HCV serotypes in Brazilian patients

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Objective: To investigate the prevalence of the different types of hepatitis C virus (HCV) in a population of chronic HCV carriers using the Murex HCV serotyping 1–6 assay.

Methods: All serum samples from these patients had a positive nested PCR HCV reaction. The sera were submitted to ELISA, modified, for the identification of antibodies against HCV serotypes 1, 2, 3, 4, 5 and 6 (Murex HCV serotyping 1–6 assay).

Results: The viral serotype was identified in 166 (75.8%) of the 219 patients, 108 (65.11%) males and 58 (34.9%) females. Patient age ranged from 12 to 73 years, with a mean of 41.1 years. The form of acquisition of the disease most frequently reported was blood transfusion. The results showed a predominance of type 1 (70.0%), followed by type 3 (22.3%) and type 2 (4.2%).

Conclusion: Samples presenting low and very close optical density readings may lead to discrepant diagnoses concerning HCV serotypes and should be confirmed by genotyping. The serotyping can be useful in clinical practice and can be of help in establishing the prognosis of the disease, also favoring epidemiologic studies independently of the technology required for genotyping tests.

Key words: hepatitis C, HCV serotype, Brazil


INTRODUCTION

The agent responsible for most cases of parenterally transmitted hepatitis non A non B (HANAB), i.e. hepatitis C virus (HCV), was discovered by Choo et al in 1989.1 HCV is considered to be a flavivirus with an envelope and a diameter of 30–38 nm. The virus has a single-stranded RNA genome, with positive polarity and approximately 10,000 nucleotides, which presents the 3'−NC and 5'−NC regions (non-coding) at its extremities. A long open reading frame containing almost the entire genome codes for a polyprotein of approximately 3010−3011 amino acids. These amino acids are subdivided into nine proteins in the following order: nucleocapsid or core (C) and two viral envelope glycoproteins, E1 and E2/NS1, which contain the structural domain of the genome, and NS2, NS3, NS4a, NS4b, NS5a and NS5b, which are nonstructural domains.2

Several studies have recently reported that HCV exhibits considerable heterogeneity in its molecular structure, probably as a result of small replication errors and of spontaneous mutations occurring in the genome.3−4 Analysis of the nucleic acid sequences of HCV isolated in different parts of the world has demonstrated up to 30% diversity and, on the basis of this variability, the following four categories have been established: types, subtypes, isolates, and quasi-species. A unified system of HCV genotype classification has been proposed in order to standardize the various nomenclatures described in different research centers in the world. The sequences of the NS5 region of HCV have been compared in variants from different parts of the world, and it has been established that a similarity of less than 72% with some known genomic sequence of HCV demonstrates the existence of a new viral type. Among those considered to belong to the same subgroup (subtype), the degree of similarity ranges from 75% to 86% (mean: 80%). Thus, six types (1, 2, 3, 4, 5, 6) presenting 2−3 subtypes (a, b, c) are currently considered for some of these genotypes. New HCV variants have been reported in Vietnam, Thailand, Burma and Indonesia, and were first classified as genotypes 7, 8, 9, 10 and 11. However, complementary analyses have suggested that types 7, 8, 9 and 11 should be classified within genotype 6a, and type 10 as a subtype of type 3a.4−6

The clinical-pathologic importance of each genotype is currently the subject of intense investigation. Several studies have discussed the different types of prognosis for hepatitis C and the response to treatment with interferon-alpha, theoretically related to the different types of HCV.7,8 Different methods are available for the identification and differentiation of genotypes, each with
advantages and disadvantages. Several aspects should be taken into account, such as specificity and sensitivity, cost, handling time, and facilities available. In addition to genotyping techniques, a new alternative for the identification of HCV types is serotyping, i.e. the identification of type-specific antibodies against the NS4 regions and also of the HCV core, without the need for the presence of the viral particle or for molecular biology techniques.9,10

There are some important geographic variations in the prevalence of genotypes. Types 1a, 1b, 2a, 2b and 3a are those most commonly found in Brazil, western Europe and the USA, whereas 1b, 2a and 2b are more frequently found in Japan and Taiwan. Type 3 is more prevalent in India, Bangladesh and other parts of Asia, type 4 in the Middle East, type 5 in South Africa, and type 6 in Hong Kong and Macao.3,5,11

Competitive ELISA (Murex HCV serotyping 1–6 assay), which uses synthetic peptides based on the NS4 region of the HCV genome, identifies the six HCV types described thus far. The great advantage of this serologic method over molecular biology methods is the ability to process a large number of samples on a single day, as well as the possibility of execution in routine laboratories not equipped with the facilities needed for molecular biology techniques.12

The objective of the present study was to investigate the prevalence of the different types of HCV in a population of chronic HCV carriers using the Murex HCV serotyping 1–6 assay.

MATERIALS AND METHODS

Patients

We evaluated 219 patients seen at the Hepatitis Outpatient Clinic of the University Hospital, University of São Paulo, from 1990 to 1997. All patients had a clinical and laboratory diagnosis (second-generation ELISA) and a positive PCR HCV for hepatitis C. Data concerning age, sex and mode of transmission were obtained from the medical records of the patients.

Serum samples

Blood was collected from the patients by vacuum venipuncture using a dry 10-mL tube. The serum was separated, centrifuged, aliquoted and stored at −20°C until the time of use. Some of the samples were used after thawing for PCR HCV.

Nested PCR

We used the external primers NCR2 and PTC1 and the internal primers PTC3 and NCR4. Results were considered to be positive for PCR HCV according to the proposed protocol.13–15

Serotyping analysis

Serotypes were determined by the Murex HCV serotyping 1–6 assay. This assay is used for the detection of type-specific antibodies against NS4-derived epitopes, presented as branched peptides in a microliter 96-well format. Serotyping was performed according to the manufacturer's instructions. The results were scored as specific serotype, multiple serotypes, no type-specific antibodies, or non-reactive, according to the interpretation criteria established.16

Statistical analysis

Since the samples with serotypes 1, 2 and 3 are independent and random, the three groups were compared for age by analysis of variance (F statistic). The presumed modes of HCV acquisition were compared among the three groups by the χ² test. It was not possible to compare certain variables, because the expected frequencies were lower than 1. In the final analysis, we considered a history of blood transfusion and hemophilia in the same group, as well as unknown forms of transmission and patients whose medical records did not contain the information (unknown).

The patients were divided into three groups for statistical analysis of epidemiologic data, i.e. serotype 1, serotype 2 and serotype 3 groups.

The level of significance was set at α=0.05 in all tests.

RESULTS

Among the 219 patients included in the study, HCV type was diagnosed by serotyping in 166, corresponding to 75.8% sensitivity for the Murex HCV serotyping 1–6 assay. The results showed a predominance of type 1 (70%), followed by type 3 (22.3%) and type 2 (4.2%). Infection with two or more types was detected in 3.6% of the samples (Table 1).

Of the 166 patients, 108 (65.1%) were male and 58 (34.9%) female. Patient age ranged from 12 to 73 years (mean: 41.1 years).

The serotype 1 group consisted of 74 males (60.7%) and 48 females (39.3%) ranging in age from 12 to 73 years (mean: 40.6 years). The serotype 2 group consisted
Table 2. Patient distribution by presumed form of HCV acquisition

<table>
<thead>
<tr>
<th>Mode of acquisition/antecedent</th>
<th>Total no. (%)</th>
<th>Type 1 no. (%)</th>
<th>Type 2 no. (%)</th>
<th>Type 3 no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfusion</td>
<td>52 (31.33)</td>
<td>38 (31.15)</td>
<td>3 (33.33)</td>
<td>12 (30.77)</td>
</tr>
<tr>
<td>Intravenous drug use</td>
<td>18 (10.84)</td>
<td>12 (9.84)</td>
<td>0 (0.00)</td>
<td>6 (15.38)</td>
</tr>
<tr>
<td>Hemophilia</td>
<td>1 (0.60)</td>
<td>1 (0.82)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Tattoo</td>
<td>8 (4.82)</td>
<td>5 (4.10)</td>
<td>1 (11.11)</td>
<td>2 (5.13)</td>
</tr>
<tr>
<td>Acupuncture</td>
<td>1 (0.60)</td>
<td>1 (0.82)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Sexual</td>
<td>6 (3.61)</td>
<td>4 (3.28)</td>
<td>1 (11.11)</td>
<td>1 (2.56)</td>
</tr>
<tr>
<td>Health professional</td>
<td>2 (1.20)</td>
<td>1 (0.82)</td>
<td>0 (0.00)</td>
<td>1 (2.56)</td>
</tr>
<tr>
<td>Needle accident</td>
<td>3 (1.81)</td>
<td>3 (2.46)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (36.75)</td>
<td>45 (36.89)</td>
<td>2 (22.22)</td>
<td>16 (41.03)</td>
</tr>
<tr>
<td>Not found in the medical record</td>
<td>14 (8.43)</td>
<td>12 (9.84)</td>
<td>2 (22.22)</td>
<td>1 (2.56)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>166 (100)</strong></td>
<td><strong>122 (100)</strong></td>
<td><strong>9 (100)</strong></td>
<td><strong>39 (100)</strong></td>
</tr>
</tbody>
</table>

Some patients reported more than one antecedent.

Table 3. Serotypes detected and respective sequencing

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1b</td>
</tr>
<tr>
<td>1, 3</td>
<td>1b</td>
</tr>
<tr>
<td>2</td>
<td>1b</td>
</tr>
<tr>
<td>2 and 2.5</td>
<td>2b</td>
</tr>
<tr>
<td>3</td>
<td>3a</td>
</tr>
<tr>
<td>3 and 6</td>
<td>3a</td>
</tr>
<tr>
<td>6</td>
<td>1b</td>
</tr>
</tbody>
</table>

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DISCUSSION

Mapping of the epitopes of protein NS4 of the HCV genome revealed two important antigenic regions (1691–1708 and 1710–1728) which proved to be highly variable among genotypes 1, 2, 3, 4, 5 and 6 and whose type-specific antibodies could be detected. Type-specific peptides corresponding to these two regions were synthesized and later used for indirect ELISA, in which a complete peptide line for the six HCV types was projected and included in the assay (a total of 21 peptides).12

The sensitivity projected in our experiment may perhaps have been impaired by the fact that the reaction steps were not fully automated, with a consequent increase in total reaction time and probably impaired quality of the washings. The immunologic competence of the patients studied should also be considered, since we did not exclude those who were being treated with interferon-alpha or any other type of medication, or even those with associated diseases.

Comparative data reported in the literature for genotyping and serotyping suggest that the sensitivity of serotyping methods is significantly lower than that of molecular biology techniques. The sensitivity of serotyping tests can also be limited by the immunocompetence of the host.i6

The results obtained revealed a predominance of type 1, in agreement with the literature surveyed. It should be emphasized the studies citing Brazilian samples used genotyping techniques, showing that the results of this new method for the diagnosis of HCV types agree with the results reported here.3,18

The epidemiologic parameters evaluated were age, sex and mode of HCV transmission. For six patients who mentioned probable sexual transmission, no other antecedents that would explain acquisition of the disease were found, except for the sexual partner, who presented the same HCV serotype. No significant differences between types 1, 2 and 3 of HCV were detected with respect to these parameters. In the USA, Mahaney et al19 and Watson et al20 reported no significant differ-
ences between HCV genotypes and patient age or sex distribution in their studies.

In the present study, in relation to the samples for which it was not possible to determine the HCV serotype, there were frequently extremely low optical density (OD) readings for all HCV types tested, including the controls. Thus, we may assume that the volume of anti-NS4 or HCV type-specific antibodies may have been very low during that phase of infection, impairing the performance of this serologic test.

Van Doorn et al reported that the immunocompetence of the infected host can also interfere with the sensitivity of the assay.

The mixed infection results may be explained by the serologic detection of previous infections with different HCV types. However, the presence of cross-reactions should not be ruled out and would probably explain most of these results, and therefore these diagnoses, especially when OD readings showed values >2.5 in control wells or when the values of negative reference wells persisted at >4.0. This may represent the presence of excess antibodies in the sample and consequently positive results for more than one HCV type.

Serotype 6 was not confirmed for any of the samples tested. Type 6a appears to be one of the types most confined to a determined geographic region and is found only in the Hong Kong, Macao and Vietnam regions.

Serotype 5 was confirmed for only one patient. Serotype 4 was present in two patients. The possibility of cross-reaction could be considered; also, the origin of this patient and his form of HCV acquisition should be investigated.

Campiott et al genotyped 705 Brazilian samples and detected a 0.9% prevalence of types 4 and 5. All of them were located in the State of São Paulo.

We had the opportunity of sequencing seven samples, which were selected in order to confirm serotypes 1, 2 and 3, to clarify some mixed infections, and to investigate a serotype 6 sample. Thus, the high percentage of discordant results was expected. The sample of serotype 2, retested three times, presented results for the 1b genotype during sequencing. For the serotyping tests, the OD readings were very low, although they fulfilled the requirements of the test, which were followed rigorously. Perhaps these OD values indicate a small volume of type-specific antibodies and the possibility of cross-reactions or nonspecific reactions.

Serotyping was based on epitopes of the NS4 region and on the sequencing of the 5'NC region of the HCV genome. The hypothesis of discordance as a function of the different HCV regions explored should also be considered.

The discussion about the sensitivity of the Murex test is not really relevant, as this test is not an HCV diagnostic test. This is a subtyping test applied to samples already shown by other more sensitive tests to be infected with HCV, so it is not expected to have the levels of sensitivity that PCR or second/third-generation ELISA tests have to diagnose HCV infection.

Despite the limitations pointed out in the present report and in view of the results obtained, which were compatible with those of other studies based on molecular biology for the investigation of HCV types, we conclude that serotyping can be useful in clinical practice and can be of help in establishing the prognosis of the disease, also favoring epidemiologic studies of regions or countries independently of the technology required for genotyping tests.

REFERENCES