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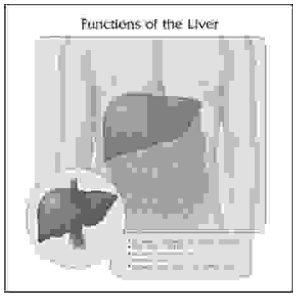


HCV INFECTED PATIENTS;
ASSESSMENT OF MAJOR GENOTYPES AND SUBTYPES OF HEPATITIS C VIRUS

DR. SAJJAD IQBAL
Shalamar Hospital
Shalimar Link Road Lahore-15

DR. RASHID AHMED

DR. MUHAMMAD HAROON YOUSAF

Dr. Asim Mumtaz

Dr. Dawood Amin

Dr. Ghulam Rasool

Dr. Azmat Manzoor

ABSTRACT... sialbrothers@yahoo.com **Objectives:** To assess the prevalence of different hepatitis C virus (HCV) major genotypes and subtypes in HCV infected patients. **Design:** A cohort prospective study. **Place and Duration:** At Shalamar Hospital Lahore, From Dec 2002 to Dec 2005. **Patients and methods:** 1652 patients, 697 females and 955 males infected with hepatitis C virus confirmed by ELISA were included in the present study for the analysis of HCV genotypes. Infection was reconfirmed by HCV RNA detection with qualitative PCR. Gentyping was done with multiplex PCR using type specific primers. **Results:** Among 1652 genotyping was done with multiplex 3 was seen most common 1220 (73.85%). Second and third common genotypes were 2 and 1 , 154 (9.33%) and 80 (4.84%) respectively followed by genotype 4 which was 41 (2.84%). In this study 106 (6.42%) cases remained unclassified and mentioned as untypable. In 51(3.09%) cases more than one genotype was seen which reported as mixed genotypes. **Conclusion:** Patients with chronic liver disease due to hepatitis C virus infection in this part of Pakistan had predominantly HCV genotype 3 but the presence of genotype 1 and 4 could not be excluded. So before therapy genotyping must be taken under consideration.

INTRODUCTION

The term genotype refers to different genetic variation of hepatitis C virus (HCV). HCV demonstrates a high degree of sequence variation, which provides the basis for grouping the virus into six major genotypes numbered 1 to 6 and multiple substypes mentioned as 1a, 1b and so on[1,2]. These grouping have served as simportant tool for studying the geographic distribution of HCV genotypes, their routes of transmission and their association with particular risk groups. Associations between viral genotype, interferon responsiveness, progression of disease and the likelihood of developing hepatocellular carcinoma have been demonstrated[3,4].

Hepatitis C virus (HCV) is a single stranded RNA virus whose genomic structure resembles that of the flaviviruses[5]. The viral genome has one large open reading frame, with three regions that appear to encode structural proteins at the 59 end (the core, E1 and E2/NS1 regions). These are followed by four regions that

probably encode nonstructural proteins at the 39 end (the NS2, NS3, NS4 and NS5 regions). Since the cloning of the HCV genome, markedly divergent sequences have been found among distinct isolates, suggesting the existence of HCV genotypes[6,7,8]. In the currently proposed nomenclature for HCV genotyping[1]. HCV can be classified into six major genotypes on the basis of extensive sequence comparisons of the HCV core, E1 and NS5 regions.

At least 6 different major genotypes or varieties of HCV have been identified as well as about 70 subcategories. The major genotypes 1,2 and 3 are found in most countries, while the other three (4,5 and 6) are found mainly in certain geographic areas. Genotypes 1 (1a , 1b) and 2 (2a, 2b) are predominance in USA and Western Europe with lower percentage of genotype 3 (3a, 3b). Genotype 4 is commonly found in Africa and genotype 5a in South Africa. Type 1b predominates with types 2,3 and 6 also represented through out the rest of the world[9].

HCV genotype has emerged as an important tool because of its important role factor in predicting the response to interferon therapy and determining the duration of antiviral therapy, such as interferon in combination with ribavirin, with genotype 1 infections having less favorable response rates and requiring longer treatment. Patients infected with HCV of genotypes 2 and 3 show better responses to interferon therapy than those infected with genotypes 1 and 4[10,11]. HCV genotyping thus is firmly established as part of the pretreatment evaluation of patients with chronic infections as proposed by the European Association of Study of Liver consensus conference in 1999[12] and the NIH consensus conference in 2002[13].

The following study was arranged to seek out the existence of different HCV genotypoes in our area because of its importance in regional distribution, clinical manifestation response to treatment, treatment duration and prognosis of HCV infection.

MATERIAL AND METHODS

Patients

1652 HCV infected patients, 697 females and 955 males with positive anti HCV confirmed by ELISA were included in the present study. Before genostyping the HCV RNA detection was compulsory to reconfirm the current HCV infection. The patient participated in the present study were from different areas of the Punjab. All the patient information including age, sex, address, contact number, previous HCV infection related investigations and family history was registered. All the samples were collected and saved at requiring temperature in study area to avoid cross contamination and degradation of HCV RNA due to improper temperature.

Methods

HCV RNA was extracted from 200µl of serum, using the protocol of gentra kit (PURESCRIPT ® , Minneapolis and MN 55441 USA. The extracted RNA was rehydrated in 50µl hydrated solution. To confirm the current infection HCV RNA detection was performed as follows. Reverse transcription (RT) was carried out to convert RNA to complementary DNA (cDNA) with 10µl of RNA using 1µM of downstream primer and 100U of Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) enzyme (Fermentas) in a final volume of 20µl. amplification of cDNA was done in two PCR rounds using nested PCR. In first round a big fragment of 375 bp from a selected region of HVC genome was amplified. In second round an inner portion of 250 bp from first round fragment was amplified using a nested pair of primers. The PCR product were submitted to electrophoresis using a 1.8% agarose gel in TBE buffer and visualized by ethidium bromide staining under ultraviolet light.

For HCV genotyping RNA was extracted according to the kit protocol of Gentra kit (PURESCRIPT ® , Minneapolis, MN 55441 USA) as mentioned above. 10µl of isolated RNA was converted to two rounds of PCR amplification. The first round of PCR was done with outer primers specific for core region of HCV. The second round of PCR was performed with one universal inner sense and 11 genotype-specific anti-sense primers in a multiplex PCR as described previously (Okamoto et al, 1993 and

Tomoyoshi et al, 1997)[14,15]. The amplified PCR products were electrophoresed on 2% agarose gel stained with ethedium bromide with 50 bp Marker and evaluated on UV light.

RESULTS

In order to better characterize Hepatitis C genotypic variants, which are important for defining more effective treatment options for chronic Hepatitis C virus (HCV) infection, 1652 patient results were analyzed at Shalamar Hospital laboratory, Lahore. This study was established to classify 6 major HCV genotypes (1,2,3,4,5and 6) together with 9 subtypes (1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a). In 1945(90.50%) out of 1652 HCV infected patients Hepatitis C Virus could be genetically classified into four major genotypes (1,2,3,4) or seven specified subtypes (1a, 1b, 2a, 2b, 3a, 3b and 4). In 106 (6.42%) patients Hepatitis C Virus (HCV) remained unclassified that was mentioned as untypable and in 51 (3.09%) patients more than one genotypes or subtypes were identified, which were reported as mixed genotypes. All the data was collected regarding to HCV subtypes (Table I & II).

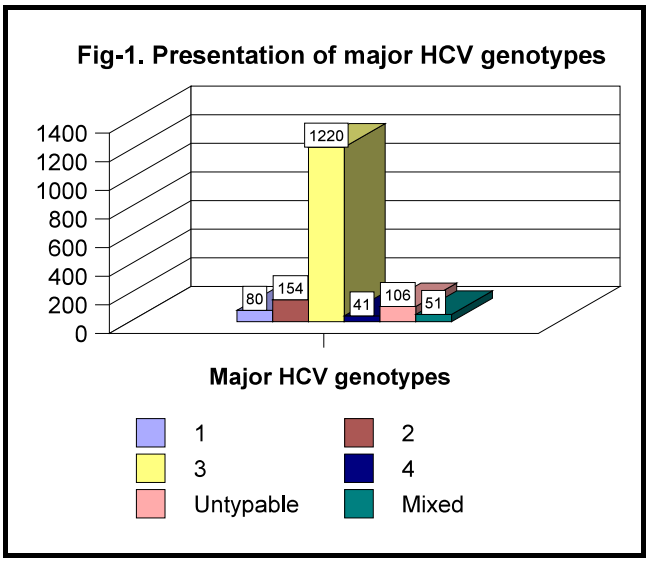
As demonstrate in (Table I) and (Fig 1) genotype 3 was seen prominent among the patients (1220 or 73.85%) in our findings. Second and third most common genotypes were 2 and 1, which were 154(9.33%) and 80(4.84%). Genotype 4 was seen less common 41(2.48%) but no case of genotype 5 and 6 was seen in the present study. Out of 1652 cases 106(2.48%) remained unclassified and in 51(3.09%) cases more than one genotype was seen. In subtypes 3a was most common 792(47.94%) followed by 3b, 428(25.91%) and 2a, 124(7.51%). Subtypes 1a, 1b, 2b and 4 were less common (2.78%, 2.06%, 1.81% and 2.48% respectively). Presence of interferon resistence genotypes (genotypes 1 and 4)was not so frequent as compared to sensitive genotypes (genotype 2 and 3). In mixed genotypes mostly the subtype 3a was seen associated with other subtypes. As mentioned in Table II most frequently subtype 3a was seen with 2a and 3b (29.41% and 23.52%). Subtype 1a was also seen combined with 3a and 3b (19.61% and 15.69%). Proportion of subtype 1b was less common in mixed genotypes, which was 11.77% showing combination only with subtype 3a. There was seen no combination of subtype 2b and 4 with any other subtype. It was also important to note here that trend of mixed genotypes was frequently seen in females as compared to males which was 58.82% in females and 41.18% in males (Table II).

Table-I. Distribution HCV Genotypes.

Genotype	Females	Males	Total	% Age
1a	16	30	46	2.78%
1b	11	23	34	2.06%
2a	77	47	124	7.51%
2b	11	19	30	1.81%
3a	220	572	792	47.94%
3b	250	178	428	25.91%
4	12	29	41	2.48%
5a	00	00	00	00%
6a	00	00	00	00%
Untypable	70	36	106	6.42%
Mixed	30	21	51	3.09%
Total	697 (42.19%)	955 (57.81%)	1652	100%

Table-II. Distribution Mixed HCV Genotypes.

Genotype	Females	Males	Total	% Age
3a & 2b	09	06	15	29.41%
3a & 3b	07	05	12	23.52%
1b & 3a	04	02	06	11.77%
1a & 3b	05	03	08	15.69%
1a & 3a	05	05	10	19.61%
Total	30 (58.82%)	21 (41.18%)	51	100%



DISCUSSION

Hepatitis C Virus (HCV) presents considerable nucleotide variations and has many genotypes. The different genotypes of HCV may possess some relationship with regional distribution, clinical manifestation, response to treatment and prognosis of HCV infection[16]. Thus to study the genotyping trend for HCV in our region was of practical value.

HCV is known to have marked genetic hetrogeneity, and it was estimated to have a nucleotide substitution rate of between $1.44x10^{-3}$and $1.92x10^{-3}$ substitution per site per year[16,17]. Accumulation of necleotide substitution in the HCV genome results in diversification and evolution into different genotypes. Presently, HCV can be classified into at least six major types and a series of subtypes[18]. There is an increasing evidence that patients infected with different HCV genotypes may have different clinical profiles, severity of liver disease, and response to alpha interferon therapy[14,19].

Six distinct but related HCV genotypes and multiple subtypes have been identified on the basis of molecular relatedness. In the United States and Western Europe Genotype 1 is most common, followed by genotypes 2 and 3. The other genotypes are virtually never found in these countries but are common in other areas, such as Egypt in the cases of genotype 4, South Africa in the case of genotype 5 and Southeast Asia in the case of genotype 6 (Poynard et al, 1998 and McHutchison et al, 1998)[20,21]. Knowledge of the genotype is important because it has a predictive value in terms of the response to antiviral therapy, better response is associated with genotypes 2 and 3 than with genotype 1. (Farchi et al, 1999)[2].

We found HCV genotypes in 1495 (90.50%) out of 1652 by PCR assay using type-specific primers. The present study was arranged with a view to study the distribution of HCV genotypes in Hepatitis C infected patients. There are some differences in distribution of HCV genotypes in different regions. In America, infection of HCV genotype 1 is predominated, but in China and Japan,, HCV genotype 2 is dominant over HCV genotype 3. Our study showed that the infection rate of HCV genotypes ? and 3 was high (9.33% and 73.85%) as compared to type 1 and 4 (4.84% and 2.48%), which is common in U.S.A and European countries (Georg et al., 2001)[9]. In previous studies done in Pakistan the same trend was noted (Shah et a!, 1997; Nousbaum, 1998; long et al, 1996 and Mehmood, 2001)[22,23,24,25].

As illustrated by the figure, the population of our study area was dominated by HCV genotype 3, (73.85%). The next most frequently observed genotype was 2 (9.33%) followed by genotypes 1 and 4 (4.84% and 2.48%). The prevalence of untypable and mixed genotypes was also consequential. The genotype distribution seen in our study is not different than the distribution seen in the last analysis in that both analyses demonstrated that the population of chronic HCV patients has a higher distribution of genotype 3 (Khokhar et al, 2002)[26]. The presence of HCV genotype 4 in our finding was unexpected because in Pakistan this genotype was not previously observed. This genotype is commonly found Middle East, North, Central and South Africa. (Poynard et al, 1998)[20].

Our findings revealed that the prevalence of genotype 3 in the population was relatively stable but the increasing trend of resistant genotypes 1 and 4 was very terrorizing. Therefore, future prevention and treatment strategy

should be directed towards type 2 and 3 mainly, but not neglecting type 1 and 4.

In our study, no HCV genotype 5 and 6 were found. Out of 1652 cases 106 (6.42%) were positive for HCV RNA by repeated qualitative PCR assay, but these could not be classified into any major genotype or subtype, indicating that there might be other HCV genotypes in our area, which need more study to evaluate new existing HCV genotypes in this region. The infection rate of mixed type was 3.09%, indicating repeated blood or blood product transfusion may be contributory.

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Wise men say nothing in dangerous times.

John Selden