 43 patients. Twenty-seven samples were obtained from a cohort of patients who had been identified in 1985; serum samples analyzed in this study were collected 6 to 9 years after onset of illness. Twenty-one serum samples were obtained from a second cohort of patients identified in 1991. Approximately half of the samples were collected immediately following onset of illness and the remaining samples were obtained during 2 years of follow-up. Each cohort had previously been tested for the presence of HCV RNA by different laboratorians by using nested PCR. Samples were blinded and reanalyzed in duplicate using TaqMan PCR, and results were compared to those previously obtained. The concordance between the results obtained by TaqMan PCR and nested PCR was 91% for those samples found to be positive by nested PCR and 77% for those samples found negative by nested PCR. Testing revealed that 32 samples were nested PCR positive and TaqMan positive, 3 samples were nested PCR positive and TaqMan negative, 3 samples were nested PCR negative and TaqMan positive, and 10 samples were nested PCR negative, and TaqMan negative. Discrepant samples were retested in duplicate under code by a third laboratorian using nested PCR. Retesting resulted in 100% concordance. Among the six discordant samples, three were initially reported as negative by nested PCR but were positive upon retesting. These false negatives may reflect losses that can occur during sample preparation presumably at either the organic extraction or precipitation step. For this reason, it is advisable to test samples in duplicate unless internal controls such as RNA transcripts are included during processing to control for such losses. Three of the discordants were initially scored as positives but retested negative by nested PCR. While it is possible that these results may represent false positives, it is also possible that these samples may be of very low titer, which may be only intermittently positive when the methods described here are used.

DISCUSSION

PCR can provide extraordinary sensitivity in the detection of viral nucleic acids in clinical samples; however, to achieve the necessary specificity and sensitivity, a second round of amplification or post-PCR hybridization is necessary. These manipulations are laborious and, in the case of second-round amplification, can significantly increase the likelihood of cross-contamination of samples resulting in false positives. In addition, detection is generally achieved by ethidium bromide staining of electrophoretically separated PCR products, which is time-consuming. A typical second round of amplification followed by agarose gel electrophoresis can take up to 6 h to complete. Single-round amplification reduces the opportunity for cross-contamination; however, if Southern hybridization with a radioactive probe is performed, detection can take up to 3 days. A single-round combined RT-PCR assay for HCV has been developed by Roche Molecular Systems (AMPLICOR HCV test). In this assay, PCR products are labeled by using a

biotinylated downstream primer. Following PCR, products are denatured, transferred to an enzyme-linked immunosorbent assay-type microtiter plate, and hybridized to a DNA capture probe. The plate is monitored for a color change produced by the addition of an avidin-horseradish peroxidase conjugate followed by a tetramethylbenzidine substrate. This method is more rapid that other single-round detection procedures employing Southern hybridization, but it still requires multiple post-PCR pipetting, washing, and incubation steps. Detection can be accomplished in about 2 h.

The TaqMan assay represents a novel technology that exploits the inherent 5′ nuclease activity of *Taq* DNA polymerase. In this assay, specific PCR primers bind to and amplify the target nucleic acid; however, a signal is not generated unless the internal fluorogenic probe hybridizes to the accumulating product and is subsequently cleaved by *Taq* polymerase; thus, an additional level of specificity, provided by the probe, is built into the assay. In addition, the hybridization reaction occurs concurrently with amplification and the unhybridized probe does not contribute substantially to the signal, obviating the need to physically separate the amplicons from the unbound probe after PCR. In the TaqMan assay, postamplification manipulations are reduced to a single pipetting step which is followed by a scan of the emission spectra of the products. A total of 92 samples (plus three no-nucleic-acid controls) can be scanned in a single microtiter plate in under 15 min. Thus, this detection methodology offers a significant time savings.

The analysis software exports the final results into an Excel spreadsheet, which assigns a yes or no decision to each sample; the threshold ΔRQ for this decision is based on a 99% confidence level using the standard deviation of three no-nucleic-acid controls. While the yes-no assignment is generally accurate, occasionally when the threshold ΔRQ was 0.05 or less, some negative control samples were assigned a yes. To address this problem, all PCR experiments included a set of limiting dilutions of well-characterized, known-positive samples that are tested in duplicate. Since all normal human plasmas tested had ΔRQ values below 0.10, any experimental sample that had a ΔRQ value lower that the end-point dilution sample and greater than 0.10 was either retested or the product was confirmed by Southern hybridization.

Application of TaqMan PCR greatly simplified the detection of amplified HCV RNA and allowed for increased throughput while retaining the exquisite sensitivity of nested PCR. These features make this assay a useful tool for the detection of HCV RNA and can aid in our understanding of the natural history and epidemiology of HCV as well as monitoring treatment for HCV infection.

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