

## LOW PREVALENCE OF HEPATITIS C VIRUS GENOTYPE -1b IN CHRONIC LIVER DISEASE PATIENTS IN INDIA

Thakur V, Guptan RC, Geeret\* M, Das BC\*\*, Sarin SK

Department of Gastroenterology G.B Pant Hospital New Delhi,

\* Innogenetics Belgium and

\*\* ICPO, Maulana Azad Medical College New Delhi, India.

(For Correspondence and reprint requests: - Dr. S. K. Sarin, Professor and Head, Department of Gastroenterology, G. B. Pant Hospital, New Delhi, India Phone:91-11-23232013; FAX:91-11-26468691 Email: [sk\\_sarin@nda.vsnl.net.in](mailto:sk_sarin@nda.vsnl.net.in))

### ABSTRACT

**Background:** The Hepatitis C Virus (HCV) genome shows significant heterogeneity due to a high rate of mutation; this has a potential bearing on the outcome of interferon therapy. Genotype-1b is known to be less responsive to interferon. We studied the spectrum of HCV genotypes in chronic liver disease (CLD) patients in India .

**Material and Methods:** HCV RNA was extracted from the serum of 44 randomly selected cases of HCV-related CLD, proven by liver biopsy, (mean age of patients  $40 \pm 15$  yr., cirrhotic: 32%) and RT-PCR was carried out. The amplicon of 240 bp (second nested PCR) was hybridized to the probes (type specific) coated on to a nitrocellulose membrane. Following this, streptavidin, labeled with alkaline phosphatase, was added to bind with biotinylated hybrid, which with chromogen type-specific band formation resulted. Patients were classified on the occurrence of one (Group I), two (Group II) or multiple genotypes (Group III).

**Results:** Genotypes 1 and 3 were the commonest genotypes, followed by type 2 and the rare genotype 4b in a lone patient. Genotype 1 was seen in 39% (1a 23%, and 1b 16%) while genotype 3 in 45% (3a 23% and 3b 7%) patients. Eighty percent (35 of 44) patients had the single genotype (Group I, mean age  $46 \pm 8$  yr.), 14% had two genotypes (Group II, mean age  $36 \pm 16$  yr. ) and the remaining (Group III, mean age  $22 \pm 9$  yr.) had multiple genotypes. Serum ALT levels in these three groups of patients were  $117 \pm 92$ ,  $85 \pm 45$  and  $49 \pm 7$  IU/L respectively.

### Conclusions:

1. Genotypes 1 and 3 are common in India, with subtype 1b not so common,
2. A unique genotype 4 b was detected in one patient ,
3. Indian patients have the possibility of good antiviral response to interferon therapy in chronic HCV infection.

### INTRODUCTION

Hepatitis C virus (HCV) is a major cause of post-transfusion and sporadic non A–non B hepatitis all over the world 1,2. A chronic indolent course often leading to end stage liver disease, is the hallmark of hepatitis C virus infection 3,4. Studies in India have revealed a seroprevalence of HCV in around 1.87% of the general population 5. Hepatitis C virus is a positive stranded RNA virus which consists of 5' and 3' non- coding regions (NCR) that flank a single open reading frame (ORF). The ORF encodes three structural proteins at the amino-terminal end and six non-structural (NS) proteins at the carboxyl-1 terminal end 6,7. Sequencing of different HCV isolates has revealed that 5' NCR core is a highly conserved region and envelope protein E1, E2, NS1 are the most variable 8. Phylogenetic analysis of the virus distinguishes viral genotypes and subtypes. The sequences of six major genotypes of HCV have been described, all differ from each other by 30% over the complete genome. Each genotype comprises of several more closely related (>20% variation) subtypes 9,10. In a series of studies<sup>11-13</sup> new genotypes 7,8,9,10 and 11 were described in 1996 14. It was recently shown that types 7,8,9 are subtypes of genotype 6. Similarly types 10 and 11 are now described as subtype of genotype 3 15.

HCV genotypes have distinct geographical distribution<sup>16</sup> and can alter the course of natural infection and outcome to antiviral regimes 17,18. HCV genotype 1b is specially implicated in accelerated progression of chronic liver disease and is described to be the least responsive to conventional antiviral regimes. Limited information is available about the HCV genotypes in the Indian subcontinent 19-21. We undertook this study in a tertiary care referral hospital for chronic liver disease in North India, to investigate the prevalence of various HCV genotypes and thereby to rationalize the therapeutic approach for chronic HCV infection.

## **MATERIALS AND METHODS**

Forty-four chronic HCV related liver disease patients diagnosed by liver biopsy were randomly selected by the sealed envelope technique. All the patients were seen at the outpatient department of the G.B. Pant Hospital, New Delhi. Detailed medical histories, including the risk factors for viral acquisitions, were taken. Investigations including biochemical, radiological, endoscopic and histological tests were done on each patient. An experienced pathologist carefully studied the liver biopsy for the evidence of chronic liver disease. After obtaining informed consent, blood was drawn for investigations.

### **Serology**

Serum samples were separated and stored in aliquots at –70° C for the various analyses. Repeated freezing – thawing was avoided. Third generation ELISA (UBI 4.0, Organon Teknika, The Netherlands) was used to establish the presence of hepatitis-C virus antibodies in the blood. ELISA-positive samples were subjected to more specific third-generation immunoblot assay (Liatek, Organon Teknika, The Netherlands). All the subjects were found negative for markers of hepatitis B: HBsAg by third generation polyclonal ELISA , total anti-HBc and anti-HBe (Organon Teknika, The Netherlands).

Commercially available kits were used to exclude autoimmune activity: anti-nuclear antibodies (ANA), anti-smooth muscle antibody (ASMA), anti-mitochondrial antibody (AMA) were tested for by immunofluorescence at a dilution of 1:80.

### **Molecular biology techniques:**

Specimens that were positive or indeterminate with HCV immunoblot assay were analyzed for the presence of HCV-RNA by RT PCR.

Extraction of RNA : RNA was isolated by using the standard guanidium isothiocyanate – phenol method<sup>21</sup>. Precisely 100 ml of patient serum was treated with 600 ml of lysis solution (4M GITC / 0.75M sodium citrate / 2% sarcosyl / 0.1M betamercaptoethanol). RNA was extracted with water-saturated phenol and 49:1 chloroform:isoamyl alcohol. RNA was precipitated with isopropanol that had been washed with 70% ethanol and dissolved in 30 ml diethylpyrocarbonate-treated deionized water.

Primers: Sequence from highly conserved 5' noncoding region (5'NCR) was used for amplification 23.

Outer sense (position base 295)

CTG TGA GGA ACT ACT GTC TT.

Outer antisense (position base10)

GTG CTC ATG GTG CAC GGT CTA CGA GAC CTC CGG.

Inner sense (nested base position 276)

TTC ACG CAG AAA GCG TCT

Inner antisense (nested base position, 26)

CAC TCG CAA GCA CCC TAT CAG GCA TGC.

Amplification of RNA : PCR was carried out in 50 ml reaction volume. The mixture contained 5 x AMV buffer, 25mM Mg SO<sub>4</sub>, 10 mM, NTP, 25 pmoles of each external primers 5 units, AMV reverse transcriptase (Promega) and 5 units Taq uDNA polymerase (Promega) . Reverse transcription was carried out at 42°C for 1 hour followed by hot start at 94°C for 4 minutes followed by 35 cycles at 94°C – 30 sec. 58°C -20 sec, 72°C- 20 sec, with final extension of 5 minutes at 72°C. 5 ml of the first PCR product was reamplified using 25 pmoles internal primer of 35 cycle at the same condition. Amplicon of 251 bp was separated on 3% agarose gel.

Genotyping of HCV: Genotyping was carried out using line probe assay (Inno-Lipa HCV II, Innogenetics, Belgium). In the first part RT PCR was carried out by using biotinylated oligonucleotide primers from the 5' untranslated region (5' U R' ) according to the manufacturer's instructions. Amplicon of 300 bp in the first PCR and of 240 bp was obtained after nested PCR.<sup>24</sup> The second part of the assay makes use of reverse hybridization principle. Biotinylated amplicon of RT PCR was hybridized with type specific probe according to the manufacturer's instructions. The genotype and subtype were defined descriptively based upon the 5'NCR sequence using type specific probes

### Statistical analysis

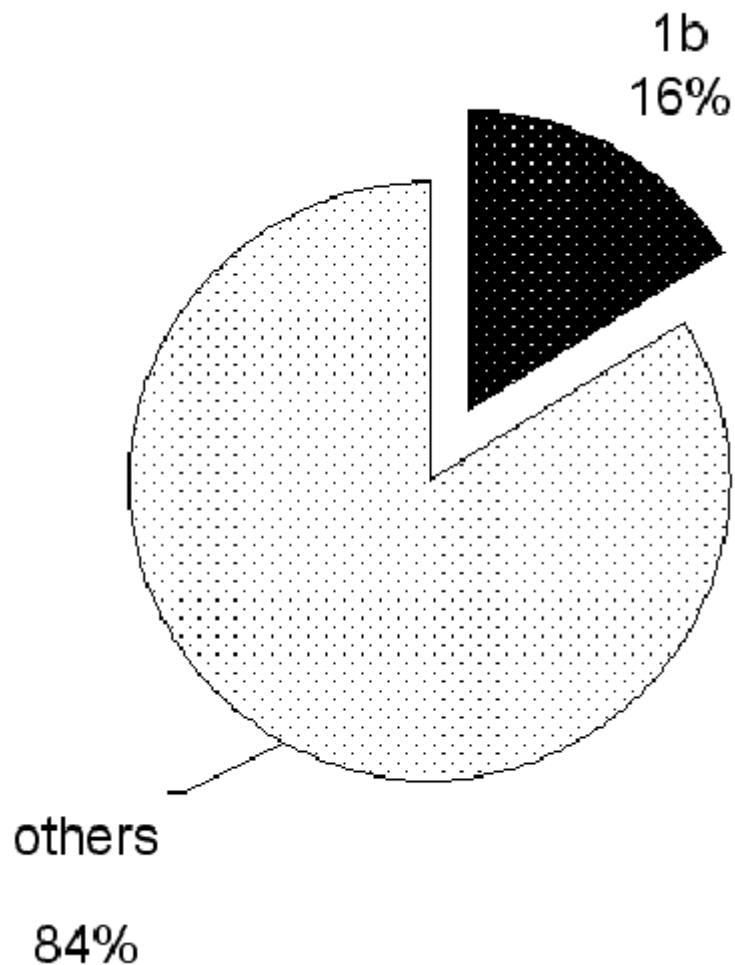
The data were expressed as mean  $\pm$  SD; the significance of continuous variables was determined by student's- t test. Chi-square test or Fisher's exact test was used to analyze the discontinuous variables.

### RESULTS

All the forty-four patients included in the study had histopathologically proven chronic liver disease due to HCV infection. Thirty four percent of the patients (15 of 44) were found to be cirrhotics. Number of male patients predominated in the study group and their mean age was 40 $\pm$ 15 years. Table- I shows the distribution and pattern of different genotypes. Genotype 1 (39%) and genotype 3 (30%) were the most prevalent genotypes seen in HCV related chronic liver disease patients. The subtype distribution in these genotypes was 1a (22.7%), 1b (16%), 3a (22.7%) and 3b (6.8%) respectively. Genotype 2 was present in 14% of the patients. An unusual genotype 4 and subtype b a new variant, was found in a single specimen (2%). Over all, genotype 1b alone was detected in only 16% of the samples (Fig. 1).

Genotype / subtype	Number	Percentage
1a	10	22.7
1b	7	16
2a	1	2.3
2b	3	6.8
3a	10	22.7
3b	3	6.8
4b	1	2.3
2a, 2b	1	2.3
2b, 3a	1	2.3
3a, 3b	5	11.4
2b,3a,3b	1	2.3
1b, 3a, 3b, 10a	1	2.3

**TABLE – I : Distribution and pattern of different genotypes/ subtypes of HCV in chronic liver disease patients (n = 44)**



**Fig 1 : DISTRIBUTION OF GENOTYPE 1b IN HCV RELATED CHRONIC LIVER DISEASE IN INDIA**

The different genotypes of HCV were correlated to the severity of chronic liver disease. There was no significant difference between the different genotypes in respect of the age of presentation with liver disease, mean serum ALT or mean serum albumin levels. However, about 59% of the patients infected with genotype 1 had advanced cirrhosis, which was significantly higher than patients infected by genotype 3 ( $p < 0.05$ ). The differences might also be higher as compared with other genotypes, but the subgroups were too small for comparison ( Table II).

Features	Genotype 1	Genotype 2	Genotype 3	Genotype4
N	17	6	20	1:0
Male : Female	12:5	5:1	12:8	1:0
Mean age (yr.)	49±17	39±16	40±17	37
Distribution	39%	14%	45%	2%
S.ALT (IU/L)	104±75	168±146	87±60	80
S. albumin(g/dl)	3.1±1.2	3.3±8	3.4±1.4	3.2
Histology				
Chronic hepatitis	7(41)	5 (83)	17 (85)	1(100)
Cirrhosis	10 (59)*	1 (17)	3 (15)*	

**TABLE – II : Demographic profile of different genotypes of HCV : \*  $p < 0.05$  Genotype 1 vs. 3, Figures in parenthesis denote percentages**

Patients were grouped into 3 categories based on the presence of one genotype (Group 1), two genotypes

(Group 2) or more than two genotypes in one patient (Group 3). About 80% of the patients were found to be infected with only one genotype of HCV, whereas about 16% of the patients had two genotypes of the virus present together (Table III). About 5% of the patients had multiple genotypes. Thus over all, about 21% of the patients had mixed variants. An attempt was made to compare the liver histology between single or mixed variants but the numbers were too small for comparison. There was however, a trend towards milder liver disease in patients with multiple virus genotype infection.

Features	Single (n = 35)	Dual (n = 7)	Multiple (n = 2)
Mean age (yr.)	46±8	36±16	22±9
Distribution	80%	16%	5%
ALT (IU/L)	117±92	85±45	45±7
S. albumin (gm/dl)	3.9±0.76	3.7±0.46	4.4±0.14
Histopathology			
Chronic hepatitis	22 (63)	6 (86)	1
Cirrhosis	13 (37)	1 (14)	1

**TABLE – III : Demographic profile of patients with single and mixed genotypes of HCV ( Figures in parenthesis denote percentages)**

## DISCUSSION

Patients chronically infected with hepatitis C virus showed genetic diversity. Line probe assay typing, based on 5' NCR showed that type 1 and type 3 were more prevalent than genotype 2. This is consistent with the known geographical distribution and prevalence of HCV genotypes. However genotype 1b, which is known to cause severe liver disease and has poor response to interferon therapy 25,17,18,26 was found in only a small percentage (16%) of patients. There are only two earlier reports available on the prevalence of HCV genotypes from the Indian subcontinent 20,21. However, in these studies only a small number of patients were studied, probably due to the rather cumbersome technique of direct sequencing of amplification product of 5' NCR and core region. Type specific oligonucleotide amplification 27 and RFLP analysis 28 are the two other techniques used for studying genotypes with sensitivity comparable to direct sequencing method. The line probe assay as used in this study and the Restriction Fragment Length Polymorphism (RFLP) analysis use type specific primers and hence are less cumbersome and are practical for large number of clinical samples. In an American study by Mahaney et al.,<sup>29</sup> 99% correlation of RFLP and line probe assay was observed.

Our data of higher genotype 3 in chronic liver disease is in conformity with another series from North India,<sup>21</sup> where prevalence of genotype 1b was reported to be in 27% patients. On the other hand, a study from the South of India reported 77% prevalence of genotype 1b 20. It is not clear why such differences in the genotypes could occur between north and south. The probable explanation for this difference could be the geographical and racial background of people in north and south India. Isolates from southern India have sequences similar to Indonesia and Japan where Type 1 b is more prevalent. According to some studies, the genotype patterns are a result of endemic spread, which took place during the last 50 years in Europe, North America, Japan and Australia. The involved genotypes were predominantly 1a, 1b, 2a, 2b, and 3a 30,31. Such endemic spread appears to have taken place in the Indian subcontinent also with genotype 3 31. The mechanism of genotype 1b influencing the antiviral outcomes is not clear. Recent studies have shown that:

- mutations in the NS5a region, the interferon sensitivity determining region, could influence a favorable response,<sup>35</sup>
- HCV genotype 1b produces higher levels of HCV RNA as compared to other genotypes specially compared to genotype 2, which determines its unfavorable antiviral outcomes 36.
- Low prevalence of genotype 1b in the North Indian chronic HCV patients might be the reason for the good end of treatment response to interferon plus ribavirin combination observed in these patients 37.

In this study we also observed relatively higher prevalence of mixed genotypes, a rare phenomenon reported in only 5% cases in other series 29,32. In our study, mixed genotypes were observed in 21% of the specimens. It was interesting to note that patients with mixed genotypes had a younger age of presentation, a milder biochemical and histological disease. The exact significance of this observation needs to be determined in larger studies. Co-existence of different types of variants can be a result of multiple infection with different strains or spontaneous mutation of the HCV genome to maintain a persistent infection 33,34. A new genotype 4b was also found in one of the isolates, which has not been reported earlier from India but which is common in Africa 16.

In conclusion, our results demonstrate that HCV genotypes 1 and 3 are more common in chronic liver disease patients in India. Genotype 1b was found in only few isolates. This information could be favorable for planning strategies to treat HCV related chronic liver disease in India. The influence of relatively higher frequency of mixed genotypes on natural progression of liver disease and response to antiviral therapy needs to be investigated further.

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