Abstract

Present study reports the standardization and use of polymerase chain reaction (PCR) followed by RFLP (PCR-RFLP) implicating HCV-core as a target region and its comparison with PCR-RFLP based on the use of 5'NCR for HCV genotyping / sub-typing. The new assay produced 399bp PCR product from core region and used a different set of restriction enzymes (MboI, AccI and BstNI) to digest these products for HCV sub-typing. This assay allows a quick determination of HCV types / sub-types. HCV was typed / sub-typed using these two assays in patients with liver and renal diseases. Analysis of results demonstrated the two assay system to be comparable with some difference in few cases. Whereas genotype-3 was a common finding in liver disease groups, genotype-1 was more frequent in chronic renal failure cases. The PCR product from core based assays was also subjected to sequence analysis for genotypes/sub-types and the findings were slightly different. This was further analysed by phylogenetic analysis which supported our findings.

Introduction

Hepatitis C virus (HCV), a major etiological agent of post-transfusion non-A non-B hepatitis was first characterized by Choo et al in 1989 [1]. It is an enveloped virus and belongs to genus Hepacivirus of the family Flaviviridae [2]. HCV genome consists of single-stranded, positive sense RNA of 9.6-kb length and a single open reading frame (ORF) of 9033 to 9099 nucleotide flanked by two non-coding regions (NCR) i.e. 5' NCR and 3' NCR. The ORF codes a long polypeptide of approximately 3000 amino acids and produces three structural proteins (core, envelope E1 and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [3]. HCV infects approximately 200 million people globally [4], has 12-13 million carriers in India [5]. Approximately 80% of HCV infected patients develop chronic hepatitis and 20% of them develop cirrhosis [6]. The reported prevalence of HCV in India is 15% to 25% in chronic liver disease (CLD) patients [7, 8]. HCV infection is an important cause of morbidity and mortality among patients with end stage renal disease (ESRD), particularly among renal transplant recipients also.

HCV-RNA has a high degree of heterogeneity over the entire genome. It varies 30-35% and 20-25% within types, and subtypes respectively [9]. Six major genotypes and more than 120 subtypes of HCV have
been characterized [10]. These HCV genotypes have distinct geographic distribution worldwide. Genotypes 1, 2 and 3 are widely distributed while other genotypes are much more restricted to certain geographical areas. In India, genotype 3 is reported to be the most prevalent, followed by genotype 1 [11, 12]. These genotypes of HCV have important epidemiological implications. The duration and response to interferon therapy in HCV infected patients depends and varies according to HCV genotypes [13, 14].

Real-time PCR and nucleotide sequencing are the most reliable methods for HCV genotyping and often used as gold standard method [15]. Sequencing is a complex and time consuming step. Other genotyping methods include restriction fragment length polymorphism (RFLP) [16, 17], type-specific polymerase chain reaction (PCR) [18], hybridization based line probe assay (LiPA) [19] and serotyping using type-specific peptides of the HCV polyprotein [20]. Although these methods are sensitive, they can identify only fewer subtypes and cannot differentiate subtypes accurately in most of the cases.

350 bases long fragment of 5' non-coding region (5' NCR), is the most conserved part (~95%) of HCV genomes and contain genotype specific motifs [21]. However, it has been analyzed that this region alone cannot accurately distinguish genotype 1 and 6 and subtypes of genotype 1 [22, 23]. The nearby core region is also well conserved and has 81 to 88% nucleotide sequence similarity [21]. Recent reports indicate that sequence information of core region is sufficient to differentiate all genotypes and several subtypes [24].

Thus, present study is an attempt to develop a method for HCV typing based on a newly designed RT-PCR, followed by restriction fragment length polymorphism (RFLP) analysis using HCV-core as the target region. This newly developed assay, simultaneous with existing assay based on PCR-RFLP of 5' NCR region, was used in patients with liver failure and renal diseases for HCV-genotyping and a comparison was made. The procedure allows a quick identification of nearly all major types and subtypes of HCV, and is more accurate and easier to perform than similar methods so far described. Present report also describes the relation of HCV-core expression as indicated by the presence of HCV-core protein in serum, with viral types / sub-types in different disease groups.

Material and Methods

Patients and blood samples

A total number of 63 patients comprising 14 patients with chronic liver diseases (CLD), 4 patients with cirrhosis, 12 patients with hepatocellular carcinoma (HCC) and 17 patients with chronic renal failure (CRF) before dialysis were included in this study plan. 16 of these were excluded for genotyping as these could not be found positive for HCV-RNA on conventional PCR due to low viral load. The above case number is based on the statistical analysis using SPSS Statistics 16.0. All these patients were HCV positive as detected and confirmed by Real Time PCR.

The venous blood (6-10 mL) was drawn and transferred in plain tubes without anticoagulant from HCV positive patients diagnosed with chronic liver diseases (CLD), cirrhosis of liver, Hepatocellular carcinoma (HCC) and chronic renal failure (CRF). The patients were in adult age group and represented both the sexes. They attended either Out Patient Department or admitted to the Gastroenterology Unit/Haemodialysis Unit of All India Institute of Medical Sciences, New Delhi. Blood samples were collected after getting consent from the patients. These patients were evaluated clinically and biochemically and their sera were tested for various hepatitis markers (HCV-antibody and HCV-core antigen) and test parameters including liver function tests and hemogram. The diagnosis of different types of diseases was based on accepted clinical, biochemical and histological criteria as outlined elsewhere [25]. The study protocol was approved by the Institutional Ethical Committee.

HCV- RNA extraction and cDNA synthesis

HCV-RNA was extracted from 200 μL serum sample using High Pure Viral Nucleic Acid Kit (total nucleic acid isolation Kit-Roche Applied Science, Germany) following manufacturer’s instructions. RNA was eluted into 50 μL Elution buffer, aliquoted and stored at -70°C. The extracted RNA was denatured at 65°C for 10 min before the reverse transcription step. cDNA was synthesized by cDNA synthesis kit (Transcriptor High Fidelity cDNA Synthesis Kit-Roche, Germany) at 42°C for 30 minutes using 60i M of random hexamer primer.

HCV- RNA detection on real time PCR

Patient sera were screened for HCV-RNA on real time PCR using primers and probe of a highly
conserved region of HCV genome. HCV RNA was detected using 5’ NCR region specific primers (5’-CGG GTG TAC TCA CCG GTT CCG-3’ and 5’-AGC GTC TAG CCA TGG CGT-3’) and fluorescent labeled probe (MCY CCC CCT YCC GGG AGA GCAT_ _DB). All positive and negative controls were tested in parallel with test samples throughout the entire procedures, starting with RNA extraction.

Amplification of 5’ NCR on conventional PCR
cDNA of HCV-RNA positive samples were subjected to conventional PCR to amplify approximately 240 bp fragment of 5’ NCR region according to following steps: initial denaturation at 95°C for 7 min followed by 30 cycles of denaturation at 95°C for 15 sec, annealing at 54°C for 30 sec, and extension at 72°C for 45 sec with final extension at 72°C for 5 min. The second round of PCR was performed with internal primers under same thermal conditions as used for first round amplification except annealing at 50°C for 30 sec. Three μL of the PCR product was electrophoresed on 2% agarose gel containing ethidium bromide and was visualized under U.V. transilluminator.

HCV genotyping by RFLP method
PCR products obtained by above method were subjected to HCV genotyping using RFLP method. These amplicons were first purified using QIAquick Gel Extraction Kit (Qiagen, Germany) and then digested using two sets of restriction enzymes: Rsal+HaeIII and Mval+HinfI (from New England Biolabs, MA, USA) at 37°C for over night, to differentiate all six genotypes of HCV (Fig. 1A & B) according to McOmish et al, 1993 [26]. The subtypes 1a, 1b and 2a, 2b, 3a & 3b were differentiated using enzymes BstUI (at 60°C for 3hrs) and ScrF1 (at 37°C for 3hrs), respectively (Fig. 2A & B). Band pattern was analyzed on either 4% high quality agarose gel or 10% polyacrylamide gel.

Amplification of core region
Sequences with the sequence Id’s (FJ795713, EU420986, D11443, D16808, AJ291279, EU420985, FJ795704, AJ291257, D16761, D10077, AJ231496, FJ159776, AJ231497, NC_009824, D00831, D00574, D00944, Z29471, U10198, AB079076, AB079077, AB008441, AB008447, D14853, AY651061, AY051292) were analysed to design degenerate primers to pick all the HCV-RNA positive cases. First round amplification was performed with primers 5’-TAC TGC CTG ATA GGG TGC TT- 3’ and 5’-AA GAT AGA G/AAA A/GGA GCA ACC- 3’ at 95°C for 5 min (initial denaturation) followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 46°C for 30 sec and extension at 72°C for 60 sec with final extension at 72°C for 5 min. The nested PCR was performed with second round internal primers 5’-TCC TAA ACC TCA AAG AAA AAC C- 3’ and 5’-ATG TAC/T CCC ATG AGG/A TCG-3’ under same conditions as used for first round amplification. Three μL of PCR product was run on 2% agarose gel and visualized under U.V. transilluminator. The 399bp PCR product was obtained. These amplicons were purified from gel and then sequenced on automated sequencer from Ms Ocimum Biosolutions Ltd, Hyderabad, AP, India. Amplicon sequences of HCV-core region were compared to an online database for the best possible match using the BLAST (Basic Local Alignment Search Tool) program of National Center for Biotechnology Information (www.ncbi.nlm.nic.gov).

HCV Genotyping by RFLP of CORE region
Digestion pattern of 399 bp core region was analyzed with restriction enzymes Mbol, Accl and BstNI. Production of fragments 307 bp and 75 bp with Mbol are predictive of genotype 1b (G1b). Restricted fragments 210 bp, 115 bp & 75 bp are predictive of genotype 2a (G2a), fragments 142 bp, 75 bp, 67 bp & 38 bp are predictive of genotype 2b (G2b), fragments 180 bp, 77 bp, 75 bp & 68 bp are predictive of genotype 3b (G3b) and fragments 325 bp & 75 bp and 245 bp, 80 bp & 75 bp are predictive of genotype 1a, 3a & 5a (G1a, G3a & G5a) and genotype 4a & 6b (G4a &6b) respectively. Similarly, digestion pattern with Accl producing two fragments of either 231 bp & 169 bp or 309 bp & 90 bp size shows the presence of genotype 1a (G1a) and genotype 3a (G3a), respectively. Restricted fragments of 203 bp & 196 bp show the presence of genotype 4a (G4a) while genotype 5a and genotype 6b do not have any restriction site for enzyme Accl. Enzyme BstNI produces the fragments of 214 bp & 186 bp and 214 bp, 165 bp & 20 bp for genotype 5a and genotype 6b, respectively.

Nested PCR product was added into reaction mixture containing 10 Units of each restriction enzyme [Accl, Mbol and BstNI (New England Biolabs, MA, USA)] individually at 37°C for overnight. Digestion products were electrophoresed on 2.5% agarose gel to determine the genotypes. The RFLP pattern was then evaluated under UV light.
**Phylogenetic analysis**

Amplicon sequences of HCV core region were compared to an online database for the best possible match using the BLAST (Basic Local Alignment Search Tool) program of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) [27] and CLUSTAL-X version 2.0. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [28]. The tree with the highest log likelihood (-1730.3173) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 299 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [29].

Subgenomic sequences of known HCV genotypes were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/157781216?report=fasta&from=340&to=9405). Only those sequences, which have our target region of HCV core, were taken for Phylogenetic study.

**HCV Core Ag Assay**

Sera samples were assayed for HCV core protein according to the manufacturer’s instructions using EIA kit from Ortho Diagnostics, UK. One hundred µL of samples and controls were mixed with 100 µL of a pre-treatment buffer. For the ELISA reaction, 200 µL of pre-treated samples and controls were incubated for 95 minutes at 37°C with continuous shaking in the antibody-coated wells of a microtiter plate. The plates were washed and incubated for 30 minutes at 37°C with 200 µL of conjugate, washed again, and incubated for 30 minutes at 37°C with 200 µL of substrate. The optical densities (ODs) were read in a spectrophotometer at 490 nm using a 620 nm reference. The samples and controls were tested in duplicate and the mean OD of each duplicate testing was used. The samples that exhibited more than 25% variation between the two ODs were considered invalid and retested. As recommended by the manufacturer, the lower detection cutoff was established for each run and corresponded to the mean OD of the 2 negative controls plus 0.040. A sample was considered positive only when the mean OD was higher than the cutoff OD of the corresponding run.

**Diagnostic Criteria**

Patients with CLD and cirrhosis of liver were diagnosed by histopathological criteria laid down by International Study Group on Chronic Hepatitis [30].

**Table 1: Prevalence and distribution of hcv genotypes / sub-types in different category of liver and renal diseases.**

<table>
<thead>
<tr>
<th>Disease Group</th>
<th>HCV genotyping using 5'NCR</th>
<th>HCV genotyping using CORE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype detected</td>
<td>Sub-type detected</td>
</tr>
<tr>
<td></td>
<td>Type No. Percent</td>
<td>Type No. Percent</td>
</tr>
<tr>
<td>Chronic liver disease</td>
<td>14 G1 2 14.2</td>
<td>1 G1a 7.7</td>
</tr>
<tr>
<td>(CLD)</td>
<td>G3 7 50.0</td>
<td>1 G3a 4 28.7</td>
</tr>
<tr>
<td></td>
<td>Mixed 4 28.7</td>
<td>2 G3b 1 14.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 G4 7.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 G1b 2 1 7.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 G3a+3b 1 7.1</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>4 G1 2 50.0</td>
<td>G1a 2 50.0</td>
</tr>
<tr>
<td>(CIR)</td>
<td>G3 2 50.0</td>
<td>G3a 2 50.0</td>
</tr>
<tr>
<td></td>
<td>Mixed 1 33.3</td>
<td>Mixed 1 33.3</td>
</tr>
<tr>
<td>Hepatocellular Carcinoma</td>
<td>12 G1 3 25.0</td>
<td>G1a 1 16.7</td>
</tr>
<tr>
<td>(HCC)</td>
<td>Unsubtype-G1* 1 8.3</td>
<td>G1b 1 41.7</td>
</tr>
<tr>
<td></td>
<td>G3 7 58.3</td>
<td>G3b 2 16.7</td>
</tr>
<tr>
<td></td>
<td>G4 1 8.3</td>
<td>G4a 1 8.3</td>
</tr>
<tr>
<td>Chronic Renal Failure</td>
<td>17 G1 13 76.5</td>
<td>G1a 13 76.5</td>
</tr>
<tr>
<td>(CRF)</td>
<td>Mixed 4 23.5</td>
<td>G1a+1b 3 17.6</td>
</tr>
<tr>
<td></td>
<td>G1a+3b 1 5.8</td>
<td>G3 2 40.0</td>
</tr>
</tbody>
</table>

these CLD patients had persistent elevation of transaminases level (at least twice the upper limit of normal range) for more than six months and histological evidence of chronic hepatitis on liver biopsy at the beginning of follow-up. The diagnosis of Hepatocellular Carcinoma is based purely on histological criteria. The CRF was diagnosed using criteria as detailed elsewhere [31].

Results

Present investigation reports the development and application of PCR-RFLP using HCV-core as the target region and its comparison with PCR-RFLP based on use of 5' NCR for HCV genotyping in different disease groups. This study also describes possible relation between HCV-core expression and HCV-genotypes. Analysis of results achieved after determining HCV genotype and their isotypes by PCR-RFLP assay in patients infected with HCV and belonging to different category of liver diseases and chronic renal failure are shown in Table 1. A total number of 14 patients with CLD indicates the presence of G-1 in 2 cases (14.2%), G-3 in 7 cases (50%), G-4 in 1 case (7.1%) and mixed genotypes in 4 (28.7%) cases, respectively while using 5'NCR as the target region of HCV-genome. Of four patients with cirrhosis, G-1 and G-3 were detected in 2 cases (50%) each. Twelve cases with HCC were analysed and found with a similar pattern of G-1, G-3, G-4 and mixed genotypes. In 17 patients with CRF, G-1 was detected in 13 (76.5%) cases and mixed genotypes in 4 (23.5%) cases, respectively. The pattern of subtypes against each genotype is shown in Table 1. In order to make an

Figure 1: A) Digestion pattern of 5' NCR with enzyme Rsa I+Hae III. L: Low molecular weight DNA marker; G: Genotype. B) Digestion pattern of 5' NCR with enzyme Mva I+Hinf I. L: Low molecular weight DNA marker; G: Genotype.

Figure 2: A) Digestion pattern of 5' NCR with enzyme BstUI. L: Low MW DNA marker; G1a: Genotype 1a; G1b: Genotype 1b. B) Digestion pattern of 5' NCR with enzyme ScrF I. L: Low MW DNA marker; G3a: Genotype 3a; G3b: Genotype 3b.
attempt for determining HCV-genotypes and their subtypes in these cases with more accuracy, we amplified, 399bp region of HCV-core, confirmed for genotype by phylogenetic analysis (Fig. 4) and then digested it with separate sets of restriction enzymes (AccI, MboI and BstNI) as outlined in methods section. Using defined pattern of bands on gel for different subtypes (Fig. 3A & 3B), we analysed results in each case and found the results as given in Table 1. The pattern of genotypes and subtypes achieved in these cases was not much different from the one observed with use of 5’ NCR by PCR-RFLP.

We could have two major findings by the use of HCV-core as compared to 5’NCR: the first, less number of subtypes of same category were picked up with use of core as compared to 5’NCR and second, there was a predominant presence of G-3 in 83.3% in HCC as compared to its prevalence in mere 58.3% cases with use of 5’NCR. In all other cases, there was not a significant difference of type detection with the use of either of two sub-genomic regions (Table 1). When same set of sera were comparatively analysed using these two assays, the difference was not very significant (Table 2). These data indicate the new assay, to be another simple and quick assay for HCV-genotyping. Of-course, a large number of cases need to be analysed before reaching a conclusion.

On comparing the genotype pattern in these disease groups with those obtained by sequencing, we could find the presence of genotypes that further supported by sequencing (Table 2). Moreover, 5’NCR region could help in the detection of more number of cases as compared to HCV-core. However, the results of genotyping using core-region were better substantiated by sequence analysis of core region as compared to those using 5’NCR.

A comparison of genotypes (1,2,3 and mixed) with HCV–core expression, as indicated by the presence of HCV-core protein in these disease groups, demonstrated G-3 to be associated with more prevalence of HCV in liver disease groups. At the same time, G-1

**Multiple alignment and phylogenetic analysis**

![Phylogenetic tree analysis](image)

Figure 4: Molecular Phylogenetic analysis by Maximum Likelihood method (Ref. 28,29): Phylogenetic tree analysis of 399 bp fragment of core region from 11 HCV isolates obtained in the studied population. The accession number of standard genotypes used in the construction of the tree: D00831_G1a, D00574_G1b, D00944_2a, D10077_2b, D16761_G3a, D11443_G3b, D16808_4a, Z29471_5a, U10198_6a.
types had more expression in CRF patients, when severity of disease in CLD patients was assessed in relation to genotypes (Table 3).

Discussion

Hepatitis C virus (HCV) infection is a major public health problem of the world. HCV infection leads to not only serious liver diseases in majority of patients but at the same time, remains non-responsive to antiviral therapy in high proportion of cases. Whereas very little success has been made in producing an effective vaccine against HCV [32], antiviral treatment is the only way of treating HCV infection. Unfortunately, majority of patients either do not respond to the antiviral treatment or develop the infection once again after a gap of time. As a result, its early diagnosis and effective treatment remains a major goal to be still achieved.

The genome of HCV is highly variable. Till date six genotypes and more than 120 isotypes have already been characterized [10]. The molecular forms originate from frequent variations in certain sub-genomic regions of HCV genome. The HCV-genotypes and their subtypes have important implications, particularly, when response of patients to antiviral therapy and its outcome is totally dependent on the presence of HCV-genotype [13, 14].

HCV infection is a serious problem of India also where a high proportion of chronic liver disease are caused by HCV [7, 8]. Present study was planned to address the problem of HCV infection by developing a simple and easy assay for determination of HCV-genotypes and find out the status of different genotypes and their subtypes in different disease groups in north India. Simultaneously, the present study was also aimed to investigate some possible relation of HCV-core expression, as indicated by the presence of HCV-core protein in blood, with HCV-subtypes so that the presence of this protein could be used as a simple and alternate to foresee the possible presence of certain HCV subtypes in human blood.

Earlier, the determination of HCV-genotypes was done by methods, such as automated reverse hybridization [33] and semi automated sequencing [34, 35]. These methods need a previously amplified genomic product as starting material. 5'NCR, a highly conserved region is conveniently used for genotyping by these two methods. However, differentiation of subtypes is not

<table>
<thead>
<tr>
<th>Disease Group</th>
<th>Total No. analysed</th>
<th>5' NCR</th>
<th>PCR – RFLP</th>
<th>HCV – CORE</th>
<th>Sequencing</th>
<th>(HCV-Core)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Liver Disease (CLD)</td>
<td>7</td>
<td>G3a 3</td>
<td>42.8</td>
<td>G3a 2</td>
<td>28.6</td>
<td>G3a 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G3b 2</td>
<td>28.6</td>
<td>G3a 2</td>
<td>28.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G3a+b1</td>
<td>14.3</td>
<td>G3a 1</td>
<td>14.3</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>G4a 1</td>
<td>14.3</td>
<td>G4a 1</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis (CIR)</td>
<td>2</td>
<td>G3a 2</td>
<td>100</td>
<td>G3a 2</td>
<td>100</td>
<td>G3a 1</td>
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<td>Hepatocellular Carcinoma (HCC)</td>
<td>6</td>
<td>G3a 4</td>
<td>66.7</td>
<td>G3a 4</td>
<td>66.7</td>
<td>G3a 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G3b 1</td>
<td>16.7</td>
<td>G3a 1</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G3b+x2</td>
<td>16.7</td>
<td>G1b 1</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>Chronic Renal Failure (CRF)</td>
<td>5</td>
<td>G1a 5</td>
<td>100</td>
<td>G1a 3</td>
<td>60.0</td>
<td>G1a 1</td>
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<td></td>
<td></td>
<td>G3a 2</td>
<td>40.0</td>
<td>G3a 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Comparative status of HCV - subtypes using PCR - RFLP and sequence analysis.

Table 3: Relation between genotypes and core expression in different disease groups.

<table>
<thead>
<tr>
<th>Disease Group</th>
<th>Total cases Analysed (No.)</th>
<th>Genotype 1</th>
<th>Genotype 3</th>
<th>Genotype 4</th>
<th>Mix infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Liver Disease (CLD)</td>
<td>14</td>
<td>3</td>
<td>0</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Cirrhosis (CR)</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Hepatocellular Carcinoma (HCC)</td>
<td>12</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Chronic Renal Failure (CRF)</td>
<td>17</td>
<td>16</td>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Above table indicates the presence of HCV-core protein in relation to genotypes in different disease groups. No., Indicates the number of sera analysed; No. **”, Indicates the number of sera positive for given parameter; ND, Not done.
always possible using this region and quite often leads to sub-typing error [23, 36-38]. Nucleotide sequencing followed by phylogenetic analysis of regions like NS5B and HCV-core has also been recommended for HCV-genotyping [23]. However, this procedure is impractical and time consuming. It also does not detect mixed genotypes infection [39].

RT-PCR followed by RFLP has been often used for HCV-genotyping and their subtypes. In all these assays the 5'NCR has been frequently used for amplification followed by digestion by different sets of restriction enzymes to detect different subtypes. However, like in other assay systems it is not possible to detect all subtypes by using 5'NCR [12]. Attempts have been made to include the conserved part of other sub-genomic regions also both in PCR-RFLP as well as Real Time PCR [40]. Present study was one more attempt to develop assays for determining HCV sub-types using HCV core region for amplification followed by RFLP with different sets of restriction enzymes. This assay was found to be simple, quick and an alternate assay for HCV-genotyping.

When the results achieved by this newly developed assay, were compared with those found with PCR-RFLP based on the use of 5'NCR, we could not find a remarkable difference in all disease groups. However, use of core produced no confusion in distinguishing HCV genotypes in compared with the use of 5'NCR. In fact, HCV core is another alternative region for HCV genotyping. It is supported also by sequencing of amplicon. This method is single step method producing fast results. The difference of percent change was more attributed to less number of cases included in some disease groups. In liver disease patients, HCV-core protein had a frequent association with G3, whereas in CRF patients, it was common in G1 positive cases. Although there appears to be an unlikely impact of organ disease on expression of a particular HCV-genotype, however, it cannot be ignored and needs extensive experimentation and discussion in context of other contributing factors ascribing to gene expression.

In conclusion, present study reports standardization of another PCR-RFLP assay using HCV-core as the target region and a different set of restriction enzymes to digest the PCR products. This assay was compared with PCR-RFLP based on 5’NCR region and shown concordance in results achieved by two assays. Use of these assays demonstrated the predominance of genotype-3 as the major genotype in Indian patients’ population. HCV-core expression appears to depend on HCV-genotype and the disease condition, however, it needs further confirmation by including more number of cases in this study plan.

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