Jpn. J. Infect. Dis., 53, 70-72, 2000

Short Communication

Simultaneous Detection of Hepatitis B, C, and G Viral Genomes by Multiplex PCR Method

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(Received March 7, 2000. Accepted May 9, 2000)

SUMMARY: We established a multiplex polymerase chain reaction (PCR) method for simultaneous detection of hepatitis B, C, and G viral genomes. The levels of concordance with the data obtained by conventional single PCR method were 100% for single infection, 98 to 100% for double infections, and 92% for triple infections. This method is not only suited to rapid, large-scale epidemiological screening and clinical diagnosis of those virus infections occurring alone or in combination, but is also time- and cost-effective.

Multiplex polymerase chain reaction (PCR) is a demanding technique used for genetic screening and other applications where it is necessary to amplify several products simultaneously (1-3). This technique often requires extensive optimization because primer-dimers and other nonspecific products may interfere with the amplification of specific products (3). Although primer-dimer formation can usually be avoided using hot-start PCR, amplification specificity is also influenced by other factors, such as the PCR buffer and primer concentration. At present, PCR is the only reliable method that allows early detection of hepatitis virus infections. Further, detection of hepatitis C virus (HCV) RNA and hepatitis G virus (HGV) RNA by PCR represents the only means of direct detection of viremia. The current methodology for the screening and detection of those hepatitis viruses in numerous clinical samples is inadequate and requires numerous PCR reactions per blood sample. In the present study, to detect hepatitis B virus (HBV), HCV, and HGV infections occurring either alone or in combination, we developed a combined nested PCR and multiplex PCR for the simultaneous detection of HBV DNA and HCV and HGV RNAs in a serum specimen. Our findings indicate that this method is simple, rapid, and highly sensitive, and could be useful for the screening of blood-borne hepatitis virus infections using serum samples.

Both DNA and RNA were extracted simultaneously from 100 μl of serum using a nucleic acid extraction kit (SepaGene RV-R, Sanko Junyaku Co., Ltd., Tokyo), precipitated with isopropanol, and washed with ethanol. The resulting pellet was resuspended in 50 μl of RNase-free water. In order to optimize the multiplex PCR, the concentrations of the deoxynucleotide mixture (100 to 500 μM), $MgCl_2$ (0.5 to 3.0 mM), and individual primer pairs (20 to 100 ng) were optimized. After establishing the best condition for the reaction, we further optimized the annealing temperature in the first (40 to 55°C) and second round (50 to 55°C) PCR, respectively. Based on these optimization results, we designed a protocol for the multiplex PCR for HBV DNA, HCV RNA, and HGV RNA amplifications with 300 μM each dATP, dGTP, dCTP, and dTTP, 1.5 mM $MgCl_2$, and 40 ng of each primer concentration; annealing at 50°C for the first round PCR, and employing a two-step annealing temperature at 53°C and 55°C for the second round PCR. In fact, the multiplex PCR was performed in a one-step process that combines cDNA synthesis and PCR in a single tube. That is, for HCV and HGV RNA, the first PCR was combined with the reverse transcriptase (RT) step in the same tube containing 50 μl of a reaction buffer prepared as follows: 10 units of RNase inhibitor (Promega, Madison, Wis., USA), 100 units of RT of Moloney murine leukemia virus (Promega), 40 ng of each outer primer for HBV, HCV, and HGV, 300 μM of each of the four deoxynucleotides, 2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, Conn., USA), and 1 × reaction buffer containing 1.5 mM $MgCl_2$. To obtain an automatic hot-start reaction, we used AmpliTaq Gold DNA polymerase instead of regular thermostable DNA polymerase. The sequences of primers used for the multiplex PCR were listed in Table 1 along with the nucleotide position and PCR product size. We previously reported the sensitivity of these primer combinations in the conventional PCR assay for HBV, HCV, and HGV (4-6). The thermocycler was programmed first to incubate the samples for 50 min at 37°C for the initial RT step and then preheat them at 95°C for 10 min to activate AmpliTaq Gold. This was followed by 40 cycles consisting of 94°C for 30 s, 50°C for 45 s, and 72°C for 1 min using a Perkin-Elmer 2400 or 9700 Thermal Cycler (Perkin-Elmer). For the second reaction, 2 μl (1/25 volume) of the first PCR product was added to a tube containing the second set of each inner primer, deoxynucleotides, AmpliTaq Gold DNA polymerase, and PCR buffer as in the first reaction, but without RT and omitting the initial 50-min incubation at 37°C. Amplification was performed for 40 cycles with the following parameters: preheating at 95°C for 10 min, 20 cycles of amplification at 94°C for 30 s, annealing at 53°C for 45 s, and extension at 72°C for 1 min, followed by an additional 20 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min. The PCR products

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Table 1. Primer sequences used for multiplex PCR

Primer	Sequence	Primer pair	Product size
For HBV DNA (X region)			
MD24	5'-TGCCAACTGGATCCTTCGCGGGACGTCCTT-3' (nt 1392-1421)	MD24/MD26 (1st)	233 bp
MD26	5'-GTTCACGGTGGTCTCCATG-3' (nt 1625-1607)		
HBx1	5'-GTCCCCTTCTTCATCTGCCGT-3' (nt 1487-1507)	HBx1/HBx2 (2nd)	118 bp
HBx2	5'-ACGTGCAGAGGTGAAGCGAAG-3' (nt 1604-1584)		
For HCV RNA (5' untranslated region)			
19	5'-GCGACACTCCACCATAGAT-3' (nt 2-20)	19/20 (1st)	329 bp
20	5'-GCTCATGGTGCACGGTCTA-3' (nt 330-312)		
21	5'-CTGTGAGGAACTACTGTCT-3' (nt 28-46)	21/22 (2nd)	268 bp
22	5'-ACTCGCAAGCACCCTATCA-3' (nt 295-277)		
For HGV RNA (5' untranslated region)			
HG1	5'-GGTCGTAAATCCCGGTCACC-3' (nt 139-158)	HG1/HG1R (1st)	262 bp
HG1R	5'-CCCACTGGTCCTTGTCAACT-3' (nt 400-381)		
HG2	5'-TAGCCACTATAGGTGGGTCT-3' (nt 163-182)	HG2/HG2R (2nd)	188 bp
HG2R	5'-ATTGAAGGGCGACGTGGACC-3' (nt 350-331)		

Nucleotide positions deduced from HBVadr4 (from ref. 8) for HBV, HC-J1 (from ref. 9) for HCV and HGV-PNF2161 (from ref. 10) for HGV.

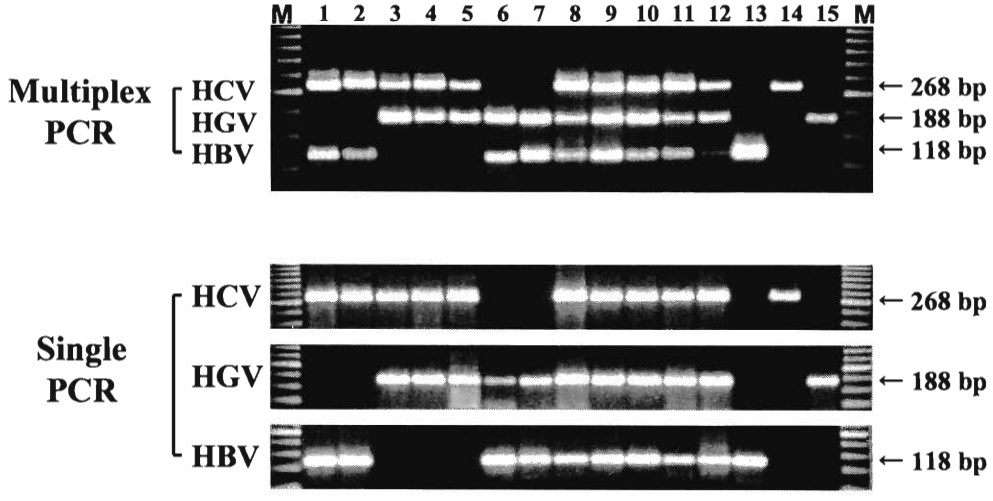

Figure. Comparison of results between the multiplex and the conventional single PCR method.

were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and evaluated under UV light. The sizes of the PCR products were estimated according to the migration pattern of a 50-bp DNA ladder (Pharmacia Biotech, Piscataway, N.J., USA). To confirm the specificity of the amplification products, sequencing analysis was performed by the method reported previously (7). To avoid the risk of false-positive results, PCR assays were done with strict precautions against cross-contamination. Furthermore, all PCR assays were performed in duplicate to confirm reproducibility.

To obtain optimization of multiplex PCR for HBV, HCV, and HGV, the reaction conditions were assessed by titration using serial dilutions of chimpanzee serum samples that had been evaluated for infectivity with HBV and HCV, and using in vitro RNA transcripts for HGV. The sensitivity of this PCR assay allowed detection of as few as 10 copies of the viral genomes of HBV, HCV, and HGV, respectively; detected by serial dilution assay of the samples. Using this method, we were able to detect all three viral genomes simultaneously in 5 of 15 serum samples examined in the first experiment. Comparison of the results between multiplex and conventional single PCR assay was shown in Figure. No discrepancy was observed between the results of the two assays. To confirm the sensitivity and specificity of the PCR, the conventional single PCR and the multiplex PCR were performed in parallel using 576 serum samples obtained from patients with liver disease. The results showed 100% (198/198) concordance in single infection, 98 to 100% (90/91) in double infections, and 92% (11/12) in triple infections (Table 2). One case showed discordance of the results in triple infections. In this case, HGV RNA was negative by the multiplex PCR. The reproducibility of the results by this PCR method was able to be confirmed. Furthermore, the specificity of the PCR products of three different expected sizes in triple infections was confirmed to correspond to the three genomes by sequence analysis in 5 cases. No positive reaction was seen in 95 serum samples from uninfected patients by this assay.

In the present study, we reported multiplex PCR assay used to detect HBV DNA, HCV RNA, and HGV RNA simultaneously. Evaluation of a serologically known panel of single infected or co-infected individuals showed specific amplification of target sequences by the multiplex PCR method. These results indicate that our multiplex PCR method could be a

Table 2. Concordance of results between the multiplex and the conventional single PCR method

	Single PCR-positive						
	HBV	HCV	HGV	B+C	C+G	B+G	B+C+G
n =	198	177	98	25	60	6	12
Multiplex PCR-positive	198	177	98	25	59	6	11
Concordance rate	100%	100%	100%	100%	98%	100%	92%

useful tool for large-scale epidemiological screening, and a rapid and cost-effective method for molecular-based diagnosis of those viral infections.

ACKNOWLEDGMENT

We thank Dr. Takeshi Kurata (National Institute of Infectious Diseases) for his continuous encouragement during this study.

This study was supported in part by a grant-in-aid for Science Research from the Ministry of Education, Science, Sports and Culture of Japan and the Ministry of Health and Welfare of Japan, and by an International Medical Cooperation Research Grant.

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