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. 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

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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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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 co-infection 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 $MgCl_2$, 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.