HCV INFECTION AMONG BRAZILIAN PATIENTS WITH LIVER DISEASE

ABSTRACT

Anti-HCV detection was performed on patients with chronic liver disease from the Federal University Hospital of Rio de Janeiro, Brazil; 59 (43%) out of 137 tested were positive and were further analyzed for HCV RNA by a transcription-nested polymerase chain reaction (RT-nested PCR). HCV RNA was detected in 45 samples which was subsequently and quantitatively titrated. Genotyping was performed by a system that consists of a two-stage amplification using core region derived type-specific primers. Genotypes 1a, 1b, and 3a were identified in 22 (48%), 17 (39%), and 4 (9%) samples, respectively. Two sera were untypeable by this method. Quantitative HCV RNA titers were determined using a single-tube combined RT-PCR assay (Amplicor HCV monitor, Roche, USA). The highest titers were observed in genotype 1b but were not significantly different from the other genotypes (p > 0.05). There was no correlation between HCV RNA titers or genotypes with clinical status or route of transmission. The high prevalence of genotype 1 confirms the importance of genotype testing for ideal treatment regimen.

Key words:
HCV, viral load, genotype
INTRODUCTION

Hepatitis C virus (HCV), a single stranded RNA virus with a high rate of establishing chronic infection, has been responsible for most post-transfusional and sporadic non-A, non-B hepatitis (Choo et al., 1989; Alter et al., 1989). In order to prevent further transfusional transmission, most blood banks throughout the world have been routinely screening for HCV.

Comparative nucleotide sequence analysis within the core gene of different HCV isolates initially allowed HCV to be classified into four types I to IV (Okamoto et al. 1992). However, on the basis of NS5 sequence analysis, a new nomenclature has been proposed, which classifies HCV into six major genotypes with several subtypes (1a, 1b, 1c, 2a, 2b, etc.) (Simmonds et al. 1993). HCV typing has permitted molecular epidemiologic studies of the virus and has been useful for tracing routes of transmission other than transfusional-associated (Chayama et al. 1995; Zuccoti et al. 1995). Specific genotypes have also been correlated with greater severity of liver disease (Lau et al. 1995; Pozzato et al. 1994) and response to antiviral therapy (genotype 1 less responsive) (Hino et al. 1994; Mita et al. 1994; Yuki et al. 1995).

The amount of HCV RNA also plays an important role in predicting therapeutic response in patients with chronic hepatitis (Lau et al. 1993) and has an important role in monitoring its efficacy. Differences in viral load among the different genotypes could explain the poor antiviral responsiveness in certain viral subtypes.

In the present study, a profile of HCV infection in patients with liver disease was conducted in a University Hospital in Rio de Janeiro. HCV genotypes were determined and viral loads for HCV RNA were measured and correlated to possible clinical and genotype associations.

PATIENTS & METHODS

Patients

Blood samples were collected from 137 consecutive volunteer patients from the outpatient liver clinic, Federal University Hospital, Rio de Janeiro, Brazil, between July and December 1993. All patients had given oral consent for blood testing (Brazilian Ministry of Health's policy) and answered an epidemiologic questionnaire. The questionnaire included information on alcohol intake (with more than 60mg ethanol/day considered as positive for alcoholism), past history of blood transfusion, number of transfusions, date of last transfusion, and other risk factors such as: IVDU, homosexuality, sexual promiscuity, and tattooing. Each serum sample was separated within 3 hours and stored at -20°C.

Serological Testing

Anti-HCV was detected using a commercial kit (HCVII, Ortho Diagnostic Systems) according to the manufacturer’s instructions. Anti-HCV testing was also carried out by a third generation “in-house” EIA with recombinant antigens NS3, NS5, and core, with previous validation of specificity and sensitivity (Ginabreda et al. 1997). These antigens
were kindly supplied by the Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan.

**HCV RNA**

All samples reactive to commercial and “in-house” EIA for anti-HCV were tested for HCV RNA by RT-PCR. HCV RNA extraction and detection were carried out as previously described (Okamoto et al. 1990).

**HCV Genotyping**

Anti-HCV positive sera were genotyped according to Okamoto’s system (Okamoto et al. 1993). This system is based on a nested PCR amplification of the HCV genome and uses three primers located in the core region. During the first round of PCR, a pair of universal (non-type-specific) primers were used; in the second round type-specific primers were used for genotypes 1a, 1b, 2a, 2b, and 3a. PCR products were electrophoresed in agarose gel and the HCV genomes were identified according to the length of the amplified sequence.

**HCV RNA Quantitation**

Aliquots of anti-HCV positive sera were tested and quantified for RNA by Amplicor HCV Monitor (Roche), according to the manufacturer’s instructions. This combined RT-PCR method adds an internal control called quantitation standard (QS) to each patient’s sera. The QS which is a synthetic RNA molecule with primer sites identical to the HCV target is used to monitor assay efficacy. The patient’s HCV RNA and the QS are extracted with guanidine thiocyanate and are then recovered by isopropanol precipitation. HCV RNA and QS RNA are together reverse transcribed and amplified by PCR in a single tube reaction using the enzyme rTth polymerase. Following amplification, the products are then serially diluted and hybridized in microwells coated with HCV-specific and QS-specific probes. Detection of HCV RNA is performed by an avidin-horseradish peroxidase system. Since this method was demonstrated to be less sensitive for genotype 3, a correction factor (multiplying viral load by a factor of 12) was used according to Hawkins and collaborators (Hawkins et al. 1997) to compensate for this reduced sensitivity.

**Statistical Analysis**

Statistical analysis was performed using STATA 6 (Stata Corporation, College Station, TX). Chi-square with Yates correction was used to compare frequency data of clinical characteristics in the different groups. The Mann Whitney U-Test and Kruskal-Wallis test were used for comparing viral load of HCV and ALT titers with different genotypes. T-test, ANOVA, and regression analysis were also used whenever appropriate.

**RESULTS**

Anti-HCV was present in 59 samples (43%) of which 45 (76%) were positive for HCV RNA. Different factors were assessed between presence of anti-HCV and HCV RNA [table 1]. No differences were observed (not shown) as to the number of blood transfusions and time since last transfusion.
Among the 45 HCV RNA-positive samples which were genotyped, the two most common genotypes were 1a and 1b (Table 2). HCV genotypes and HCV RNA copies are outlined in figure 1. Even though genotype 1b had the highest (median), it was not significant (p>0.05).

DISCUSSION

The significant lower mean ages observed among the anti-HCV negative group (Table 1) was likely due to present liver diseases (auto-immune hepatitis, Wilson’s disease, congenital liver fibroses, sclerosing cholangitis) common to younger individuals.

Previous studies have demonstrated the predominance of genotype 1 in Brazil (Bassit et al. 1994; Krug et al. 1996; Stuyver et al. 1993). Our findings were not different, with genotype 1 detected in 87%. However, differences were observed between genotype 2 and 3. Detection of genotype 3 (20% to 37%) and genotype 2 (1.5% to 8%) were more frequently observed by other investigators. These differences are most probably due to the small number of individuals studied and geographic distribution.

All HCV RNA positive samples were quantified by the Amplicor Monitor HCV assay. This assay however has a reduced sensitivity for genotypes 2 and 3, and to compensate for this, the correction factor (proposed by Hawkins and collaborators) was used for genotype 3a values. The corrected titers showed no significant differences among the genotypes, consistent with recent data (Hawkins et al. 1997; Lau et al. 1995; Smith et al. 1996). The median viral load in this study (1.02 x 10^5 copies/ml), determined through the Amplicor Monitor HCV assay, was lower compared to other studies that used other methods (competitive PCR or branched DNA). This finding has been similarly observed by other authors (Kessler et al. 1996; Trabaud et al. 1997) and is most probably due to its lower detection limit when compared to the branched DNA assay. If one had used the branched DNA assay (Quantiplex HCV RNA 2.0 assay) in this study, 22% of the samples would have remained undetected considering its detection limit to be 2.0 x 10^5 equivalents/ml and its ten-fold titer difference (Trabaud et al. 1997). In spite of the Amplicor Monitor HCV assay’s lower sensitivity (500 copies/ml), it is nonlinear for samples greater than 10^7 (Gretch et al. 1996) and lacks sensitivity for genotypes other than 1 (Hawkins et al. 1997). Optimal quantitative HCV RNA assays still need to be developed for standardized results.

Since HCV viral load and specially genotyping helps predict outcome of interferon therapy, the Brazilian National Health Program has included in their legislation (MS 639/2000) sponsorship of HCV genotyping. This test, which is presently restricted to research laboratories, should be included in central public laboratories throughout Brazil to indicate appropriate therapeutic regimen among those infected with HCV.
Table 1: Comparisons between anti-HCV +/- and HCV RNA +/- patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>anti-HCV</th>
<th>anti-HCV</th>
<th>HCV RNA</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Number of patients</td>
<td>137</td>
<td>78</td>
<td>59</td>
<td>45</td>
<td>14</td>
</tr>
<tr>
<td>Age: mean ± sd</td>
<td>49 ± 14</td>
<td>45± 15</td>
<td>55 ± 12</td>
<td>56 ± 12</td>
<td>52 ± 12</td>
</tr>
<tr>
<td>Gender: (M/F)</td>
<td>95/41</td>
<td>53/25</td>
<td>41/18</td>
<td>29/16</td>
<td>122</td>
</tr>
<tr>
<td>Blood transfusion:n (%)</td>
<td>77 (56)</td>
<td>35(45)</td>
<td>42(71)</td>
<td>33 (73)</td>
<td>9 (64)</td>
</tr>
<tr>
<td>Alcoholism: n (%)</td>
<td>42 (30)</td>
<td>22(28)</td>
<td>15 (26)</td>
<td>12(32)</td>
<td>5 (36)</td>
</tr>
<tr>
<td>ALT (IU/L): median (range)</td>
<td>55(12 - 450)</td>
<td>46(12 - 272)</td>
<td>60(14 - 450)</td>
<td>64(19 - 450)</td>
<td>48(14 - 230)</td>
</tr>
</tbody>
</table>

a. Anti-HCV negative versus anti-HCV positive;
b. HCV RNA positive versus HCV RNA negative;
NS: p values >0.05.
Table 2: Clinical status and HCV viral load among the different genotypes:

<table>
<thead>
<tr>
<th></th>
<th>TOTAL HCV RNA +</th>
<th>GENOTYPES</th>
<th>UNTYPED</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of patients</td>
<td>46</td>
<td>1a</td>
<td>1b</td>
</tr>
<tr>
<td></td>
<td>Age: mean ± sd</td>
<td>55 ± 12</td>
<td>58 ± 11</td>
<td>54 ± 12</td>
</tr>
<tr>
<td></td>
<td>Gender (M/F)</td>
<td>30/16</td>
<td>14/8</td>
<td>13/5</td>
</tr>
<tr>
<td></td>
<td>Blood transfusion: n (%)</td>
<td>33 (72)</td>
<td>17 (77%)</td>
<td>12 (67%)</td>
</tr>
<tr>
<td></td>
<td>Alcoholism : n (%)</td>
<td>11 (24)</td>
<td>5 (23%)</td>
<td>3 (17%)</td>
</tr>
<tr>
<td></td>
<td>Anti-HBc: n (%)</td>
<td>11 (24)</td>
<td>9 (41%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td></td>
<td>HBsAg (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ALT (IU/L) median (range)</td>
<td>63 (19-450)</td>
<td>66 (34-450)</td>
<td>54 (19-336)</td>
</tr>
<tr>
<td></td>
<td>Clinical status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chronic liver disease (no cirrhosis)</td>
<td>25</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>19</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular carcinoma</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>HCV RNA (copies/ml)</td>
<td>91,685</td>
<td>88,385</td>
<td>167,096</td>
</tr>
<tr>
<td></td>
<td>median (range)</td>
<td>(393 - 1,005,556)</td>
<td>(393 - 1,005,556)</td>
<td>(1,424 - 1,000,405)</td>
</tr>
</tbody>
</table>

NS: p values >0.05.
Figure 1: HCV RNA titer distribution among the different genotypes
REFERENCES


19. SMITH, D. B.; DAVIDSON, F.; YAP, P-L.; et al. 1996. Levels of
hepatitis C virus in blood donors infected with different viral genotypes. *Journal of Infectious Diseases* 173: 727-730.


ACKNOWLEDGMENTS

We are indebted to Cleber F. Gineuno for his assistance in sample withdrawing and handling, Anthony V. Carella, Maria G P. Ginebreda and Marcia L. Baptista for laboratory assistance, Dr. Pedro H. Cabello for statistical orientation, Dr. Christian M. Niel and Dr. Ana M. C. Gaspar for their helpful discussions.