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Research Article

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Genotypic distribution of hepatitis C virus among hospital based general population in Vijaypur, India

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ABSTRACT

Hepatitis C virus (HCV) has emerged as a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Genotypes of HCV can vary in pathogenecity and can impact on treatment outcome. To study the prevalence and different genotypes of HCV among hospital based general population in Vijaypur, Karnataka. Study subjects were those attending various Out-Patient Department (OPD) and In-Patient Department (IPD) of Shri B.M. Patil Medical College, Hospital and Research Centre, Vijaypur, Karnataka. 5ml of blood sample was aseptically collected in plain vial from the study subjects. Serum was separated and aliquoted in different vials. Serum was tested for HCV antibodies by Enzyme Linked Immunosorbent Assay (ELISA) and HCV RNA by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Serum sample positive for HCV RNA was subjected to genotyping by RFLP (Restriction Fragment Length Polymorphism) and direct sequencing. Of the 1,200 samples tested, 32 (2.6%) were positive for anti-HCV antibody and 25 (2.1%) were positive for HCV RNA. HCV genotype 1 (68%) was found to be the most predominant type followed by genotype 3 (32%). The subtypes of genotype 1 were 1a and 1b, where as subtypes of genotype 3 were 3a and 3b. In this study, HCV genotype 1 and genotype 3 were found to be the predominant genotypes respectively. For physicians, knowing the genotype of hepatitis C virus is helpful in deciding type and duration of therapy. In addition, knowledge of circulating genotypes could impact on future vaccine formulations.

Key words: Seroprevalence; RT-PCR; RFLP; Sequencing Genotype; Subtype

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INTRODUCTION

Hepatitis C virus (HCV), since its discovery in 1989, has been considered a leading cause of chronic hepatitis which can progress to liver cirrhosis and hepatocellular carcinoma.[1] According to World Health Organization (WHO), there are 180 million people infected with HCV worldwide[2] and about 12.5 million carriers in India.[3]

HCV is an enveloped positive strand ribonucleic acid (RNA) virus belonging to genus hepacivirus in the family flaviviridae.[4] The RNA genome comprises of about 9500 nucleotides with a single open reading frame that encodes a polypeptide precursor of 3000 aminoacids and is flanked by the noncoding regions at both the 5' and the 3' termini.[5]

The polyprotein precursor is cotranslationally processed by host signal peptides to yield the structural (core [c] and envelope [E1 and E2]) and the non-structural proteins (NS1, NS2, NS3, NS4A, NS4B, NS5A and NS5B).[6]

HCV has been classified into six major genotypes and into more than 90 subtypes distributed across the world.[7] The HCV genotypes too have distinct geographical distribution and may have a bearing on the duration of treatment and outcome, [8] although the impact of HCV genotype in progression of disease is still controversial.[9]Studies in India have revealed a seroprevalence of 1.8% of HCV infection among general population.[1][10]

The conventional antiviral therapy against HCV is either monotherapy with interferon (IFN) or in combination with ribavirin.[7] The response to therapy differs across genotypes, while upto 80% of the genotypes 2 and 3 can be cured with standard of care treatment consisting of pegylated or standard IFN-α and ribavirin, [11] genotypes 1, 4, 5 and 6 have been reported to show poorer response. In the present study, we analyzed the genotypes of HCV among hospital based general population in Bijapur. Our study will help in knowing the genotypes prevalent in this region and helps to choose appropriate treatment for HCV infection.

EXPERIMENTAL SECTION

The study was conducted in Department of Microbiology, Shri B.M. Patil Medical College (SBMPMC), H & RC, Vijaypur, Karnataka. All patients attending Out-Patient Department (OPD) and In-Patient Department (IPD) of SBMPMC, H & RC from April 2011 to July 2014 were included in the study. A detailed medical history including risk factors was taken. The study protocol was approved by Institutional Ethics Committee of BLDE University, Vijaypur. Informed consent was taken from all the patients. Proforma was maintained for each patient containing clinical information about his/her previous exposure to risk factors.

5ml of blood sample was aseptically collected in plain vial from the study subjects. Serum was separated and aliquoted in different vials and stored at -70^{0}C until tested. Repeated freezing and thawing was avoided. The following tests were performed on serum samples:

Serological studies

Anti-HCV antibodies-using commercially available third generation ELISA Kits which comprised of Core, E1, E2, NS3, NS4 and NS5 antigens of HCV (SD HCV ELISA, Bio Standard Diagnostics Pvt Ltd, India), as per manufacturer instructions. Anti-HCV antibodies positive samples were further processed for next step evaluation.

Viral RNA extraction

HCV viral RNA was extracted from the serum samples positive for HCV antibodies using Qiagen (Germany) RNA according to manufacturers' instructions. HCV RNA was extracted from serum and eluted in 50 µl of elution buffer. Eluted RNA was stored at -70°C until further processed.

Detection of HCV RNA by RT-PCR

RT-PCR was carried out by modified method of Mellor et al. The RNA was denatured by heating at 70°C for 3 min prior to RT-PCR, and reverse transcribed at 42°C for 60 min, in a PCR tube containing 1X RT buffer, 10 mM deoxyribonucleoside triphosphates, 20 U RNase inhibitor, 50 U murine leukemia virus reverse transcriptase, 20 pmol primer (core region P1: 5 × ATGTACCCCATGAG/TA/ GTCGGC 3 × anti-sense) to a final volume of 20 µl. The c-DNA product was denatured at 95°C for 5 min, then cooled at 4°C for 5 min and used for direct PCR.

Direct polymerase chain reaction

Direct PCR was performed in the reaction mixture containing PCR buffer (10×), 2 mM MgCl2 , 10 mM deoxynucleotide triphosphates (dNTPs), 20 pmoles primers (sense P2: 5′ACTGCCTGATAGGGTGC TTGCG AG 3′) and anti-sense (P1: 5′ ATGTACCCCATGAG/ TA/GTCGGC 3′) for 5 × NCR core region, 0.75 U Taq Deoxyribonucleic Acid (DNA) polymerase, in a total reaction volume of 25 µl.

Nested-PCR was performed in a reaction mixture containing PCR buffer (10×) 2 mM MgCl2 , 10 mM dNTPs, 20 pmoles primers (sense P3: 5′ ACTGCCTGATAGGGTGCTTG CGAG3′) and anti-sense (P4: 5' ATGTACCCCATGAG/TA/GTCGGC 3′) for 5 × NCR core region, 0.75 U Taq DNA polymerase, in a total reaction volume of 25 µl.

Amplified PCR product was electrophoresed in ethidium bromide stained 2% nusieve agarose gel with commercially available 100 bp DNA marker (Fermentas). Specific bands of HCV were visualized under ultraviolet light of Wealtec gel doc system. Positive and negative controls were also included.

HCV RNA quantification

Quantitative HCV RT-PCR was performed using the light cycler taqman master mix kit (Roche Diagnostics GmbH, Mannheim, Germany) on Roche Light cycler as per manufacturer's instructions. The unit of the HCV RNA quantification was copies/ml. The range of standard used in quantitative analysis was 10^{2}-10^{8} copies/ml.

HCV genotyping

The restriction fragment length polymorphism (RFLP) analysis was carried out using the nested PCR product of RNA positive samples (20-30 µl). The amplified nested PCR product was digested with three enzymes Accl, Mbol and BstN1 and incubated at 37°C for overnight in a specific endonuclease buffer. The digested product was loaded onto 3% nusieve agarose gel and the restriction pattern was analysed using Wealtec gel doc System (Figure 1 and 2). The RFLP was followed by direct sequencing for determination of HCV genotype. The nested PCR product and sense primer were used for sequencing reaction.

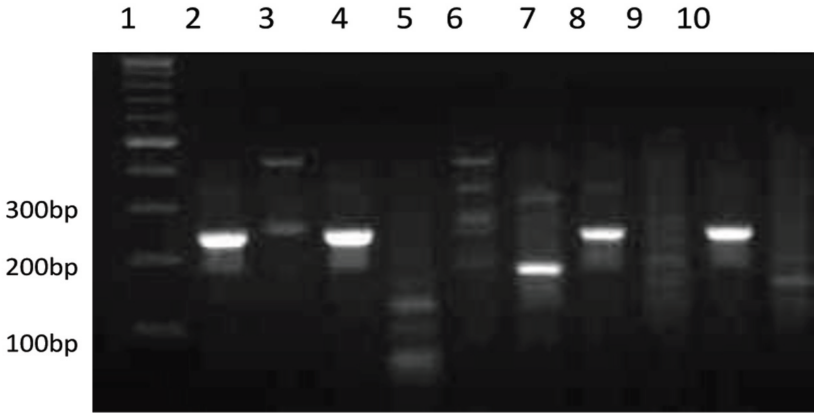

Figure 1: The RFLP pattern of HCV genotype. Lane 1: 100 bp marker, Lane 2 and 4: genotype 1b (234 bp), Lane 5: Negative control, Lane 7: genotype 3b (176 bp), Lane 8 and 10: genotype 3a (232 bp)

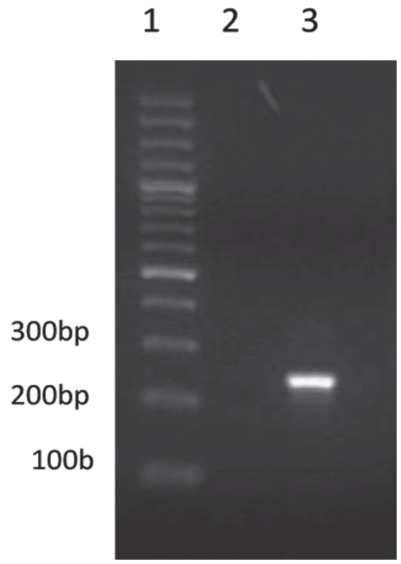

Figure 2: The RFLP Pattern of HCV genotype. Lane 1: 100 bp DNA marker, Lane 2: Negative control, Lane 3: genotype 1a (209 bp)

RESULTS

A total of 1,200 patients were recruited in the study. Majority of these patients belonged to Bijapur city and the neighboring small towns and villages. Of the 1,200 patients screened, 32 (2.6%) were found positive for ant-HCV antibody.

25 (2.1%) out of 32 were found to have active infection (HCV RNA positive). 25 HCV RNA positive samples were subjected to genotype determination using RFLP followed by direct sequencing. Genotype 1 was the commonest type observed in 17 (68%) patients followed by genotype 3 in 8 (32%) patients. Among genotype 1, subtypes 1a and 1b were most commonly isolated. In genotype 3 subtypes 3a and 3b were most frequently isolated.

Genotypes 1a and 1b were observed in 10 (58.8%) and 7 (41.2%) patients respectively whereas types 3a and 3b were seen in 3 (37.5%) and 5 (62.5%) patients respectively. Out of 25 PCR positive patients, 15 (60%) were males and 10 (40%) were females. Distribution pattern of HCV genotypes according to the gender is given in Table 1. Distribution pattern of HCV genotypes according to the age is given in Table 2.

Viral load quantification was carried out in HCV RNA positive patients. The average viral load of patients infected with genotype 1 was significantly higher than those infected with genotype 3.

Table 1: Distribution pattern of HCV genotypes according to age

Gender	Genotype 1a	Genotype 1b	Genotype 3a	Genotype 3b
Male (N=15) (60%)	6 (40%)	4 (26.7%)	2 (13.3%)	3 (20%)
Female (N=10) (40%)	4 (40%)	3 (30%)	1 (10%)	2 (20%)
Total (N=25) (100%)	10 (40%)	7 (28%)	3 (12%)	5 (20%)

N=Number

Table 2: Distribution pattern of HCV genotypes according to age

Age	Genotype 1a	Genotype 1b	Genotype 3a	Genotype 3b
< 18 (N=4) (16%)	2 (50%)	2 (50%)	0 (0%)	0 (0%)
18-40(N=9) (36%)	4 (44.4%)	2 (22.2%)	0 (0%)	3 (33.3%)
41-60(N=12) (48%)	4 (33.3%)	3 (25.0%)	3 (25.0%)	2 (16.7%)
Total (N=25) (100%)	10 (40.0%)	7 (28.0%)	3 (12.0%))	5 (20.0%)

N=Number

DISCUSSION

Overall a seropositivity of 2.6% was observed among hospital based general population in this study. Currently India harbors an estimated 10-15 million chronic carriers of HCV, which is a major cause of liver related mortality and morbidity. Knowledge of genotype is crucial for management of HCV infection and prediction of prognosis. [12]

HCV genotypes show differing distributions in different geographic regions. In the United States, about 70% of cases are caused by genotype1, 20% by genotype2 and about 1% by each of the other genotypes.[13] Genotype 1 is also the most common in South America and Europe.[14] In India, genotype 3 has been predominant in the Northern, Eastern as well as Western region,[15] while in South India, genotypes 1 and 3 have been reported in decreasing order of frequency.[16] Genotype 3 has also been reported to be the commonest type from the neighboring countries of Nepal and Pakistan, while in the eastern countries of Thailand, Vietnam and Japan, genotype 1 is the most prevalent.[17]

The most common genotypes isolated in our study were 1 and 3 respectively. Our finding correlates well with the study conducted by Raghuraman S et al in 2004. Knowledge of regional distribution of HCV genotypes is important since this could influence configuration of diagnostic assays as well as vaccine designs. [16] Within genotype 1, we observed that subtype 1a (58.8%), the current most prevalent subtype.

According to our study in the group of patients of age less than 18 years 50% of patients were found to be infected with HCV genotype 1a and also 50% 0f patients with 1b. In the age group 18-40 years, 44.4% had 1a genotype infection, 22.2% showed 1b genotype infection while 33.3% showed 3b genotype infection of HCV. The patients of age group 41-60 years showed the percentage to be 33.3% of 1a, 25% of 1b, 25% of 3a and 16.7% 0f 3b. Genotypes 3a and 3b were not found in age group of less than 18 years and 3a genotype was not found in age group 18-40 years.

Our study showed no significant difference in genotype distribution in relation to gender. Various genotypes were equally distributed in relation to gender.

HCV plasma viral load, also called HCV viremia, is expressed in copies per milliliter. In our study we used PCR to quantify HCV viremia. In our study, mean HCV RNA were higher in patients infected with HCV type 1 than in patients infected with HCV type 3 ( ). Our result correlated well with a study conducted by Nabi et al in 2003. [18]

For physicians, knowing the genotype of hepatitis C is helpful in deciding type and duration of therapy. [19] Several clinical trials of pegylated interferon/ribavirin therapy have revealed significant differences in response rates for the various HCV genotypes. Individuals with genotypes 2 and 3 are more likely than individuals with genotype 1 to respond to therapy with alpha interferon or the combination of alpha interferon and ribavirin.[20]

One probable reason for more treatment failures with HCV genotype 1 could be its efficient replication ability enabling it to establish higher viral RNA compared to other genotypes. [21] In the present study, patients with HCV genotype 1 had significantly higher viral load as compared to genotype 2 and 3. Patients with high viral load present a poor response to interferon therapy than those with lower levels. The probability of a relapse after cessation of therapy is higher in patients with high HCV RNA copy numbers prior to therapy. [22]

CONCLUSION

There is an association between different types of genotypes and viral load. Further studies should be carried out to determine the association of viral load with different genotypes. The information provided by the present study provides valuable information to physicians in clinical decision making. For physicians, knowing the genotype of hepatitis C is helpful in deciding type and duration of therapy. A continued monitoring of HCV genotypes is essential for the optimum management of chronically infected patients. In addition, knowledge of circulating genotypes could impact on future vaccine formulations.

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