Distribution of the different genotypes of HCV among patients attending a tertiary care hospital in south India

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Abstract

Background: Genotyping of the hepatitis C virus (HCV) and assessment of viral load is important for designing therapeutic strategies and region specific diagnostic assays. Objectives: To determine the distribution of HCV genotypes among patients attending a tertiary care hospital in south India, and to correlate this with viral load. Study design: Ninety HCV RNA positive patients were recruited for the study. HCV genotyping was carried out using type-specific primers from the core region of the viral genome [J. Clin. Microbiol. 35 (1997) 201]. Viral load estimations were carried out using the Amplicor HCV Monitor (Versions 1.5 and 2, Roche Diagnostics, Branchburg, NJ, USA). Clinical details were elicited from patients’ hospital records. Results: Genotype 3 was detected most frequently (62.2%) followed by infection with HCV genotype 1 (18.8%). There was no significant difference seen in alanine aminotransferase (ALT) values between the two genotypes. Genotype 1 was associated with a significantly higher viral load as compared with genotype 3 (P = 0.001). Parenteral transmission accounted for 61% of all infection caused. Infection with genotype 1 was significantly associated with a history of haemodialysis (P = 0.01). Genotype 3 was detected more frequently in patients from east India, as compared with its detection in patients from south India (P = 0.004). Similarly, genotype 1 was detected with greater frequency in individuals from south India as compared with patients from east India (P = 0.004). The concordance between Ohno’s genotyping assay and nucleotide sequencing, for genotypes 1 and 3, was 75%. Conclusions: HCV genotypes 1 and 3 accounted for 81% of HCV infections in patients from this geographical region. HCV genotype distribution showed regional differences and genotype 1 was associated with higher viral loads. Parenteral transmission was the major route for acquisition of HCV infection. Ohno’s type-specific primer based genotyping assay can be used for distinguishing between HCV genotype 1 and non-1 HCV genotypes in laboratories that do not possess nucleotide sequencing facilities. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: HCV genotypes; HCV viral loads; India

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1. Introduction

Infection with the hepatitis C virus (HCV) is the major cause of non A- non-B hepatitis following transfusion (Alter et al., 1989). HCV can cause chronic liver disease, with chronic active hepatitis, cirrhosis and hepatocellular carcinoma. (Nishioka et al., 1991; Tong et al., 1995). Parenteral transmission accounts for nearly 60% of HCV infections, with recipients of blood and blood products, hemodialysis/renal transplant patients and intravenous drug abusers facing a significant risk (Makris et al., 1990; Giammaria et al., 1992; Zeuzem et al., 1996). Mother to child, interfamilial, and sexual transmission are other postulated modes of transmission for this virus (Thaler et al., 1991; Da Porta et al., 1992; Salleras et al., 1997).

The HCV is a positive stranded RNA virus with a genome that is approximately 9400 nucleotides long. HCV is genetically a very heterogeneous virus with at least six major genotypes. These differ in nucleotide sequence by more than 30% over the complete virus genome. A number of subtypes, which differ in nucleotide sequence by more than 20%, have also been described (Simmonds et al., 1994a). They are designated 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 3g, 4a to 4f, 5a and 6a (Simmonds et al., 1994b; Stuyver et al., 1995; Panigrahi et al., 1996).

The genotypes of HCV show a distinct geographical distribution. Genotypes 1a, 1b, and 2a are the predominant genotypes in the United States and Western Europe. Genotype 4 is the predominant genotype of the Middle East. Types 5 and 6 are largely confined to South Africa and South East Asia, respectively, (Davidson et al., 1995).

Genotyping of HCV strains is important for epidemiological purposes, and furthermore it is thought that the rates of response to interferon therapy may vary according to HCV genotypes (Poynard et al., 1998; McHutchison et al., 1998). High pre-treatment levels of serum HCV RNA has been correlated with the pathogenicity and non responsiveness of certain genotypes, like HCV genotype 1, to anti-viral therapy. Therefore, understanding the distribution and properties of HCV genotypes may have important implications for prognosis and therapy (Kohara et al., 1995; Zein et al., 1996; Izopet et al., 1998).

Determination of the HCV genotype by sequencing is considered the ‘gold standard’ for typing methods. A number of other techniques are currently being used to type large patient groups. These include the technique of restriction fragment length polymorphism (RFLP) (Nakao et al., 1991), polymerase chain reaction (PCR) using subtype specific primers (Okamoto et al., 1992; Ohno et al., 1997), hybridization with sub-type specific oligonucleotide probes (Stuyver et al., 1993) and studying the serological response to synthetic peptides (serological typing) (Schroter et al., 1999).

Previous studies have suggested that in India, genotype 1 and genotype 3 are the predominant genotypes (Valliammai et al., 1995; Panigrahi et al., 1996). These two studies were comparatively limited in scope and reported on 24 and 11 patients, respectively. These two studies were also restricted to geographically selected patient populations.

The primary aim of the current study was to identify the distribution of HCV genotypes among patients attending a tertiary care hospital in south India. Additional objectives were to determine whether there was any association of genotype with certain factors including gender, age, viral load and alanine aminotransferase (ALT) levels. We believe that the relationship of viral loads to different genotypes of HCV in Indian patients is reported here for the first time.

2. Materials and methods

2.1. Patients

This cross sectional study was performed on 90 patients with chronic HCV infection, attending the Christian Medical College and Hospital in south India, between the period January 1999 and March 2001. The study population included 64 (71.1%) men and 26 (28.8%) women, in the age range of 9–70 years (Mean ± S.D., 43 ± 15). None of the patients had been on anti-viral ther-
apy for HCV at the time of sampling for the study. These patients were identified as HCV infected by a qualitative PCR earlier standardized in this laboratory (Radhakrishnan et al., 2000). The study group included both patients presenting with chronic liver disease, as well as those being screened for HCV infection in high risk groups. They comprised of 20 (22.2%) patients who were either on or had been on haemodialysis, 33 (36.6%) had received multiple blood transfusions, 30 (33.3%) had no attributable risk factor for acquiring infection and seven (7.7%) patients had other risk factors which included tattooing, a past history of surgery and intravenous drug abuse.

All patients were also tested for HBsAg (AxSYM V2, Abbott Laboratories Abbott park, IL, USA) and HCV antibody. (UBI HCV EIA 4.0, United Biomedical Inc, NY, USA/AxSYM HCV 3.0, Abbott Laboratories Abbott park). These patients came from different parts of India. Clinical information, inclusive of ALT levels, and possible risk factors were obtained for analysis from patients’ hospital records.

2.2. Genotyping of HCV

For HCV RNA detection, blood was collected in EDTA containing tubes and plasma was obtained within 3 h of collection. Aliquots were stored at −60 °C until further testing.

In HCV RNA positive samples, genotypes were determined by performing PCR using primers specific for the core region of the HCV genome, using two separate reaction tubes containing different primer mixes, as described previously (Ohno et al., 1997). This method allows for the determination of genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a in two separate reaction tubes.

2.2.1. First round amplification

Briefly, 2 μl of the cDNA was amplified in a 50 μl reaction volume containing 1.5 mM MgCl₂, 10 mM Tris–HCl, 50 mM KCl, and 2.5 pmol each of sense primer Sc2 and antisense primer Ac2 (outer primers). The first round of amplification was performed under the following conditions: twenty cycles of amplification at 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min followed by an additional 20 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min.

2.2.2. Second round amplification

For this round of PCR, two mixes were made. One mix contained primers for the specific detection of HCV genotypes 1b, 2a, 2b and 3b. The second mix contained primers for the selective amplification of genotypes 1a, 3a, 4, 5a, and 6a. One microlitre of first round product was taken as input for the second round PCR.

The products of the second round PCR were electrophoresed on a 2% agarose gel. ΦX174 DNA cleaved with HaeIII (Boehringer Mannheim, Roche Diagnostics, Germany) was used as the molecular weight marker. The products were viewed on an UV transilluminator (Mighty Bright, Hoefer Scientific Instruments, USA). The molecular weights of the bands of representative samples from all detected genotypes were analyzed with the aid of a gel documentation system (Gel Doc 2000, BioRad). Samples were assigned genotypes based on the band size of the final amplified product, as recommended (Ohno et al., 1997).

2.3. HCV RNA quantitation

This was done on RNA positive samples with the help of a commercially available quantitation kit—the Amplicor HCV Monitor test Version 1.5 and 2.0, as available. (Roche Diagnostics, Branchburg, NJ, USA). Briefly, this uses a quantitation standard (QS), a non-infectious RNA transcript, that contains primer binding regions similar to that of the HCV target and additionally, an unique probe binding region that allows for the differential detection of the QS during the detection stage. Reverse transcription and amplification of the 5′ non-coding region occurs simultaneously using the enzyme Thermus thermophilus DNA polymerase (rTth pol). Biotinylated primers are used for amplification. Following amplification, serial dilutions of the resultant HCV and QS are performed. Microtiter plates coated with probes specific for the two amplicons are used to determine concentrations. The concentration of HCV RNA is calculated.
using a formula. The lower detection limit of the kit is 2000 HCV RNA copies per ml of plasma (for the 1.5 Version) and 600 IU/ml of plasma (for the 2.0 Version). All samples with HCV RNA titers above the upper limit of the assay (500 000 copies per ml for the 1.5 version and 850 000 IU/ml for the 2.0 version) were diluted one in 100 with negative human plasma and retested as recommended. The dilution and retesting was performed at a later point of time on stored plasma samples. Samples which had been tested in the earlier version of the Amplicor kit and had titers in copies per ml were converted to IU/ml using a conversion factor provided, for two lots of the kit, by the manufacturer.

2.4. Statistical analysis

The $\chi^2$-test, and Fisher exact test was used to analyze significance of differences between proportions, while the Mann–Whitney U-test (Wilcoxon rank sum test) was used to determine the significances of differences between numerical data. The NCSS/PASS 2000 Dawson Edition and the EPI INFO (version 6.04b) statistical packages were used for analysis as appropriate.

3. Results

Samples were classified as belonging to genotype 1, genotype 3, genotype 4 or as untypeable using the type specific primer based PCR.

The genotype profile most frequently detected was genotype 3 seen in 56 patients (62.2%) followed by infection with genotype 1 seen in 17 patients (18.8%). Genotype 4 was seen in five patients (5.5%), and one patient had infection with HCV genotype 2. The HCV strains in 11 patients (12.2%) could not be typed using this genotyping technique.

Viral load testing was carried out on all 90 patients and was compared between genotypes. The average viral load of patients infected with genotype 1 was significantly higher than the average viral load of patients infected with genotype 3 ($P = 0.001$, Mann–Whitney U or Wilcoxon Rank sum-test).

The mean and median ALT values and their ranges across the two most commonly detected genotypes are shown in Table 1. A statistically significant difference was not seen between the mean ALT of patients infected with genotype 1 and the mean ALT of those infected with genotype 3 ($P = 0.55$).

There was no significant difference in the male to female ratio across genotypes 1 and 3 ($P = 0.41$).

The risk factors and the possible routes of acquisition are shown in Table 2. A history of past blood transfusion was elicited in 5 of 17 (29.4%) patients with genotype 1, compared with 20 of 56 (35.7%) patients with genotype 3 ($P = 0.631$). A history of haemodialysis was elicited from eight of 17 (47%) patients with HCV geno-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of mean age, gender ratio, median ALT levels and plasma HCV RNA values between genotype 1 and genotype 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infecting genotype</td>
<td>Gender (M/F)</td>
</tr>
<tr>
<td>Genotype 1 (n = 17)</td>
<td>13/4</td>
</tr>
<tr>
<td>Genotype 3 (n = 56)</td>
<td>37/19</td>
</tr>
</tbody>
</table>

* Infection with genotype 1 was associated with a significantly higher viral load as compared with infection with genotype 3 ($P = 0.001$).
Table 2
Routes of transmission of the different genotypes of HCV

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Haemodialysis no (%)</th>
<th>Blood transfusion no (%)</th>
<th>Unknown no (%)</th>
<th>Miscellaneous b no (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype 1 (n = 17)</td>
<td>8 (47)</td>
<td>5 (29.4)</td>
<td>3 (17.6)</td>
<td>1 (5.8)</td>
</tr>
<tr>
<td>Genotype 2 (n = 1)</td>
<td>–</td>
<td>1 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Genotype 3 (n = 56)</td>
<td>10 (17.8)</td>
<td>20 (35.7)</td>
<td>21 (37.5)</td>
<td>5 (8.9)</td>
</tr>
<tr>
<td>Genotype 4 (n = 5)</td>
<td>–</td>
<td>2 (40)</td>
<td>3 (60)</td>
<td>–</td>
</tr>
<tr>
<td>Untypeables (n = 11)</td>
<td>2 (18.1)</td>
<td>5 (45)</td>
<td>3 (27.2)</td>
<td>1 (5.8)</td>
</tr>
</tbody>
</table>

a Infection with genotype 1 was significantly associated with a history of haemodialysis as compared with infection with genotype 3 (P = 0.01).
b The miscellaneous routes of transmission included tattooing, intravenous drug abuse, a past history of surgery and a possible sexual mode of transmission.

type 1 as compared with ten of 56 (17.8%) patients with genotype 3 infection. This difference was statistically significant (P = 0.01, Fisher’s exact test).

The geographical distribution of HCV genotypes in this patient population is shown in Table 3. Of the 26 patients from south India, ten (38.4%) were infected with HCV genotype 1, 11 (42.3%) were infected with genotype 3. Genotype 4 was seen in two South Indian patients and three HCV strains from this region were untypeable.

Of the 50 patients from east India, five (10%) were infected with HCV genotype 1, 38 (76%) with HCV genotype 3, two (4%) patients with genotype 4 and four strains (8%) were untypeable. Genotype 3 was detected in a significantly higher number of individuals from east India as compared with its detection in individuals from south India (P = 0.004, χ²-test). The detection of genotype 1 was significantly higher in south Indian individuals as compared with the detection of genotype 1 in individuals from east India (P = 0.004, Yates corrected χ²).

4. Discussion

This study confirms the preponderance of HCV genotypes 1 and 3 in Indian patients with chronic liver disease. Similar studies in patients exclusively from Northern and Southern India have reported the predominance of either genotype 1 or 3. The results of other studies on circulating HCV genotypes in the Indian subcontinent are summarised in Table 4.

Additionally genotype 4 has been observed in this group of patients and may reflect the migration of Indians, in search of employment, to the Middle East—where four is the predominant HCV genotype (Shobokshi et al., 1999).

The route of acquisition of HCV in this group of patients is well defined with 59% of patients having a history of haemodialysis or blood transfusion. Other documented risk factors included tattooing, a past history of surgery, and a history of promiscuous behavior. Overall, parenteral transmission accounted for 61% of all infection caused. Genotype 1 was significantly associated with a history of haemodialysis. It is

Table 3
Geographical distribution of HCV infected individuals and their genotype distribution

<table>
<thead>
<tr>
<th>Genotype</th>
<th>North India no (%)</th>
<th>East India no (%)</th>
<th>South India no (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype 1</td>
<td>l(11.1)</td>
<td>5 (10)</td>
<td>10 (38)</td>
</tr>
<tr>
<td>Genotype 2</td>
<td>–</td>
<td>1 (2)</td>
<td>–</td>
</tr>
<tr>
<td>Genotype 3</td>
<td>4 (44.4)</td>
<td>38 (76)</td>
<td>11 (42)</td>
</tr>
<tr>
<td>Genotype 4</td>
<td>–</td>
<td>2 (4)</td>
<td>2 (7.6)</td>
</tr>
<tr>
<td>Untypeable</td>
<td>4 (44.4)</td>
<td>4 (8)</td>
<td>3 (11.5)</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>50</td>
<td>26</td>
</tr>
</tbody>
</table>

a Detection of genotype 1 was significantly higher (P = 0.004) in south Indians as compared with its detection in individuals from east India.
b Detection of genotype 3 was significantly higher (P = 0.004) in patients from east India as compared with its detection in individuals from south India.

In all, five of the patients with genotype 1 (n = 1), genotype 3 (n = 3) and genotype 4 (n = 1) were from outside India (Maldives).
Table 4
Results of other studies, as compared with the present study, on the distribution of HCV genotypes in the Indian subcontinent

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Number of samples genotyped/sequenced</th>
<th>Region of HCV genome used for study</th>
<th>Genotype 1</th>
<th>Genotype 3</th>
<th>Others</th>
<th>Major finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valliammai et al., 1995</td>
<td>24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS5</td>
<td>21</td>
<td>3</td>
<td>–</td>
<td>Genotype 1 predominates in chronic liver disease patients from southern India</td>
</tr>
<tr>
<td>Panigrahi et al., 1996</td>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Core and NS5</td>
<td>3</td>
<td>7</td>
<td></td>
<td>Genotype 3 predominates in HCV infected patients from northern India.</td>
</tr>
<tr>
<td>This study (2001)</td>
<td>90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Core</td>
<td>17</td>
<td>56</td>
<td>17</td>
<td>(1) Genotype 1 more frequently detected in patients from south India. (2) Genotype 3 predominates in patients from eastern India. (3) The viral load in patients with genotype 1 was found to be higher than in patients with genotype 3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Genotypes identified by nucleotide sequencing.

<sup>b</sup> Genotypes were identified using type specific primers and sequencing of a representative number of samples ($n = 28$) was also carried out.
possible that genotype 1 was introduced into this group due to the dependence on developed countries, previously, for anticoagulation factors, as earlier suggested (Shobokshi et al., 1999). This phenomenon also indicates that there is a close association between certain HCV genotypes and routes of transmission in high-risk groups. Intravenous drug abuse, which is the major cause for HCV infection in developed countries, was responsible for only two cases of infection in this group of studied patients. In 33.3% of patients, no history of high-risk behavior could be elicited.

In this study, genotype 1 was associated with a significantly higher viral load when compared with genotype 3, corroborating reports by other investigators from other regions of the world (Zein et al., 1996; Izopet et al., 1998). It is believed that the higher baseline viral load is one of the factors that contribute to the aggressive form of disease characterizing infection with genotype 1. It was also seen that patients infected with genotype 1 were younger (Mean ± S.D., 36.6 ± 13) compared with patients infected with genotype 3 (Mean ± S.D., 43.6 ± 14.8) though this difference was not significant (P = 0.08). Whether this reflects a change in the known risk factors of HCV transmission biasing the spread of certain HCV genotypes or a faster and more aggressive progression of disease in individuals infected with genotype 1 as earlier suggested (Pozzato et al., 1991) and thereby making them seek medical attention earlier, remains to be investigated with larger sample sizes and long term follow-up.

Five patients (two infected with genotype 1, and three with genotype 3) were found to be additionally positive for hepatitis B surface antigen (HBsAg). However the viral loads in these patients was not significantly different (P = 0.187, Mann–Whitney U-test) from the average load in the other groups. (Data not shown).

The geographical distribution of genotypes was also studied. Genotype 1 was most frequently detected in south Indian patients while genotype 3 was most frequently detected in patients from east India. However, a proportion (11/90, 12.2%) of HCV strains could not be typed. This finding can be explained by (i) an intrinsic insensitivity of Ohno’s type specific primer based PCR genotyping assay based on the core region of the HCV genome or by (ii) the more interesting possibility of the emergence of a new subtype undetectable by this technique.

In order to validate the genotyping method used, a representative number of samples from this study were sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Twenty-eight samples that were typed as genotype 1 or 3 were further sequenced. All samples typed as genotype 1 (n = 9) by Ohno’s primers, were confirmed by sequencing. However, of the 19 samples typed as belonging to genotype 3 by Ohno’s primers, only 12 were confirmed as genotype 3 by sequencing. The overall concordance rate in this study between Ohno’s type specific primer based genotyping assay and sequencing was 75%. This degree of concordance suggests that Ohno’s HCV genotyping technique can serve as an alternative, to laboratories that do not have access to sequencing facilities, for distinguishing between HCV genotype 1 and non-1 HCV genotypes.

The prevalence of different genotypes of HCV in different parts, of a large country like India, also raises the possibility of designing tailor-made diagnostic assays for each geographical region. Currently available diagnostic assays use HCV antigens derived from the prototype HCV strain 1a. In a study conducted by Dhaliwal et al. in 1996, it was seen that donors infected with genotype 1 showed 4–4.5 times greater serological reactivity by the Ortho EIA 3.0 (Ortho diagnostic Systems, Raritan, NJ, USA) assay than those infected with a genotype other than genotype 1. (Dhaliwal et al., 1996). Introduction of fourth generation HCV antibody detection assays, which are able to detect most genotypes with equal efficiency, is highly recommended for screening blood and blood products, especially in the Indian sub-continent, where there is a preponderance of genotypes other than genotype 1.

In conclusion, genotype 3 was most frequently found in this geographical region. Genotype 1 was associated with higher viral load and younger age at presentation. There was no significant association of any genotype with elevated ALT values. Twelve percent of HCV isolates could not be
typed, though the typing method used is able to identify all commonly prevalent genotypes. The emergence of a new subtype peculiar to this geographical region cannot be disregarded. Targeting another region of the HCV genome, with alternative techniques like RFLP and sequencing, will have to be pursued to identify putative new subtypes of HCV. Though haemodialysis and blood transfusion accounted for a high proportion of HCV infection across all genotypes, in a good number of cases no major risk factor could be elicited. The presence of genotype 4, currently thought to be a poor responder to IFN (El-Za-yadi et al., 1996), in this patient population is an additional cause for concern. Improved surveillance and periodic epidemiological studies will have to be undertaken to monitor and prevent the spread of virulent and interferon resistant strains of HCV.

References


