Multiplex Reverse Transcriptase-PCR for Simultaneous Detection of Hepatitis B, C, and E Virus

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Introduction: The hepatitis B virus (HBV), HCV, and HEV may occur as singly or concurrently in patients of different kind of liver disease. The rapid, reliable, and cost-effective screening of these pathogens is required for the large epidemiological studies. Therefore, a study has been planned to develop a multiplex Reverse Transcriptase-PCR assay which can be used for the screening of maximum number of pathogens at a time. **Methodology:** To develop multiplex Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assay for simultaneous detection of HBV, HCV, and HEV; the serum samples of 54 patients who were positive either singly or in co-infection with for HBV, HCV, and HEV serologically were screened by uniplex PCR/RT-PCR followed by multiplex RT-PCR for HBV, HCV, and HEV using specific primers. These primers can detect most genotypes of these viruses. Multiplex RT-PCR was done in one tube for the identification of viral DNA/RNA using a mixture of three pairs of specific primers for hepatitis B, C, and E viruses. Representative positive samples of these viruses by uniplex/multiplex RT-PCR were also confirmed by sequencing followed by alignment with reference strains sequence. **Results:** The specificity of multiplex PCR was 100% with high sensitivity 89%, 87%, and 74% for HBV, HCV, and HEV respectively. The sensitivity and specificity of RT-multiplex PCR demonstrated a good correlation with that of uniplex PCR. **Conclusion:** The study suggests that multiplex RT-PCR can serve as a simple and reliable assay for rapid, sensitive, and cost-effective method for simultaneous detection of super-infections with HEV particularly in Asian countries as a cause of decompensation of chronic liver disease. (J CLIN EXP HEPATOL 2016;6:33–39)

The diagnosis of viral hepatitis poses a unique problem, since causative agents belong to both DNA (hepatitis B) and RNA (hepatitis A, C, D, E, and G) viruses. It is extremely important to establish the exact etiological agent for better prognostication and management, especially with the advent of anti-viral therapies. The clinically most important viruses that need frequent detection are hepatitis B virus (HBV), HCV, and HEV which may occur as single or multiple infections. Nucleic acid amplification technologies (NATs) are reliable tool for the detection of various infectious agents, particularly viruses which are otherwise difficult to detect by standard methods.

Keywords: multiplex PCR, hepatitis B virus, hepatitis C virus, hepatitis E virus, liver diseases

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Abbreviations: ELISA: enzyme linked immunosorbent assay; HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus; PCR: polymerase chain reaction; RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction; PPV: positive predictive value; NATs: nucleic acid amplification technologies; NPV: negative predictive value
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Amplification of up to several million fold of low-copy-number DNA or RNA genome allows making of an early diagnosis of viral infections.^{1,2}

HBV causes post-transfusion acute viral hepatitis as well as chronic hepatitis, and viral DNA has been detected in a small but appreciable percentage of chronic HBsAg-negative hepatitis cases. HCV, on the other hand, is an important cause of chronic hepatitis. HEV has been established as the sole cause of endemic hepatitis in Afro-Asian countries and the most important cause for fulminant hepatitis, particularly in pregnant women from developing countries.^{3,4} Serological methods involving detection of HBsAg, anti-HBc IgM, and anti-HCV is widely used for clinical diagnosis, while molecular detection of HBV and HCV is the gold standard for diagnosis. Hepatitis E is regarded by many health care professionals as a typical travel-associated disease. A considerable proportion of autochthonous infections likely remain undiagnosed, and hepatitis of unknown etiology is in fact often caused by HEV.⁵

At present, RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) is a sensitive and specific method that is commonly used with success to accurately define the true burden of disease due to virus infections. Recently, RT-PCR with specific primers individually or in combination for detection of multiple human pathogens has proved to be

comparable to or better than cell culture or other immunodiagnostic methods for virus detection.^{6,7} Conventional uniplex PCR with a single pair of primers that can detect only one target virus genome at a time is expensive. In contrast, multiplex RT-PCR with multiple pairs of specific primers for amplifying different viral genomes in one reaction tube enables to detect two or more targets in a single test. Thus, the multiplex PCR becomes a highly cost-effective method because of the reduction in labor and reagent and faster detection.⁸⁻¹⁰ Multiplex PCR has been used for detection of HBV and coinfection with both HAV and HCV.¹¹ The present study was designed with the aim to develop a rapid, reliable, and cost-effective multiplex PCR assay for the simultaneous detection of both DNA and RNA containing hepatitis viruses which are highly prevalent in India such as HBV, HCV, and HEV.

MATERIAL AND METHODS

Study Design

The present study has been designed for the diagnosis and detection of viral hepatitis in patients having super and coinfection of HBV and HCV with HEV, which is more common in developing countries including India. Institutional review boards of Maulana Azad Medical College, New Delhi, approved the study protocol. A total of 54 patients of various kind of liver disease who were infected either with HBV, HCV, and HEV singly or co-infection cases were included in the study from OPD and Ward of Lok Nayak Hospital, New Delhi after obtaining Informed consent. Among them, 30 cases were serologically singly positive either for hepatitis B, C, or E viruses and were positive for either HBV DNA or HCV/HEV RNA. The remaining 24 patients, whose clinical, biochemical, and serological profiles were suggestive of either co-infection with HBV and HCV or superinfection with HEV were subjected to "Multiplex PCR" in order to ascertain the clinical utility and reliability of multiplex PCR in serologically positive patients. Therefore, "multiplex PCR" was standardized, using known PCR positive cases of HBV, HCV, and HEV.

To further validate the results of multiplex PCR, the 30 known positive cases, which were singly positive for either HBV DNA or HCV RNA or HEV RNA, were subjected to cross-evaluation by "Multiplex PCR" and sequenced. A few representative amplified PCR products of HBV, HCV, and HEV were amplified by "Multiplex PCR" and sequenced for validation. A complete aseptic protocol was taken for collecting, handling, and storage of the serum samples.

Serological Tests

Serological tests were performed using commercially available ELISA kits, according to the manufacturer's

instructions. The various serological tests performed in all the study samples were HBsAg (SURASE B-96 kit; General Biological, Taiwan); IgM and IgG anti-HBc (anti-coarse MB-96 kit; General Biological, Taiwan); HBeAg (EASE BN-96 kit; General Biological Corp., Taiwan); Anti-HCV (Innotest HCV Ab III, Innogenetics N.V., Ghent, Belgium); IgM anti-HEV (IgM anti-HEV ELISA kit, Genelabs Diagnostics, Singapore).

Viral Nucleic Acid Isolation

HBV DNA was extracted by QIAamp DNA Mini Kit (Qiagen Inc, Chatsworth, CA) according to manufacturer's instructions. HCV or HEV RNA was extracted from serum samples using Trizol BD reagent (GIBCO BRL, Life Technologies, Maryland, MD, USA) according to the manufacturer's protocol.

Primers

The primers for HBV DNA were obtained from the surface region, for HCV RNA from the UTR-core region and for HEV RNA from the ORF-1 region as reported elsewhere with slight modification in HCV primer.¹²⁻¹⁴ The primer sequences are given in Table 1. The PCR for HBV was semi-nested while that of HCV and HEV was nested. All these primers have been picked from regions which could detect major genotypes of the three viruses.

Multiplex RT-PCR for Simultaneous Amplification of HBV, HCV, and HEV

The cDNA was synthesized by reverse transcription (MMuLV-RT, NEB) and then followed by amplification by polymerase chain reaction in 50 µl of the PCR master mix-I containing 1× PCR Buffer (Bangalore Genei, India), 0.25 mM each dNTPs, 3 mM MgCl₂, 1.5 U of Taq DNA (Bangalore Genei, India), and 20 pmol each of first round primers for HCV (HC-1 and HC-2), HEV (#3043 and #3044), HBV (HBMF1 and HBMR1), MMuLV-RT, RNasin, HBV DNA, and HCV/HEV RNA were added. The cycling condition was 42 °C for 1 h followed by PCR profile where the first cycle starts with initial denaturation for 4 min at 94 °C, followed by denaturation at 94 °C for 1 min, annealing for 1.5 min at 50 °C, and extension for 2 min at 72 °C for 35 cycles. Final extension at 72 °C was carried out for 7 min.

For the second round PCR, another master mix-II was prepared, which contains the same constituents as described earlier for master mix-I except RNasin, MMuLV-RT, and the outer primers. In this mix, 20 pmol each of second round primers for HCV (HC-3 and HC-4), HEV (HEV-1 and HEV-3), and HBV (HBMR1 and HBMF2) was added (Table 1). To the 50 µl of the master mix-II, 5 µl of the first PCR product-1 was added that underwent 35 cycles of amplification using the same temperature and time profile as in the first PCR except 42 °C for 1 h.

Table 1 List of Primers Used for PCR/RT-PCR.

	Primers	Size (Mer)
Hepatitis B virus		
<i>Outer primer</i>		
HBMF 1(+):	5'-YCCTGCTGGTGGCTCCAGTTC-3'	21
HBMR 1(-):	5'-AAGCCANACARTGGGGGAAAGC-3'	22
<i>Inner primer</i>		
HBMF 2(+):	5'-GTCTAGACTCGTGGTGGTGGACTTACTCTC-3'	30
Hepatitis E virus		
<i>Outer primer</i>		
#3043(+):	5'-CCGGATCCACACACATCTGAGCTACATTCGTGAGCT-3'	36
#3044(-):	5'-CCGAATTCAAAGGCATCCATGGTGTGTTGAGAATGAC-3'	36
<i>Inner primer</i>		
HEV-1(+):	5'-GGAATTCGACTCCACCCAGAATAACTT-3'	27
HEV-3(-):	5'-GGAATTCACAGCCGCGCATCAGGACAG-3'	27
Hepatitis C virus		
<i>Outer primer</i>		
HC-1 (+):	5'-CTGTGAGGAAGTACTGCTT-3'	19
HC-2(-):	5'-GTGCTCATRGGTGCAACGGTCTACGAGACCTCCGG-3'	34
<i>Inner primer</i>		
HC-3(+):	5'-TTCACGCAGAAAGCGTCTAG-3'	20
HC-4(-):	5'-CACTCGCAASGCACCCTATCAGGCATGCA-3'	29

The base other than A, T, G, and C are mixed bases. Y = C and T, R = A and G, S = C and G, N = Any base (A, T, G, C).

Agarose Gel Electrophoresis

The final amplified products were detected by electrophoresis on 3% Nusieve-agarose gel containing ethidium bromide under a UV transilluminator and size of the PCR product compared with a marker of known molecular weight DNA digest (ϕ X174 Hae III digest). An amplicon of 251 bp was detected for HCV, 343 bp for HEV, and 480 bp for HBV.

Direct Nucleotide Sequencing

The few representative amplified PCR products of HBV, HCV, and HEV were purified using QIA quick PCR purification kit (Qiagen Ltd) and bidirectionally sequenced using an ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction (Perkin Elmer Cetus, Norwalk, CT, USA) with an ABI 310 Genetic Analyzer (Perkin Elmer Cetus) on commercial basis.

Statistical Analysis

Cohen's Kappa test was used for comparing more positive agreement between positive cases of the uniplex and multiplex PCR results. A kappa equals to 1 means there is perfect agreement, chance of agreement would equate to 0 and -1 means perfect disagreement. Probability test is used to measure the performance of sensitivity and

specificity for the binary classified variables like PCR and qualitative results of the uniplex and multiplex PCR among different infections.

RESULTS

Optimization of Multiplex RT-PCR

Three previously developed individual/single RT-PCRs were integrated to generate a multiplex RT-PCR for simultaneous detection of HBV, HCV, and HEV genomes. Optimum conditions for the multiplex RT-PCR assay were achieved with 200 units of reverse transcriptase/reaction for cDNA preparation in the amplification reaction. Increasing the concentration of polymerase did not significantly improve the amplification reaction. It was maintained at 1.5 U per reaction. Particular attention was paid to the concentration of $MgCl_2$ as the optimal concentrations were different in the single virus PCR. The $MgCl_2$ concentration was balanced to 3 mM that provided optimal HBV and HCV amplification whereas the efficiency for HEV was a bit reduced. Concentrations of different primers were also adjusted accordingly. Increasing the concentration of HEV specific primers did not compensate totally for the slight loss of efficiency related to the suboptimal $MgCl_2$ concentration. The specific PCR product was observed on agarose gel electrophoresis by uniplex and multiplex PCR (Figure 1).

Figure 1 Multiplex PCR showing amplification of HEV, HCV RNA, and HBV DNA. Lane 1: marker ϕ X174 Hae III digested DNA ladder; lane 2: Positive control for HBV (480 bp), HEV (343 bp), and HCV (251 bp); lane 3: Negative control; lane 4: Multiplex PCR products for HEV RNA and HCV RNA; lanes 5, 7, 8: Multiplex PCR products of HBV DNA and HCV RNA; lane 6: Multiplex PCR products for HBV DNA and HEV RNA; and lane 9: Multiplex PCR products for HEV RNA.

Comparison of Multiplex RT-PCR with Uniplex PCR

HBV DNA was positive in 12 (22.2%) cases by uniplex PCR whereas 11 (20.4%) cases by multiplex PCR. HCV RNA was positive in 11 (20.3%) cases by uniplex PCR and 10 (18.5%) cases by multiplex PCR. Similarly, a higher percentage of HEV RNA positive cases by uniplex PCR were 18 (33.3%) compared to multiplex PCR of 14 (25.9%). Co-infection of HBV and HCV was observed in 3 (5.6%) cases by uniplex as well as multiplex PCR. Co-infection of HBV and HEV was observed in 3 (5.6%) by uniplex PCR and 2 (3.7%) by multiplex PCR. Similarly, co-infection of HEV and HCV was observed in 2 (3.7%) by uniplex PCR and 1 (1.9%) by multiplex PCR. The agreement value (Kappa) between multiplex PCR and single PCR is 0.75, which shows a full concurrence between multiplex PCR and uniplex PCR (Table 2).

Comparison of Uniplex and Multiplex PCR Assay with ELISA

Out of 54 patients included in the study, 30 patients were singly positive for either HBV or HCV or HEV while 24 patients have multiple viral infections as detected by

Table 2 Comparison of Uniplex PCR with Multiplex PCR.

	Positivity by uniplex PCR, n (%)	Positivity by multiplex PCR, n (%)	Kappa value
HBV	12 (22.2)	11 (20.4)	
HCV	11 (20.3)	10 (18.5)	
HEV	18 (33.3)	14 (25.9)	
HBV + HCV	3 (5.6)	3 (5.6)	
HBV + HEV	3 (5.6)	2 (3.7)	
HEV + HCV	2 (3.7)	1 (1.9)	
PCR Negative	5 (9.2)	13 (24.1)	
Total	49 (90.7)	41 (75.9)	0.75

serology. The uniplex PCR for HBV, HCV, and HEV detected infection in 90.7% (49/54) while multiplex PCR of 75.9% (41/54). Dual infections were confirmed in 8 patients by uniplex and in 6 patients by multiplex PCR with no particular combination in predominance (Table 2).

Comparison of Sensitivity and Specificity of Multiplex PCR with Uniplex PCR Assay

The multiplex PCR for detection of HBV, HCV, and HEV showed $88.9 \pm 4\%$, $87.59 \pm 8\%$, and $73.9 \pm 8\%$ sensitivity in comparison to uniplex PCR, respectively. The multiplex PCR assay of all three viruses was found to be 100% specific with 100% positive predictive value (PPV). The negative predictive value (NPV) was found to be $94.7 \pm 7\%$ and $95 \pm 7\%$ for HBV and HCV, respectively. However, the NPV for HEV was $83.8 \pm 12\%$ less than those of HBV and HCV (Table 3).

Comparison of Multiplex PCR Results with Direct Nucleotide Sequencing

The PCR products of HBV DNA, HCV RNA, and HEV RNA obtained by multiplex PCR were sequenced and

Table 3 Evaluation of Sensitivity and Specificity of HBV, HCV, and HEV Amplification by Uniplex and Multiplex PCR.

	Uniplex PCR, n = 54 (%)	Multiplex PCR, n = 54 (%)	Quality evaluation parameters
HBV positive	18 (33.3)	16 (29.6)	Sensitivity = $88.9 \pm 4\%$ Specificity = 100% PPV = 100% NPV = $94.7 \pm 7\%$
HBV negative	36 (66.7)	38 (70.4)	
HCV positive	16 (29.6)	14 (25.9)	Sensitivity = $87.5 \pm 8\%$ Specificity = 100% PPV = 100% NPV = $95 \pm 7\%$
HCV negative	38 (70.4)	40 (74.1)	
HEV positive	23 (42.6)	17 (31.5)	Sensitivity = $73.9 \pm 8\%$ Specificity = 100% PPV = 100% NPV = $83.8 \pm 12\%$
HEV negative	31 (57.4)	37 (68.5)	

PPV: positive predictive value; NPV: negative predictive value.

Figure 2 Representative sequential alignment of the (a) HBV amplicon (accession number EU370156 & EU370145), (b) HCV amplicon (accession number EF015721 & EF015715), and (c) HEV amplicon (accession number DQ318844 & DQ318838) with the reference sequence.

aligned with the sequence of randomly selected sequence of three viral groups (Figure 2a-c) that confirm the specificity of the multiplex PCR results. The viral nucleotide sequence data reported in this paper were deposited in GenBank using the National Center for Biotechnology Information (Bethesda, MD) Sequin v. 5.26 submission tool under accession numbers [EF015721](#) & [EF015715](#) (HCV), [EU370156](#) & [EU370145](#) (HBV), and [DQ318844](#) & [DQ318838](#) (HEV) respectively.

DISCUSSION

In view of increasing multiple infections of hepatitis viruses and to diagnose these agents quickly and reliably in blood banking and for disease control, it is important to establish quick and cost-effective screening of large numbers of samples. The commonly used ELISA test, which detects antibody for the diagnosis of virus infection, is applicable only after window period (~6–12 weeks) does not provide any status of viral replication. The NATs are based on detection of the genome of infectious agent which can detect viral genome and also provide the status of virus replication. The results of several studies suggest that the use of NATs can reduce the window period to 11 days for HCV, thereby diminishing further the risk of acquiring infections from blood transfusions.^{1,15} However, a disadvantage is that the cost of NATs is 5–10 folds greater than that of the most expensive enzyme immunoassay which is being performed routinely in the laboratories. To reduce the cost of NATs, two approaches have been suggested, namely the use of pooled plasma testing so that fewer tests are required to screen large numbers of samples, and the use of multiplex PCR.

The multiplex PCR amplification technology with higher specificity and sensitivity can be a useful tool for detection of multiple infectious agents. Therefore, the screening of infectious viral genomes represents a major step for detection of infection in the blood collected during the pre-seroconversion window period and cases of immuno-silent latent infections.

Most highly developed techniques are based on real-time detection; fundamentally they cut down the period of analysis, improve the sensitivity, and also allow quantitative determinations in the same time. There are numerous marketable detection systems which are developed for real-time NAT-analysis.¹⁶ The majority of these real-time systems either use DNA binding fluorophores such as SYBER Green I or they use specific fluorescently labeled oligonucleotide probes to detect amplification.^{16–19} However, it is also hard to implement the real time detection routinely in majority of tertiary care hospitals in India as well as in other developing countries due to the unavailability of expensive real-time PCR machine, lack of well trained manpower, and the use of expensive fluorophores I or specific fluorescently labeled oligonucleotide required for real time PCR reaction.

Therefore, we have developed a sensitive, specific, and cost-effective multiplex PCR assay by end point PCR for detection of multiple infectious agents.

Firstly, we have selected primers from the target sequences of HBV, HCV, and HEV genome which showed maximum sequence conservation for simultaneous detection of all genotypes of HBV, HCV, and HEV. The development of multiplex NAT is made difficult by the different enzymes and ion optimal concentrations required by each target virus amplification and detection. There is no limit on the number of target sequences simultaneously amplified, theoretically, by multiplex PCR. But the number of target sequences to be amplified was constrained by specific conditions. Hence, false amplifications happen frequently. The other considerations are different amplification efficiencies of distinct templates and cross-interactions of the primers. In this experiment, primer pairs for the co-amplification reaction were carefully selected to obtain the similar amplification efficiency and reduce the cross-interaction with the aid of the software. We have successfully developed multiplex RT-PCR system which did not show any false-positivity. For thermal cycler program, the annealing temperature was found appropriate at 50 °C in order to decrease nonspecific priming or other artifacts as given in Method section.

In this study, the analysis of the 54 serologically characterized clinical serum samples showed an ideal relation with the expected outcome. The results of multiplex PCR of HBV, HCV, and HEV were in perfect concordance of uniplex PCR with the exception of two, two, and six samples which were found positive by uniplex PCR but negative by multiplex PCR respectively. The specificity of multiplex PCR for all three viruses was 100% and the sensitivity of HBV was $88.9 \pm 14\%$, for HCV was $87.5 \pm 18\%$, and for HEV was $73.9 \pm 18\%$ (Table 3). Simultaneous amplification of HBV DNA and HEV RNA virus using multiplex PCR system developed by Vinay et al. showed all samples of co-infection could be detected for HBV and or HEV successfully.⁸ Wang et al. demonstrated that the sensitivity of multiplex normalized PCR method was 78.6%, 75%, and 83.3% for the detection of HBV DNA, HCV RNA, and super-infection of HBV and HCV, with specificity of 80%, 90%, and 70%, respectively.⁹ The super-infection rate of HBV and HCV was very high and is a leading cause of chronic hepatitis and hepatocellular carcinoma.^{20–22} Konomi et al.²³ reported a multiplex PCR method for simultaneous detection of hepatitis B, C, and G viral genomes, where the levels of concordance with the data obtained by conventional single PCR method were 100% for single infection, 98–100% for double infection, and 92% for triple infection. In the multiplex PCR, the sensitivity and specificity depend on the rate of denaturing of the amplification targets, rate of annealing of the sequence specific primers with the desired amplification targets, and extension of primer to the desired sequence

during PCR amplification cycles. Therefore, the primers selection is strongly associated with the outcome of PCR reaction. In this developed multiplex PCR system, the primer sequence size of 30 mer or more (Table 1) for amplification of each infectious agent is increasing the specificity while compromising the sensitivity.

The commonly used ELISA test, which detects antibody for the diagnosis of virus infection, is applicable only four to ten weeks after infection, but the viral replication cannot be explained by it. PCR method could directly detect the viral genome and the status of virus replication. Out of 54 ELISA positive patients included in the study, the uniplex PCR for HBV, HCV, and HEV detected infection in 90.7% (49/54) while multiplex PCR in 75.9% (41/54). In this study, the infection was undetectable in 9.3% (5/54) of patients by PCR but detectable by ELISA maybe because the patients were under recovering period, and the virus has cleaned up by now.

Although the sensitivity of multiplex PCR is less than 100% of all three viruses, its specificity of 100% ensures promising results, when used as a routine diagnostic procedure. This implies that multiplex PCR developed by us can identify all the cases correctly which are negative for any of the viruses and does not give any false positive results when compared to results obtained by uniplex PCR. Along with its suitability for clinical diagnosis, it is also suitable for blood donors where the screening of HBV and HCV can be done to prevent the transmission of these diseases. The multiplex PCR described is a rapid, reliable, and cost-effective method for simultaneous screening out for any evidence of concomitant infection of HBV, HCV, and HEV in patients with chronic liver diseases. HEV super-infection and HBV reactivation are major causes of acute or chronic liver failure in Asia and this test could be very important in Asian countries for simultaneous screening for evidence of super-infection with HEV as a cause of decompensation of chronic liver disease.

CONFLICTS OF INTEREST

The authors have none to declare.

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