Sequence of non-structural regions 3 and 5 of hepatitis C virus genomes from Spanish patients: existence of a predominant variant related to type 1b

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Several hepatitis C viruses (HCV) have been described. In this study, the prevalence of HCV subtypes 1a, 1b, 2a and 2b has been studied by means of specific PCR in 93 serum samples of Spanish patients. Among these, the HCV-1b subtype was the most frequently detected (62%). Complementary DNA fragments from non-structural region 3 (NS3) and 5 (NS5), obtained from serum samples of three Spanish patients, were amplified by PCR and the products were cloned and sequenced. Comparison of the sequence obtained with those previously published shows the highest homology (91·7% in NS3 and 91·8% in NS5) with the HCV-1b subtype. The incidence of the local variant was analysed among the HCV-1b-infected patients. In order to distinguish between the local and HCV-1b prototype subtype, a new specific PCR assay was designed using primers from NS5. In the majority of the 76 HCV-1b-infected patients, the local variant was the only subtype detected (53%). These findings support the existence of a local variant, belonging to the HCV-1b subtype.

Since the identification of hepatitis C virus (HCV) as the major causative agent of post-transfusion non-A, non-B hepatitis (Kuo et al., 1989; Miyamura et al., 1990), several complete HCV sequences have been published (Kato et al., 1990; Choo et al., 1991; Ogata et al., 1991; Takamizawa et al., 1991; Okamoto et al., 1991, 1992a; Chen et al., 1992; Tanaka et al., 1991). Analysis of these sequences, as well as of many partial sequences, demonstrates that there are at least six types of HCV (types 1, 2, 3, 4, 5 and 6) according to Simmonds et al. (1993). The sequence variability among types may reach 40% (Chan et al., 1992). Each type also has several subtypes (1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 4a, 5a and 6a) and, among these subtypes, several different isolates have been described (Chen et al., 1991; Chayama et al., 1993; Müller et al., 1993).

There is also evidence which indicates that HCV types may have different pathogenicity (Pozzato et al., 1991; Takada et al., 1992a; Yoshioka et al., 1992), as well as distinct geographical distributions (Cha et al., 1992; Takada et al., 1992b). Previous studies show that HCV subtypes 1a and 1b seem to be prevalent in European countries (Li et al., 1991; Müller et al., 1993).

In this study, we have analysed the prevalent HCV genotypes in Spanish patients. We have also cloned and sequenced fragments of the non-structural regions 3 (NS3) and 5 (NS5) of different HCV isolates in our patients and analysed their prevalence by means of specific PCR.

HCV genotypes were analysed in 93 HCV antibody-positive patients with a histologically proven chronic hepatitis (77 chronic active hepatitis, five chronic persistent hepatitis and 11 liver cirrhosis) and abnormal alanine aminotransferase (ALT) levels (202±135 IU/l), from different parts of Spain. All patients were serum HCV RNA-positive by nested PCR, using primers from the highly conserved 5' non-coding region (Bukh et al., 1992). The possible source of HCV infection was blood transfusion in 32 patients, accidental parenteral exposure in 15, intravenous drug abuse in 19, and in 27 there was no reference to epidemiological antecedent. None of the patients had received antiviral therapy before analysis of the HCV types.

Typing of HCV sequences was done by reverse transcription and nested PCR, using primers from the core region of the HCV genome as described (Okamoto et al., 1992b). To avoid false positive results in the PCR reactions, the contamination prevention protocols of Kwok & Higuchi (1989) were followed in all PCR

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The nucleotide consensus sequence data reported in this paper have been deposited with GenBank and assigned the accession number M87630 (HCV-S5) and M87631 (HCV-S3).
Table 1. Oligonucleotide primers for HCV detection

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'-3')</th>
<th>Origin of the sequence</th>
<th>Nucleotide position* (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS5 region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1 sense</td>
<td>ATGGGGCAAGGACGTCCG</td>
<td>consensus</td>
<td>7897–7915</td>
</tr>
<tr>
<td>A2 antisense</td>
<td>TACCTAGCTACATGCTCCG</td>
<td>consensus</td>
<td>729</td>
</tr>
<tr>
<td>B1 sense</td>
<td>GACACACCATCATGGGACAATT</td>
<td>HCV-BK</td>
<td>7992–8015</td>
</tr>
<tr>
<td>B2 antisense</td>
<td>TGAAGGACCATGGGG</td>
<td>HCV-BK</td>
<td>8228–8243</td>
</tr>
<tr>
<td>B3 sense</td>
<td>AATACCCACATGGGCAAAGA</td>
<td>HCV-S5</td>
<td>7992–8015</td>
</tr>
<tr>
<td>B4 antisense</td>
<td>CGAGAAGCCCATAGGA</td>
<td>HCV-S5</td>
<td>8228–8243</td>
</tr>
<tr>
<td>NS3 region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1 sense</td>
<td>GGCTATACGGGACCTTTGA</td>
<td>HCV-BK</td>
<td>4658–4678</td>
</tr>
<tr>
<td>C2 antisense</td>
<td>AGCTGATTACAAAGCCACACAGGC</td>
<td>HCV-BK</td>
<td>4901–4921</td>
</tr>
</tbody>
</table>

* Nucleotide positions of primers are based on those of Takamizawa et al. (1991).

reactions. As negative controls, three serum samples from healthy controls were included in each PCR assay. All serum samples were tested in duplicate and analysed blind.

To investigate further the sequence of the HCV infecting our patients, a single PCR in the NS5 region was performed in serum samples from six patients infected with the 1b HCV subtype, determined as described above. This single PCR was carried out using conserved primers A1 and A2 (Table 1). The 40 cycles of PCR consisted of denaturation at 94 °C for 36 s, primer annealing at 59 °C for 42 s and extension at 68 °C for 3 min. Three patients gave positive results. These three PCR products (729 bp in length) were directly cloned using the TA Cloning Kit (Invitrogen). Five independent clones from each PCR product were isolated and sequenced by the dideoxynucleotide chain termination reaction (Sequenase; USB).

Additional HCV sequencing studies were also carried in the three NS5-positive serum samples, by analysing a NS3 fragment obtained by PCR (Table 1), performed as described (Castillo et al., 1992). A PCR product from each patient was cloned and five clones from each of them were sequenced (180 bp in length) as outlined above.

NS3 and NS5 regions were chosen for sequencing analysis because the 5' non-coding and core regions seem to be quite conserved among the different HCV types, and the putative envelope regions are highly variable, even among the same HCV subtype (Ogata et al., 1991; Tanaka et al., 1992).

HCV typing with core primers showed that the majority of the patients included in this study (58/93; 62%) were infected with HCV-1b; two (2%) were positive for HCV-1a and one patient was infected by HCV-2a. Seventeen of the 93 patients (18%) had mixed infection with HCV-1a and -1b, while another patient presented simultaneously the HCV-1b and -2a subtypes. The other 14 patients (15%) could not be typed with this method.

The analysis of the nucleotide sequences of the 15 clones derived from the NS5 region revealed among them a nucleotide homology between 96–99%. A consensus sequence of the 15 clones was obtained (HCV-S5) and compared with the published HCV sequences (Simmonds et al., 1993).

The local HCV isolate and the HCV-1b subtype had the highest nucleotide and deduced amino acid sequence homology (Table 2 and Fig. 1). In contrast, the lowest nucleotide sequence homology was found among the local HCV isolate and the HCV subtypes 3a, 3b, 4a, 5a and 6a. A more detailed analysis of the nucleotide sequences between the HCV-1b prototype and the HCV-S5 isolate revealed that, among the 56 nucleotide changes observed, 53 (95%) were transitions and 3 (5%) were transversions. Most of the changes observed in the deduced amino acid sequence were conservative. Taking
into account these conservative changes, amino acid homology reached 99.9%.

Similar results were obtained when analysing and comparing the nucleotide and deduced amino acid sequences from the local NS3 region (HCV-S3) with the NS3 sequences available (la, 1b, 2a, 2b). The highest homology was found between the HCV-S3 and the HCV-1b prototype, reaching 91.7%, while the lowest was obtained when comparing the HCV-2b and the Spanish isolate (Table 2). The same results were observed when the homology at the amino acid level was analysed. Moreover, in this region, all the amino acid substitutions were conservative.

Among the 76 patients infected by the HCV-1b subtype (including those patients who were simultaneously infected by the HCV-1a subtype), a comparison was performed to determine the incidence of the HCV-1b prototype and the HCV-S5. To achieve this, a new PCR assay was designed. The first PCR products from the NS5 region obtained with the conserved pair of primers A1 and A2 (Table 1) were subjected to a specific nested PCR. This PCR was performed in two independent reactions, using two sets of primers. Specific primers for HCV-1b detection (primers B1 and B2) were designed according to the HCV-1b prototype (Takamizawa et al., 1991). Detection of the local isolate was carried out using specific primers (B3 and B4) designed according to the local HCV sequence, HCV-S5. Both sets of primers (Table 1) were chosen at the same nucleotide position (7992–8015, 8228–8243) and presented 83% and 75% homology between sense and antisense primers, respectively. Forty cycles were

Fig. 1. (left) Comparison of the deduced amino acid sequence of the NS5 region from all the HCV subtypes. Common cysteine residues are boxed. Residues marked with an asterisk are found in all the RNA-dependent RNA polymerases of flaviviruses.

Fig. 2. Analysis of PCR products (252 bp) on an ethidium bromide-stained agarose gel (a) and after Southern blot hybridization (b). M, markers, lane M (1 kb DNA ladder; Gibco BRL); lane 1, negative control; 10 pg of a plasmid containing the Spanish-derived 729 bp NS5 fragment amplified with primers B1 and B2 (lane 2) or with primers B3 and B4 (lane 3).
performed, using different annealing temperatures (55 °C for B1–B2 and 50 °C for B3–B4). To assure the specificity of the designed primers, 10 pg of a plasmid containing the 729 bp fragment of the Spanish HCV NS5 region were amplified. PCR products were analysed by agarose gel electrophoresis and Southern blot hybridization using a 32P-labelled oligonucleotide of conserved sequence between the HCV-1b prototype and the Spanish isolate (5' GTGATGGGCTCCTCATACGG 3'). As shown in Fig. 2, a positive signal was only found using primers B3 and B4. As controls, two samples positive only for HCV-la and one for HCV-2a were also studied. None of these samples was positive in the PCR reaction.

The HCV-S5 was the sequence detected in the majority of the patients (40/76; 53%), while HCV was only amplified with primers derived from the HCV-la prototype in six cases (8%). Finally, in 30 patients (39%), HCV sequences were detected simultaneously by HCV-S5 and HCV-1b prototype sequence-derived primers. The presence of HCV-S5-related sequences was confirmed by direct sequencing of selected PCR products (Fig. 3).

No differences in either the epidemiology or the ALT levels were found among the patients infected by the different HCV types or subtypes, including those infected with our isolate (data not shown).

The results of this study have demonstrated that HCV-1b is prevalent in our patients. These results are in agreement with previous reports, which discovered that HCV-1b is the prevalent strain in southern Europe (Takada et al., 1992b; Marin et al., 1993). However, we found a high proportion of patients (18%) simultaneously infected by HCV-la and -1b subtypes. This percentage is higher than that reported for Japan and the northern European countries (Okamoto et al., 1992b), but similar to that found in southern Europe (Pozzato et al., 1993).

The existence of 14 patients that could not be typed with the core primers, although they were HCV RNA-positive using primers from the 5' non-coding region, suggests that they are infected with other HCV genotypes different from those tested. This possibility is currently under investigation.

It has been suggested that HCV genotypes have different pathogenicities (Pozzato et al., 1991; Takada et al., 1992a; Yoshioka et al., 1992). However, in this study we have not found differences in the ALT levels among patients infected by the different HCV types. This discrepancy may be explained by the small number of patients infected by only the HCV-la or HCV-2a subtype, although other causes, such as racial or epidemiological differences between our patients and those analysed in other studies, cannot be excluded.

The analysis of the nucleotide sequences from the NS3 and NS5 regions of the Spanish HCV isolate showed the highest homology with the HCV-1 type, both at the nucleotide and at the amino acid level. Among the different HCV-1 type sequences, the HCV-1b subtype

![Fig. 3. (a) Autoradiograms of the HCV-S5-related sequences HCS-17 and HCS-36, obtained from two different patients after amplification of the NS5 region using primers B3 and B4. (b) Comparison of HCV-BK (Takamizawa et al., 1991), HCV-S5 (local consensus sequence), HCS-17 and HCS-36 nucleotide sequences. Specific nucleotide changes conserved among HCV-S5, HCS-17 and HCS-36 are boxed.](image-url)
showed the highest homology with the Spanish HCV isolates. This finding indicates that the local HCV isolate belongs to the HCV-1b subtype.

Furthermore, most of the amino acid substitutions in our HCV isolate, with respect to the HCV-1b subtype, were conservative, especially in the NS3 region. Whether or not the greater conservation of the NS3 gene product as compared to the NS5 has any importance for the pathogenesis of the virus should be determined by future studies.

In the NS5 region product, most of the cysteine residues are conserved among the different HCV isolates. This observation might imply an important role of these residues in the maintenance of the tertiary structure, and possibly in the activity of the NS5 protein, the putative viral polymerase.

An interesting observation is that, although the HCV-1b subtype is prevalent among our patients, the local isolate is the variant most frequently found in our HCV-infected population. Similar findings have been described in other countries (Chen et al., 1991; Li et al., 1991; Kremsdorf et al., 1991; Müller et al., 1993). All these results suggest that, irrespective of the HCV genotype prevalent in each country, there are local HCV variants prevalent in each geographical area. In this context, it would be necessary to search for HCV proteins which can induce protective antibodies, as well as consensus sequences of these proteins, not only among the different HCV subtypes, but also among the different HCV isolates in each subtype. These findings may be of relevance in the future development of a universal anti-HCV vaccine.

References


Short communication


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