IMPROVED AMPLIFICATION SYSTEM FOR DETECTION OF HEPATITIS C VIRUS GENOME THAT SIMULTANEOUSLY DIFFERENTIATES VIRAL GENOTYPES

Sirirurg Songsivilai[1], Duangjit Kanistanon[1], Wariya Panyavinin[2], Montana Neelamek[1] and Tararaj Dharakul[1]

[1]Department of Immunology; [2]Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Abstract. An improved system for amplification of hepatitis C virus genome (HCV) was developed based on a multiplex nested polymerase chain reaction format. Two sets of oligonucleotide primers were used simultaneously. One was derived from the conserved sequences in the 5' non-coding region of the viral genome which can bind to the viral genome of all genotypes. The other set of primers was designed from a sequence in the nonstructural-5 region of HCV. HCV genotypes 1 and 3 can be differentiated by the banding patterns of amplified DNA products. All of 39 samples containing the HCV genotype 1 could be amplified with primers in the 5' non-coding region only, whereas 92% of those with genotype 3 could be amplified by both primer sets. In addition, HCV RNA can be detected in 81% of 84 anti-HCV-positive blood donors and in 0% of 34 anti-HCV-negative cases. Of the HCV RNA-positive specimens, 69% showed genotype 1-like patterns while 31% showed genotype 3-like patterns. The detection rate of HCV RNA in this study was much higher than that in our previous report due to the improvement of new primers which can detect all genotypes of the virus. In conclusion, this improved amplification system is a sensitive method for rapid identification of HCV RNA in clinical specimens that can simultaneously differentiate the two most common genotypes of HCV found in Thailand.

INTRODUCTION

Hepatitis C virus (HCV), a major causative agent for posttransfusion hepatitis, is endemic in Thailand with a prevalence in blood donor population of around 2%. More than half of HCV-infected patients develop chronic hepatitis and in some cases progress to cirrhosis and liver cancer. HCV is a member of the *Flaviviridae* family, and possesses a single-stranded RNA genome of 9,500 nucleotides in length (Houghton *et al*, 1991). The virus is transmitted mainly via parenteral routes, such as contamination in blood and blood products and intravenous drug abuse.

The detection of antibodies to HCV has been introduced for blood donor screening since 1989 and in clinical diagnosis of HCV infection, resulting in a decreased incidence of posttransfusion hepatitis. This assay is based mainly on recombinantly expressed HCV proteins or synthetic peptides as antigens. However, false-positive or false-negative results are not uncommon (Maggi *et al*, 1995). Samples testing positive to anti-HCV antibody assay need to be confirmed with tests such as recombinant immunoblot assay or polymerase chain reaction (PCR). Several systems for PCR amplification of the HCV genome have been introduced with different results depending on the system employed. PCR system for detecting HCV RNA also has advantages over antibody detection in some clinical situations, such as in the diagnosis of early HCV infection before the antibody can be detected as well as in perinatal infection. In addition, HCV RNA, not anti-HCV antibody, is a marker of infectivity. The disappearance of HCV RNA in patients treated with interferon is also a marker of viral clearance (Kanai *et al*, 1992). The sensitivity and specificity of PCR-based assays are, however, dependent on the choice of oligonucleotide primers (Bukh *et al*, 1992).

Genomic diversity among different isolates of the virus were found throughout the viral genome. HCV can be classified into 6 major genotypes and several subtypes (Simmonds *et al*, 1994). The distribution of HCV genotypes differs in various geographical areas. Recent studies showed that the outcome of infection with different genotypes of HCV differed in various aspects, such as develop-

Correspondence: Dr Sirirurg Songsivilai, Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
Tel: 66-2-4197066; Fax: 66-2-4181636; E-mail: sissv@mahidol.ac.th

ment of chronicity, progression to cirrhosis and cancer, and response to treatment. For example, European and Japanese patients with HCV genotype 2 infection responded more favorably to interferon treatment than did patients with genotype 1b infection (Kanai *et al*, 1992). Strong association between HCV genotype 1b and hepatocellular carcinoma has also been demonstrated (De Mitri *et al*, 1995; Silini *et al*, 1995).

Our group has previously reported the detection of HCV RNA using a nested polymerase chain reaction system with 2 sets of primers designed from the nucleotide sequences of HCV isolated in the United States and Japan which were later classified as the genotypes 1a and 1b, respectively (Songsivilai *et al*, 1993). Neither set of primers could amplify all cases with HCV RNA, one set could amplify the viral genome in 7 of 13 cases (53.8%) whereas the other set could amplify HCV RNA from only 4 patients (30.8%). The result suggested that at least 2 types of HCV existed in Thailand and the primers used in that study were inappropriate for detecting HCV RNA in this country. Subsequent work by our group and others has identified at least 2 major genotypes of HCV found in Thailand (Luengrojanakul *et al*, 1994; Mellor *et al*, 1995), namely genotype 1 (subgenotypes 1a and 1b), and genotype 3 (subgenotype 3a). The clinical features of genotype 1 infection have been widely studied since this genotype is common in western countries and Japan, whereas those of genotype 3 are currently not known. A system that can differentiate the genotype of infecting HCV, especially between genotypes 1 and 3, may be useful for further studies of viral behavior.

The objective of the present study was to develop a rapid PCR-based system for amplifying HCV RNA using oligonucleotide primers that can amplify HCV genomes of all genotypes, and can simultaneously differentiate between genotypes 1 and 3.

MATERIALS AND METHODS

Specimens

Plasma samples were collected from 84 blood donors at the Department of Transfusion Medicine, Siriraj Hospital, Bangkok, who were seropositive for antibodies to HCV using 2 commercial second-generation anti-HCV enzyme immunoassays (Abbott Diagnostics, USA and confirmed by that of Diagnostic Biotechnology, Singapore). Sixty-five specimens of known HCV genotypes by nucleotide sequencing or reverse hybridization were also included in this study. Specimens collected from 34 blood donors who tested negative for antibodies to HCV and had normal levels of alanine aminotransferase enzyme (ALT) were used as negative controls. The specimens were aliquoted and stored at -20°C prior to the analysis.

Design of PCR primers

PCR primers for amplification of HCV RNA were designed from nucleotide sequences of the HCV genome deposited in the GenBank database release 85.0 (National Center for Biotechnology Information, USA) and from the sequences of Thai isolates of HCV, using Mac Vector Program (Kodak Scientific Imaging System, USA) and Oligo Primer Analysis software (National Bioscience Inc, USA). Universal primers for HCV (Set A1) were designed from the conserved sequences in th 5' non-coding region of 97 isolates of HCV, including 14 complete nucleotide sequences of the virus and those of 17 Thai isolates sequenced by our group (GenBank accession numbers U23385-U23392 and U23742-U23750). These primers can bind to the HCV genome of all isolates known to date, including genotypes 1-6. The other set of primers (set K) were designed from nucleotide sequences in the NS-5 (nonstructural protein) of HCV genotype 3, of which the inner primers were genotype 3-specific. The oligonucleotide primers were synthesized by an automated DNA synthesizer (Applied Biosystems, USA). The sequences of the primers and their locations in the HCV genome are shown in Table 1 and Fig 1, respectively.

Reverse transcription and multiplex nested polymerase chain reaction amplification (RT-PCR)

RT-PCR was performed as previously described with some modifications (Songsivilai *et al*, 1993). Briefly, viral RNA was extracted from 100 μl of plasma. The RT-PCR step was carried out in a volume of 100 μl containing 10 units of AMV. Reverse transcriptase (Promega, USA), and 2.5 units of *Taq* DNA polymerase (Promega, USA).

Table 1

Nucleotide sequences of oligonucleotide primers used in this study.

Primer set	Primer name	Nucleotide sequence (5' → 3')
Set A1:		
Outer primers	A1L	CACTCCCCTGTGAGGAACTACTGT
	A10R	AAGCACCCTATCAGGCAGTACCAC
Inner primers	A11L	ACTACTGTCTTCACGCAGAAAGC
	A12R	AATCTCCAGGCATTGAGCGG
The size of nested PCR product is 174 base pairs.		
Set K:		
Outer primers	K5L	TATGATACCCGCTG(T/C)TTTGA
	K2R	ACGGAGGCTATGACCAGGTACTCC
Inner primers	K7L	CTCAACCGTCACTGAACAGGACAT
	K8R	CCACGACTAGATCATCTCCG
The size of nested PCR product is 294 base pairs.		

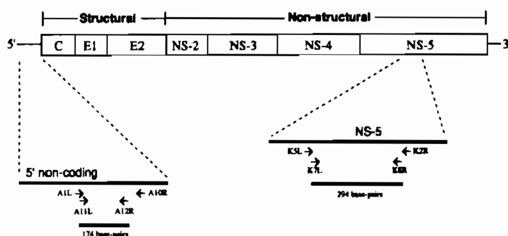

Fig 1–Organization of HCV genome and position of primers with respects to HCV genes. (C, core region; E1, envelope-1; E2, envelope-2, NS-2 to NS-5, genes encoding nonstructural proteins).

The mixture was incubated at 42°C for 30 minutes in a DNA Thermal Cycler 480 (Perkin Elmer, USA) for reverse transcription, and then subjected to PCR amplification, using 2 pairs of outer primers, for 35 cycles; each amplification comprised 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes, followed by final extension step for 10 minutes at 72°C. One-tenth of products from the first round were amplified (nested PCR reaction) using 2 pairs of the inner primers in a 50 µl reaction with the same cycle profile as for the first PCR. The amplified PCR product was electrophoresed in a 2% agarose gel, stained with ethidium bromide and visualized under an ultraviolet light. PCR products from primers set A1 and set K were 174 and 294 base-pairs in length, respectively. Strict precautions were observed during the whole process to avoid RNA degradation, contamination and carry over. Positive controls (plasma samples that contained HCV RNA) and negative controls (samples with no HCV RNA) were included in each experiment. Each assay was carried out at least twice.

RESULTS

Multiplex nested PCR amplification of HCV genome

Both regions of HCV genome (5'-NC and NS-5) can be amplified simultaneously in a multiplex nested PCR system. The two bands of PCR-amplified products can be easily differentiated in ethidium bromide-stained agarose gels (Fig 2). An upper band of 294 base-pairs represents the products of amplification by primers set K (in the NS-5 region), and a lower band (174 base-pairs in length) was a result of PCR amplification using primer set A1 (in the 5'-NC region).

Correlation between PCR patterns and HCV genotypes

Sixty-five samples were collected from blood donors who were infected with known genotypes of HCV. Of those, 39 donors were infected with HCV

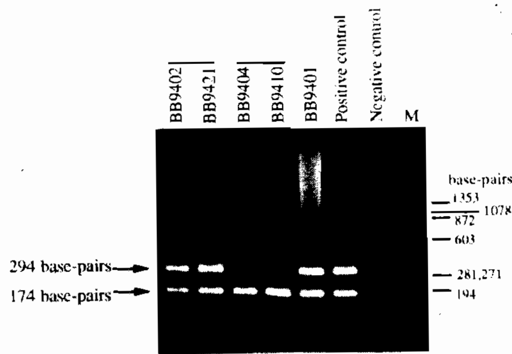

Fig 2–Ethidium bromide-stained agarose gel showed a typical result of multiplex nested PCR amplification of HCV RNA. Genotype 3 (donors BB9402 and BB9421) gave a double-band pattern, while genotype 1 (BB9404 and BB9410) gave a single-band pattern. New genotype of HCV (BB9401) had the same banding profile as of genotype 3. Positive and negative control samples were also included. M = DNA size marker ØX174 digested with *Hae*III.

genotype 1, 25 donors with genotype 3, and 1 donor with the new genotype of HCV. PCR products from all of 39 donors with genotype 1 showed a single-band pattern (amplified by primer set A1 only), as expected. For those with genotype 3, 23 of 25 samples showed a double-band pattern (amplified by both sets of primers), whereas 2 samples showed a single-band pattern. The other sample (BB9401), of which the genotype was different from any known HCV isolated to date, showed a double-band pattern similar to that of genotype 3 (Table 2).

PCR amplification of plasma samples from anti-HCV-positive blood donors

Sixty-eight of 84 samples (81.0%) from anti-HCV-positive blood donors had detectable HCV RNA. Of those, 47 samples (69.1% of the PCR-positive cases) were amplified using primers set A1 alone (single-band pattern), and 21 samples (30.9%) were amplified by both set of primers (double-band pattern). In contrast, none of 34 samples from anti-HCV-negative blood donors had detectable HCV RNA.

DISCUSSION

The design of PCR system for amplifying HCV genome has been proved difficult due to the heterogeneity of the viral genome. We have previously reported the amplification system using 2 sets of primers designed from the conserved sequences of the prototype viruses isolated in the United States (genotype 1a) and Japan (genotype 1b), whose sequences were known at that period. This test detected HCV RNA in only 53.8% of anti-HCV-positive chronic hepatitis patients. PCR amplification using either set of primers alone still missed some cases whose HCV RNA can be amplified by the other primer sets (Songsivilai *et al*, 1993). This finding was supported by the identification of a new genotype of HCV from Thai blood donors (now known as genotype 3) (Mori *et al*, 1992). The complete nucleotide sequence of this HCV genotype, which was available recently (Sakamoto *et al*, 1994), is different from that of genotype 1 used for designing the above mentioned sets of primers.

Table 2

Correlation between PCR patterns and HCV genotypes.

HCV Genotype	Total No.	No. of samples with PCR products showing	
		Single-band pattern	Double-band pattern
1	39	39 (100%)	0
3	25	2 (8%)	23 (92%)
NG	1	0	1

The most widely used system for HCV classification characterizes the virus into 6 genotypes and 11 subgenotypes based on nucleotide sequence homology in the NS-5 region of the genome (Simmonds *et al*, 1993a). Genotypic distribution of HCV varies in different geographical regions. Genotypes 1a and 1b can be found worldwide. Most of the HCV isolated in the United States are of genotype 1. Genotype 1 and 2 are common in Japan. Genotype 3 has been identified in some European populations but is less common than genotype 1. Genotype 4 is found almost exclusively in the Middle East whereas little is known about genotypes 5 and 6. Recent studies of the distribution of HCV genotypes in the Thai population showed that genotypes 3 and 1 were the most common (Luengrojanakul *et al*, 1994; Mellor *et al*, 1995). These two genotypes represent about 90% of HCV found in this country. The high representation of genotype 3 is unique for Thailand. In addition, another genotype of HCV was recently identified from a healthy Thai blood donor of which the nucleotide sequence is different from any known HCV (Songsivilai *et al*, 1993) GenBank accession number U13010), and provisionally named genotype NG. The assignment of this virus into a new genotype has yet to be reconfirmed by complete nucleotide sequencing of the entire viral genome. Based on these findings, new sets of primers for amplification of HCV RNA are needed. In addition, these primers should also allow the rapid identification of HCV genotypes, especially should be able to differentiate the genotype 1 from genotype 3.

The 5' non-coding region of HCV was selected for designing universal primers since this region is the most conserved part of the viral genome. Computer-aided comparison of nucleotide sequences of all genotypes of HCV showed that the appropriate sites for primer binding could be identified and new sets of primers (set A1) were synthesized. In addition, a set of genotype 3-specific primers was designed based on the limited nucleotide sequences available in the NS-5 region of which the type-specific region has been identified (Simmonds *et al*, 1993c). These 2 sets of primers were used simultaneously in a multiplex nested PCR system; the first round PCR contained all 4 outer primers and the second round contained all 4 inner primers. These primers were used in a PCR system combining the reverse transcription and polymerase chain reaction in the same tube, as previously reported (Songsivilai *et al*, 1993). At the optimal temperature for the enzyme AMV reverse transcriptase, the 5'-NC and NS-5 regions of HCV RNA were reverse transcribed simultaneously by the two anti-sense primers (A10R and K2R, respectively). At the end of the reverse transcription step, AMV reverse transcriptase was inactivated at high temperature and the PCR amplification reaction was initiated in the same tube without the need to reopen the tube. Both regions of the HCV genome were then amplified simultaneously by the appropriate primers. In the second (nested) reaction tube, the inner parts of the amplified genome were reamplified by the inner primers. This system is convenient and less prone to contamination and carry over.

Of 65 HCV RNA samples that had been genotyped, all of 39 samples with genotype 1 gave a single-band pattern, and 92% of 25 samples with genotype 3 gave a double-band pattern, as expected from the nucleotide sequence data. The failure to amplify the NS-5' region of two genotype 3 samples by primer set K was likely due to mutations in the viral genome resulting in the inability to bind to at least one of the primers. This drawback is similar to that of the genotyping methods using genotype-specific PCR primers in other regions of the viral genome, restriction fragment length polymorphism of the PCR products, and hybridization with genotype-specific probes (Okamoto *et al*, 1992; Simmonds *et al*, 1993b; Stuyver *et al*, 1993). In addition, one sample (BB9401) which contained a new genotype of HCV (genotype NG) also showed the genotype 3-like pattern. To date, limited information is available on the nucleotide sequence of this new genotype and further studies are needed to clarify its real classification. In summary, the present rapid genotyping method can thus differentiate the two most common HCV genotypes in the Thai population with 95.4% accuracy. However, it failed to assign the viral genotypes in 3 of 65 samples from this panel.

This multiplex nested PCR amplification system could detect HCV RNA in 81.0% of anti-HCV-positive specimens and none of specimens without antibody to HCV. The detection rate of HCV RNA in anti-HCV-positive samples in this study was higher than that in our previous report (53.8%), and was similar to that recently reported by other groups (Pawlotsky *et al*, 1994; Marin *et al*, 1994; Yamada *et al*, 1994; Ndimbie *et al*, 1995). The improvement was mainly due to the increased sensitivity of the new primers which can amplify all genotypes of the

virus. Of note, all PCR-positive samples can be detected by primers set A1, as predicted, and approximately 31% of the PCR-positive samples can be amplified by primer set K. This result suggests that genotype 1-like HCV represents about 70% of HCV in Thailand and genotype 3-like virus about 30%.

The sensitivity and specificity of the present PCR system cannot be analyzed due to the lack of a gold standard for HCV diagnosis. PCR detection and antibody assay provided different types of information. Serum HCV RNA is a marker for viremia or persistent viral infection while antibody to HCV is evidence of previous or present infection (Marin *et al*, 1994). Patients who had completely recovered from HCV infection still had anti-HCV antibody for years while the viral RNA could not be detected. It was estimated that about 75% of HCV-infected individuals failed to completely eradicate HCV (Anonymous, 1990), thus about 75% of anti-HCV-positive patients should have HCV RNA, a percentage similar to that obtained from this study.

In conclusion, an improved multiplex nested PCR amplification system employing new sets of primers has been evaluated by amplifying HCV RNA isolated in Thailand. The present system also allows simultaneous differentiation between HCV genotypes 1 and 3.

ACKNOWLEDGEMENTS

This work was supported by the National Science and Technology Development Agency of Thailand, Mahidol University, Siriraj-China Medical Board and Anandhamahidol Foundation. We thank Dr Sasitorn Bajarachandra for providing plasma samples.

REFERENCES

Anonymous. Public Health Service Interagency guidelines for screening donors of blood, plasma, organs, tissues, and semens for evidence of hepatitis B and hepatitis C. *MMWR* 1991; 40 (RR-4): 1-17.

Bukh J, Purcell RH, Miller RH. Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction. *Proc Natl Acad Sci USA* 1992; 89 : 187-91.

De Mitri MS, Poussin K, Baccarini P, *et al.* HCV-associated liver cancer without cirrhosis. *Lancet* 1995; 345: 413-5.

Houghton M, Weiner A, Han J, Kuo G, Choo QL. Molecular biology of the hepatitis C viruses: Implications for diagnosis, development and control of viral disease. *Hepatology* 1991; 11 : 381-8.

Kanai K, Kato M, Okamoto H. HCV genotypes in chronic hepatitis C and response to interferon. *Lancet* 1992; 339 : 1543.

Luengrojanakul P, Vareesangthip K, Chainuvati T, *et al.* Hepatitis C virus infection in patients with chronic liver disease or chronic renal failure and blood donors in Thailand. *J Med Virol* 1994; 44 : 287-92.

Maggi F, Vatteroni ML, Pistello M, *et al.* Serological reactivity and viral genotypes in hepatitis C virus infection. *J Clin Microbiol* 1995; 33 : 209-11.

Marin MG, Bresciani S, Puoti M, *et al.* Clinical significance of serum hepatitis C virus (HCV) RNA as marker of HCV infection. *J Clin Microbiol* 1994; 32 : 3008-12.

Mellor J, Holmes EC, Jarvis LM, *et al.* Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implication for virus classification. *J Gen Virol* 1995; 76 : 2493-507.

Mori S, Kato N, Yagyu A, *et al.* A new type of hepatitis C virus in patients in Thailand. *Biochem Biophy Res Commun* 1992; 183 : 334-42.

Ndimbie OK, Nedjar S, Kingsley L, Riddle R, Rinaldo C. Long-term serologic follow-up of hepatitis C virus-seropositive homosexual men. *Clin Diag Lab Immunol* 1995; 2 : 219-24.

Okamoto H, Sukiyama Y, Okuda S, *et al.* Typing of hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J Gen Virol* 1992; 73 : 673-9.

Pawlotsky JM, Fleury A, Choukroun V, *et al.* Significance of highly positive c22-3 "indeterminate" second-generation hepatitis C virus (HCV) recombinant immunoblot assay (RIBA) and resolution by third-generation HCV RIBA. *J Clin Microbiol* 1994; 32 : 1357-9.

Sakamoto M, Akahane Y, Tsuda F, Tanaka T, Woodfield DG, Okamoto H. Entire nucleotide sequence and characterization of hepatitis C virus genotype V/3a. *J Gen Virol* 1994; 75 : 1761-8.

Silini E, Bono F, Cividini A, *et al.* Differential distribution of hepatitis C virus genotypes in patients with and without liver function abnormalities. *Hepatology* 1995; 21 : 285-90.

Simmonds P, Holmes EC, Cha TA, *et al.* Classification of hepatitis C virus into six major genotypes and a series of subtype by phylogenetic analysis of the NS-5 region. *J Gen Virol* 1993a; 74 : 2391-9.

Simmonds P, McOmish F, Yap PL, *et al.* Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. *J Gen Virol* 1993b; 74 : 661-8.

Simmonds P, Rose KA, Graham S, *et al.* Mapping of sero-type specific immunodominant epitopes in the NS-4 region of hepatitis C virus (HCV): use of type-specific peptides to serologically differentiate infection with HCV types 1, 2 and 3. *J Clin Microbiol* 1993c; 31 : 1493-503.

Simmonds P, Alberti A, Alter HJ, *et al.* A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology* 1994; 19 : 1321-4.

Songsivilai S, Luangwedchakarn V, Kanistanon D, Louisirirotchanakul S, Dharakul T. Detection of hepatitis C virus RNA by polymerase chain reaction: Design of amplification primers. *Siriraj Hosp Gaz* 1993; 45 : 597-603.

Stuyver L, Rossau R, Wyseur A, *et al.* Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. *J Gen Virol* 1993; 74 : 1093-102.

Yamada M, Fukuda Y, Imoto M, *et al.* High rate of mixed genotypes of hepatitis C virus in patients of an epidemic area in Japan. *J Gastroenterol Hepatol* 1994; 9 : 620-3.