Journal of Virological Methods 186 (2012) 113–118

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Optimization and application of subtype specific polymerase chain reaction for detection and identification of mixed subtypes of hepatitis C virus in patients with renal disease

Prathiba K.[a], Bagyalakshmi R.[a], Malathi J.[a], Anusha H.[a], Ravichandran R.[b], Chandy G.[c], Madhavan H.N.[a,*]

[a] Larsen and Toubro Microbiology Research Centre, Kamal Nayan Bajaj Research Centre, Vision Research Foundation, No. 41, College Road, Chennai 600006, India
[b] Nephrology unit, MIOT Hospital, No. 4/112, Mount Poonamallee Road, Manapakkam, Chennai 600089, India
[c] Department of Gastroenterology and Hepatology, MIOT Hospital, No. 4/112, Mount Poonamallee Road, Manapakkam, Chennai 600089, India

Article history:
Received 10 November 2011
Received in revised form 26 June 2012
Accepted 27 June 2012
Available online 1 September 2012

Keywords:
HCV
Mixed genotypes
Patterns
Reverse transcriptase PCR
Real time PCR

A B S T R A C T

The main objective of the study is to optimize a subtype specific polymerase chain reaction (PCR) for identification of hepatitis C virus (HCV) subtypes in patients with renal disease. Thirty two peripheral blood specimens obtained from 28 patients (post renal transplant $N = 14$, chronic kidney disease $N = 14$) were subjected to HCV viral load determination followed by genotyping analysis. Based on the mixed genotypes and subtypes (>one subtype) obtained by type specific PCR, specific patterns from Pattern I through Pattern X were assigned. All the 32 peripheral blood specimens revealed mixed HCV subtype patterns (>one subtype). The detection of Pattern I in 12 (42.8%) out of 28 patients was statistically significant (Chi square test, P value < 0.001). HCV subtyping assay developed using stringent thermal profile revealed the presence of mixed subtype patterns (>one subtype) which is for the first time being reported in literature.

1. Introduction

Hepatitis C virus (HCV) infection is among the most important Flaviviridae infections with significant clinical problems throughout the world in humans (Idrees and Riazuddin, 2008a). To date at least there are six major genotypes of HCV each with multiple subtypes, which have been identified worldwide (Zeinn and Persing, 1996a). The different genotypes of HCV are relevant to epidemiology, vaccine development and clinical management of chronic HCV infection (Liew et al., 2004). Furthermore, the HCV genotype is the strongest predictive parameter of sustained virological response to antiviral therapy (Zeinn et al., 1996b). The clinical relevance of HCV genotyping is based on the influence of HCV genotypes on the clinical course of the disease since patients with different HCV genotypes respond differently to alpha interferon (Trepo, 1994). A positive sustained response to antiviral therapy occurs in patients with HCV genotype 2 and 3, as compared to HCV genotype 1 (Dusheiko et al., 1994). The clinical prognosis to combination therapy in patients infected with HCV genotype 2 and 3 is 65% whereas that of HCV genotype 1 is 30% (McHutchinson et al., 1998; Poynard et al., 1998). Hence the genotype of HCV determines the treatment with standard interferon therapy and the risk of developing hepatocellular carcinoma (Fabrizi et al., 1996). In addition, different HCV genotypes may evolve different serological responses (McOmish et al., 1993).

Several different methods of HCV genotyping have been applied which include line probe hybridization assay (Stuyver et al., 1993), DNA enzyme immunoassay (Viazov et al., 1996), type specific nested PCR (Okamoto et al., 1992) and sequencing of each HCV amplified product. The distribution of HCV genotypes varies according to geographical location and risk group for infection. The predominant genotypes of HCV within the USA, Europe, Australia, Japan, Taiwan, Thailand, China are genotypes 1, 2 and 3. Genotype 4 is largely confined to the Middle East, Egypt and Central Africa whereas genotypes 5 and 6 are found predominantly in South Africa and South East Asia respectively (Dusheiko et al., 1994).

HCV subtypes 1a and 1b are the most common genotypes in the United States contributing to 73% of cases of HCV infection. Although HCV subtypes 2a and 2b are relatively common in North America, Europe, and Japan, subtype 2c is found commonly in northern Italy. HCV genotype 3a is particularly prevalent

* Corresponding author at: Larsen and Toubro Microbiology Research Centre, Kamal Nayan Bajaj Research Centre, Vision Research Foundation, No. 41, College Road, Chennai 600006, India. Tel.: +91 044 28270229; fax: +91 44 2825 4180.
E-mail address: drhnm@snmail.org (M. H.N.).

0166-0934/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2012.06.023

in intravenous drug abusers in Europe and the United States (Zeinn, 2000).

HCV is a RNA virus and undergoes a high mutation rate resulting in extensive genetic heterogeneity (Butt et al., 2011). Humans can be co-infected with more than one genotype of HCV (Martell et al., 1992). It is difficult to determine the prevalence of mixed genotype infections by the available assays including DNA sequencing because these are designed to identify only the HCV genotype dominant in that particular population. Consequently, genotypes present at lower frequencies could be missed or mistyped by DNA sequencing (Lau et al., 1995).

A genotyping assay with precise identification has to be developed to identify the existing mixed subtypes of HCV. The present study involves the optimization and application of type specific polymerase chain reaction (PCR) to identify the HCV genotypes and subtypes in patients with renal disease.

2. Materials and methods

2.1. Patients and clinical specimens

Thirty two peripheral blood specimens obtained from 28 patients with renal disease were subjected to viral load determination by reverse transcriptase based real time PCR (RT-PCR) followed by PCR for determining the HCV subtypes. Of the 28 patients, 14 were post renal transplant patients (2 had failed graft and were on maintenance haemodialysis while the remaining 12 had previous history of dialysis), and 14 were chronic kidney disease patients (13 were on dialysis while the other patient was not on dialysis). The peripheral blood samples were collected in RNAse free pre sterilized TARSONS tube (BD, California) and processed immediately. RNA was extracted using QIAamp RNA Extraction Kit (QIAGEN, Germany) and used for molecular assays. All the RNAs were stored in liquid nitrogen till further use. The present study was approved by ethics committees of both the research institutes and informed consent was obtained from the patients enrolled in the study.

2.2. RNA extraction

RNA was extracted from the peripheral blood specimens using RNA extraction kit following the manufacturer's instructions. In brief, 280 μl of QIAGEN lysis buffer (AVL) was pipetted into the bottom of a 1.5 ml diethyl pyrocarbonate (DEPC) treated microfuge tube and 2.8 μl of carrier RNA was added. To this, 70 μl of the clinical specimen was added and mixed by pulse vortexing for 15 s. The specimen was incubated at room temperature for 15 min. Two hundred and eighty μl of ethanol (96–100%) was added to the sample and mixed by gentle pipetting for 15 s. The mixture was carefully applied to the QIAamp mini spin column (in a 2 ml collecting tube) without wetting the rim. The cap was closed and centrifuged at 8000 rpm for 1 min. The spin column was placed in a clean 2 ml collecting tube and the tube containing the filtrate was discarded. To the spin column, 500 μl of buffer AW1 was added without wetting the rim and centrifuged at 8000 rpm for 1 min. The filtrate was discarded and 500 μl of buffer AW2 was added without wetting the rim and centrifuged at 14,000 rpm for 3 min. For elution, 50 μl of buffer AVE was added and incubated at room temperature for 1 min, followed by centrifugation at 8000 rpm for 1 min. The eluted RNA was used to determine the viral load.

2.3. Determination of viral load

The viral load was estimated in the peripheral blood specimens using a commercial kit-artus HCV RG RT-PCR kit (QIAGEN, Hilden, Germany) and the assay was performed on Rotor Gene Q (QIAGEN, Hilden, Germany) real time PCR equipment. The amplification reaction was carried out following the manufacturer's instructions. Reverse transcription was carried out at 50 °C for 30 min followed by initial denaturation at 95 °C for 15 min and then 50 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 60 s and extension at 72 °C for 30 s. The viral load was expressed in IU/ml. The samples with viral load of ≥2000 IU/ml were subjected to genotyping assay (Idrees, 2008b).

2.4. Reverse transcriptase PCR targeting the 5′ untranslated region (UTR) to core region of HCV

Reverse transcriptase PCR was optimized using universal forward and reverse primers which amplify all HCV genotypes to generate a 473 bp product. For the second round, universal forward primer was used and 12 type specific reverse primers (1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 3c, 4, 5a, 6a) were used for each subtype generating a subtype specific amplified product. The primer sequences for each subtype, the corresponding accession numbers used for primer design and the amplified product size are provided in Table 1. Type specific primers were designed for 1a, 1c, 3a and 3c using Primer 3 software analyzing the nucleotide differences existing within the HCV subtypes.

2.4.1. cDNA conversion and amplification of HCV

First round PCR was carried out using QIAGEN one step RT-PCR kit (QIAGEN, Hilden, Germany). For a 30 μl reaction 12 μl RNAse free water, 4 μl 5× buffer (2.5 mM $MgCl_2$), 0.8 μl dNTPs (400 μM each), 0.8 μl enzyme mix, 1.2 μl of each primer (600 pM) and 10 μl RNA were used. The cDNA conversion was carried out at 50 °C for 30 min followed by inactivation of reverse transcriptase at 95 °C for 15 min and amplification was done using HCV universal first round primers (Table 1). The thermal profile consisted of initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 64 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 7 min. The amplified product size of HCV PCR was 473 bp.

2.5. HCV subtype specific PCR

HCV subtype specific PCR was carried out as a individual PCR reaction with specific primers for subtypes 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 3c, 4, 5a and 6a. The reaction was carried out using 5 μl water, 4 μl of 10× *Taq* buffer (3 mM $MgCl_2$), 5 μl TMAC (tetramethylammonium chloride) (1:100), 8 μl dNTPs (100 μM each), 0.3 μl *Taq* polymerase, 1.0 μl of each primer (400 pM) and 1 μl of first round amplified product. The amplification was performed as a hot start PCR by adding *Taq* polymerase to the reaction tubes after the first denaturation to avoid non specific annealing of primers. The thermal cycling consisted of 30 cycles of denaturation at 94 °C for 45 s, annealing and extension at 72 °C for 1 min 45 s and final extension at 72 °C for 10 min. A high annealing temperature (72 °C) was used for limiting the nonspecific bands considering the high sequence similarity among the subtypes. The base pair size of amplicon of HCV subtypes were calibrated using the molecular weight marker calibration software available in Vilber Lourmat Gel documentation system (France).

2.6. DNA sequencing targeting the 5′ untranslated region (UTR) to core region of HCV for identification of HCV subtypes

PCR based DNA sequencing targeting the 5′ UTR to core region of HCV was performed on the second round amplicon. Subtype specific DNA sequencing reactions were carried out on subtypes 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 3c using ABI 3100 genetic analyzer (Applied Biosystems, USA). The sequences were subjected to blast analysis (NCBI blast website) and were assigned the specific subtypes