The Scientific Temper
Vol. 12, No 1-2, January-July, 2021:pp 16-22
ISSN 0976 8653, E-ISSN 2231 6396
A Web of Science Journal
Doc ID: https://connectjournals.com/03960.2021.12.16

The Molecular Profiling and HCV RNA Quantification to Study the Distribution of Different HCV Genotypes in Accordance to Geographical Condition

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ABSTRACT

The HCV RNA quantification provides insight for treating and curing the HCV (Hepatitis C Virus) and HCC (Hepatocellular Carcinoma). This study deals with the molecular profiling of HCV RNA viral load and the molecular investigation of the HCV RNA genotypes. The distribution of various genotypes among the population is studied in this paper. Total 94 EDTA blood samples were taken from suspected HCV reactive patients from the different hospitals including OPDs and IPDs of in Dehradun, Uttarakhand, India. The RNA extraction was performed by the QIAamp Viral RNA mini kit (cat. no. 52904) and the HCV RNA quantification was performed by Rotor Gene Q 5 Plex Real Time PCR machine. The HCV genotypic characterization was performed by the Real Time PCR Technology utilizing Sansure (Korea) Biotech kit. Out of 94 samples collected for HCV RNA quantification, 44.7% (42 cases) were with target was not detected and 55.3%, i.e., 52 cases were with high viral load. HCV genotype 3 was found to be the most prevalent in 65.62% of the cases i.e. 21 cases in total. The presence of HCV genotype varies within the different geographical conditions because of the differences in the major causative factors. The HCV RNA viral load was quantified in 52 samples with high HCV RNA viral load and their titer values were observed in between the 34 IU/ml to 1.10×10^8 IU/ml and the other 42 samples were not considered for genotyping because of low viral load in them.

Keywords: Hepatitis C Virus (HCV), RNA quantification, Real-Time Polymerase Chain Reaction (RT-PCR), Genotype

INTRODUCTION

Hepatitis C Virus (HCV) is responsible for causing contagious liver disease in nearly 70-160 million people all around the world and is responsible for causing liver cancer ((El-Serag, 2012; Estes et al., 2015; de Martel et al., 2015; Smith et al., 2019). According to a study in the year 2010, it is estimated that HCV causes 0.5 million deaths every year (Lozano et al., 2012). Even after successful treatment, patients are still prone to develop hepatic carcinoma (D'Ambrosio et al., 2015; Ono et al., 2017; Romano et al., 2018; Perez et al., 2019). HCV also causes cancer and hepato-cellular carcinoma (HCC) holds the

fifth position of cancer disease and in case of malignancy-related mortality cases is ranked third globally (Jemal et al., 2011). The distribution of HCV cases varies within the different geographical condition of the world (Messina et al., 2015). Although, this census has increased significantly despite the presence of anti-HCV drugs (Omran et al., 2018). Blood transfusion, hemodialysis, multiple injections of drugs and unsterilized equipment use are the main reason of HCV transmission (Mohd et al., 2013). HCV takes nearly 20-30 years to become a progressive disease with persistent hepatic inflammation, fibrotic wound scars, cirrhosis and even HCC (El-Maraghy et al., 2020). Studies with HCC patients revealed that they contain anti-HCV antibody in the patient serum sample. There are various diagnostic approaches to detect the presence of HCV in the patients. Various globally accepted markers for HCV diagnosis are des-c carboxy prothrombin, alpha fetoprotein (AFP), imaging and liver biopsy. Although, these methods of diagnosis are non-reliable, non-sensitive, costly and also invasive due to which late diagnosis occurs and untimely treatment and delayed intervention and therapy becomes the increasing case of HCV infection (Attallah et al., 2013). Due to this eradication of this disease has become difficult without sufficient research and the development of prophylactic vaccine (Bartenschlager et al., 2019). To prevent such medical and diagnostic issues, better approaches of diagnosis are needed to introduce to assist early diagnosis, prognosis and staging (El-Maraghy et al., 2020).

Hepatocytes play a vital role in regulating the blood glucose level by regulating the carbohydrate and lipid metabolism through the process of lipid gluconeogenesis and lipogenesis. This regulation is disturbed by HCV infection through various manners leading to the development of many metabolic disorders. HCV is known for promoting the process of gluconeogenesis by FoxO1-dependent pathway (Deng et al., 2011). For promoting gluconeogenesis, Akt-JNK signaling pathway is induced by the help of non-structural protein 5A (NS5A) (Kuo et al., 2014). Similarly, lipid metabolism is disturbed by the involvement of modulating lipid regulatory factor Angiopoietin-like 3 gene which is involved in blocking the transcription of liver X receptors (LXRs) (Foka et al., 2014). Another study reveals the overexpression of SHP (a typical orphan nuclear receptor small heterodimer partner) leads to the decrease in TGF-beta and fibrotic gene expression in the HCV-infected cells (Jung et al., 2015). SHP originally is known for its importance in the regulation of metabolic homeostasis and hence is studied for hepatic metabolic homeostasis with respect to carbohydrate and lipid metabolism. The only human hepatoma cell line Hup7 and its derivatives are responsible for strongly replicating the HCV. Although, different sub-clones and passages of this single line exhibit tremendous differences in the efficiency of HCV replication (Dächert et al., 2020). A newer study related to miRNA which controls most of the coding genes and is found partly tissue-specific (Otsuka et al., 2017). The miR-122 is mainly expressed in the liver tissue where it is sequesters for its own replication by the HCV RNA which in turn depresses the normal miR-122 targets (Otsuka et al., 2017; Hou et al., 2011). This study deals with the molecular investigation of the HCV RNA genotypes. It includes a detailed study related to the molecular profiling of HCV RNA viral load. The distribution of various genotypes within the population according to the geographical condition is also discussed here.

MATERIAL AND METHODOLOGY

94 EDTA blood samples were taken from HCV-reactive patients. These samples were collected by different hospitals including OPDs and IPDs of Gastroenterology, Medicine, Gynecology, Pediatric, Tuberculosis, Chest and Surgery in Dehradun, Uttarakhand, India. The serum was separated from all of the 94 samples and RNA was extracted using QIAamp Viral RNA mini kit (cat no. 52904). HCV RNA quantification was performed by Rotor-Gene Q 5 Plex Real-Time PCR machine. The viral load was estimated in all 94 clinical samples. The master mix was prepared for the quantification of HCV RNA with the help of the usage of the Artus amplification Kit from Qiagen. The Hepatitis C Virus RG Mater A and B reagents and enzymes were utilized for the reverse transcription and specific amplification of 240 bp region of 5'-3' untranslated region (UTR) of HCV genome. HCV RNA was quantified within the range of 34 IU/ml to 1.00×10^8 IU/ml whereas HCV RNA viral loads greater than 500 IU/ml were considered for the HCV genotyping. For HCV genotyping characterization, Real-Time PCR Technology utilizing Sansure (Korea) Biotech kit was used. The diagnostic kit uses magnetic bead technology for extracting HCV-RNA from serum. By applying one-step RT-PCR technology, the kit uses several specific pairs of HCV primers to target conserved regions of different HCV genotypes, including genotypes 1b, 1, 2, 3, 4, 5 and 6 and Taqman fluorescence probes for achieving HCV RNA genotype detection through fluorescent signal changes.

RESULT

There was a total of 94 samples evaluated in the laboratory for molecular profiling and genotypic investigation. For molecular profiling, results were interpreted by Rotor-Gene Q-QIAGEN-5 Plex Real-Time Thermal Cycler. Based on

fluorescence waves, results were studied in an automated and computed manner on the screen. The visualized waves in the graph help in a comparative study with the use of different fluorescent colored graphs, each representing a different interpretation (Figure 1).

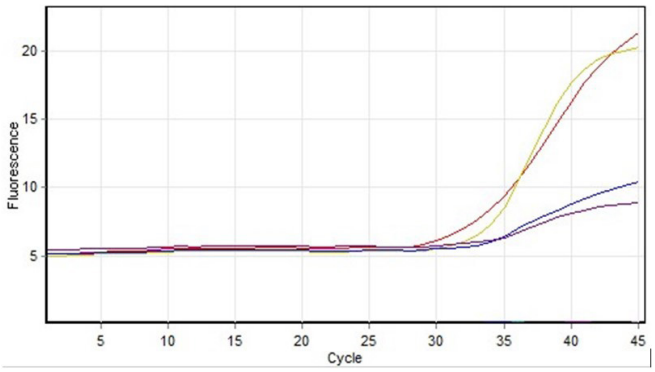

Figure 1: Different fluorescent colored graphs, each representing a different interpretation Red colour shows Qualitative Standard (Positive Control), Yellow colour shows Negative Template control (NTC), Blue and Violet and Red Colour shows samples with its Ct Values.

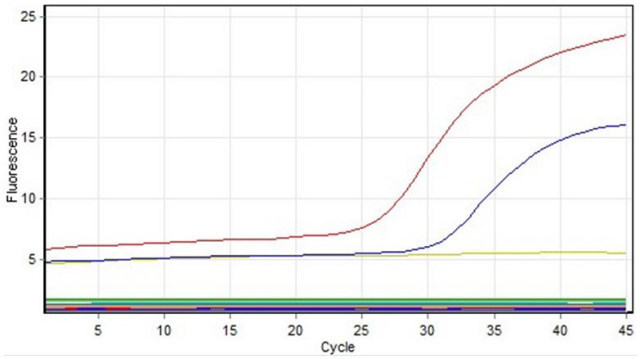

Figure 2: Amplification curve show high viral load with 28.52 Ct Value (Blue colour), Red Colour show Qualitative Standard ct value 23.07 and Yellow colour show Negatice Tamplate control (NTC).

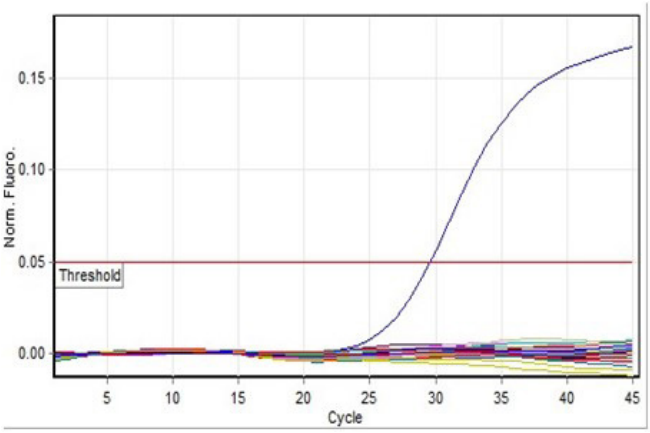

Figure 3: Amplification Curve for HCV genotype 3

The amplification curve (Figure 2) represents the results for the high viral load of HCV in R-PCR with their respective Ct (Cycle threshold) values for IC (Internal Control) and target genes. Being an automated method, RT-PCR provides the results being displayed when the amplification is still going, so that proper monitoring of the tests can be performed to better understand the results obtained. Moreover, while studying various genotypes for HCV PCR viral load, different curves for Amplification curve for HCV genotypes like 3a, 4, 1b, 6 and 1a along with its Ct value (Figure 3, Figure 4, Figure 5, Figure 6, Figure 7).

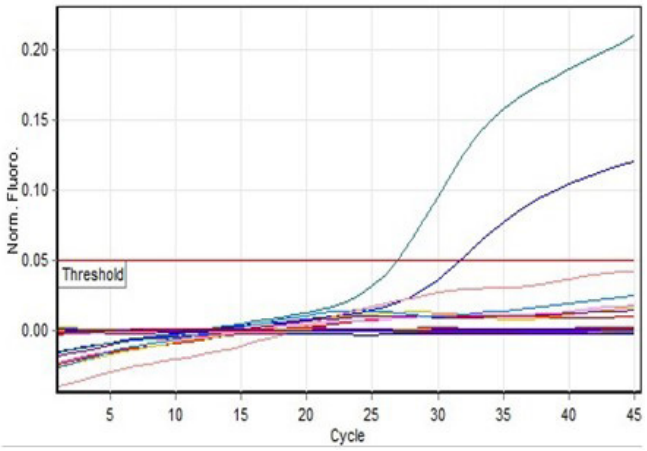

Figure 4: Amplification Curve for HCV genotype 4

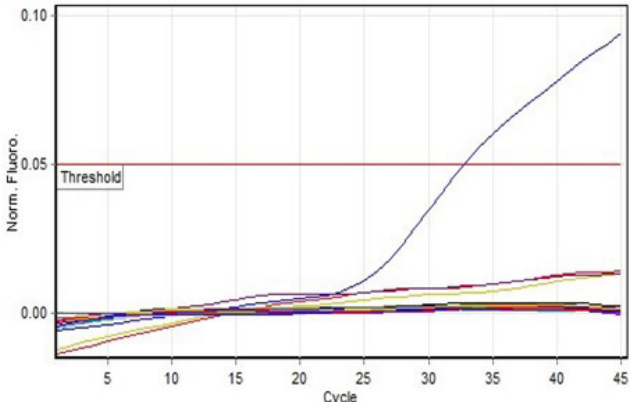

Figure 5: Amplification Curve for HCV genotype 1b

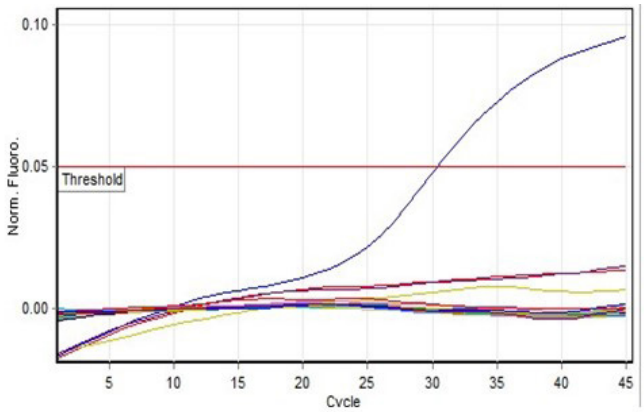

Figure 6: Amplification Curve for HCV genotype 6

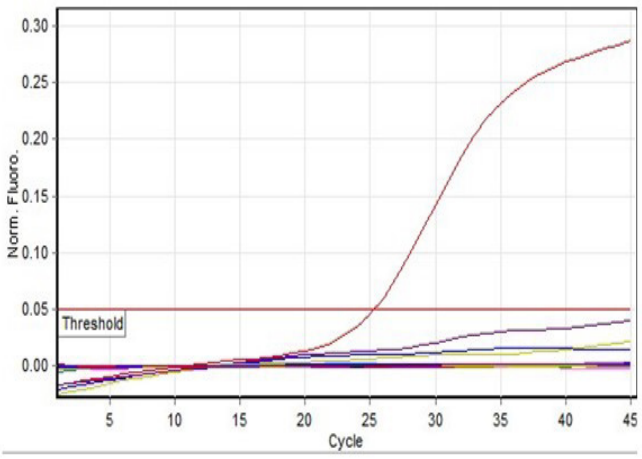

Figure 7: Amplification Curve for HCV genotype 1a

Out of 94 samples collected for HCV RNA quantification, 44.7%, i.e., 42 cases were those in which target was not detected and 55.3%, i.e., 52 cases were with high viral load (Table 1). Moreover, cases with greater than 500 IU/ml were gone for HCV genotyping which includes 32 cases. According to the study of 32 cases of HCV genotyping, HCV genotype 3 was found to be the most prevalent which was found in 65.62% of the cases which marks for 21 cases in total. After that HCV genotype 1a stands with 12.5% i.e. 4 cases in total which is followed by HCV genotype 6 with 9.37% i.e. 3 cases, HCV genotype 1b stands with 6.25% i.e. 2 cases and lastly HCV genotype 2 and 4 stands with 3.12% i.e. only one case as shown in Table 2.

The study of HCV genotype and subtype was performed in different spectrums of HCV RNA viral titer ranging from 1.00×10^3 10/ml to 1.00×10^8 10/ml. The maximum number of cases were seen in HCV RNA viral load with different ranges from 1.00×10^4 to 1.00×10^7 IU/ml. There were total 27 cases for studying this with the highest prevalence of HCV genotype 3 in 17 cases. It was followed by HCV genotype 1a and 6 with 3 cases each.

Table 3 provides the description of the gender-wise distribution of HCV RNA's high viral load and TND (Target Not Detected) in clinical samples. Out of 94 samples, 55 cases (58.6%) belonged to males of which 26 (47.3%) cases had high HCV RNA viral load whereas 29 (52.70%) cases reports target not detected. The remaining 39 cases of total 94 cases contain HCV RNA high viral load in 26 samples (66.6%) and 13 (33.4%) cases do not detect the specific target for HCV infection.

Table 1: Cases of HCV RNA high viral load and TND.

TOTAL NO. OF CASES	HIGH VIRAL LOAD	TARGET NOT DETECTED
110	65 (59.09%)	45 (40.90%)

Table 2: HCV RNA viral load and its genotype/s detected.

Range HCV RNA titer (IU/ml)	No. of cases	Total no. of cases with HCV RNA viral load > 500IU/ml	HCV Genotype/s detected	HCV Genotypic distribution						
				1a	1b	2	3	4	5	6
1.00×10^1-1.00×10^3	26	3	1a,3	*1*	-	-	2	-	-	-
1.00×10^4-1.00×10^7	34	27	3,1a,1b,2,4,6	*3*	2	1	17	1	-	3
$>1.00\times10^8$	02	2	3	-	-	-	2	-	-	-
Total	**94**	32		*04*	02	01	21	01		03

HCV genotype 3 =**65.62%**

- HCV genotype 1a = **12.5%**
- HCV genotype 1b = **6.25%**
- HCV genotype 6 = **9.37%**
- HCV genotype 2 = **3.12%**
- HCV genotype 4 = **3.12%**

Table 3: Gender-wise distribution of HCV RNA viral load and TND in clinical samples.

Gender	Total cases	HCV RNA high viral load	TND
Male	63 (57.27%)	38 (60.31%)	25 (39.68%)
Female	47 (42.72%)	27 (57.44%)	20 (42.55%)
Total	110	65 (59.09%)	45 (40.90%)

DISCUSSION

Hepatitis C Virus (HCV) is one of the major causes of liver cirrhosis and hepatocellular carcinoma. HCV causes a lot of changes in metabolic processes. HCV infection is transmitted through blood-related transfusion. According to the WHO, 180 million people are affected by HCV worldwide and about 12.5 million carriers in India (Mindolli and Salimani, 2015). There are 85-90% of primary liver cancer is caused by Hepatocellular carcinoma. Chronic hepatitis and cirrhosis mark the major pre-neoplastic conditions in the majority of HCC. It takes two or more decades to develop significant HCC from HCV infection. HCV Quantification and Genotyping depend on the target-conserved region in the HCV genome. The presence of HCC varies within the different geographical conditions because of the differences in the major causative factors. Majorly, chronic hepatitis B and C are responsible for causing HCC all around the world. HCC mainly develops in cirrhotic conditions and rarely in non-cirrhotic conditions. In the current study, out of 94 specimens, HCV RNA viral load was quantified in 52 samples and their titer values were observed and ranges in between 34 IU/ml to 1.10×10^{8} IU/ml and the other 42 samples were not considered for genotyping because the viral load in them was very low. The genotypes studied in this paper are genotype 3, 1a, 1b, 2, 4 and 6. They were found in the most frequent manner and among them, genotype 3 was found to be the most prevalent one which came in between the 1.10×10^{3} to 1.10×10^{8}. This study used a quantitative assay to measure the viral load in different individuals infected with the different HCV genotypes. This quantification and genotypic identification was studied by the most reproducible and sensitive technique called RT-PCR, which can track the putative presence of virions, passively absorbed or replicating in the cells. Total 94 clinical samples were taken in this study, in which 52 (55.4%) cases were detected with a high viral load of HCV RNA and 42 (44.6%) cases with the target not detected. This study is very important for the proper management of the patients infected with HCV and undergoing treatment. The HCV viral load quantification is very important to study as indicated the severity of the infection and the extent of the damage. According to this HCV RNA viral load, the treatment and therapy is monitored by the clinicians and doctors. An increase, decrease or no change in the HCV RNA viral load is essential for studying the drug resistance, susceptibility pattern in HCV infected patients. The high viral load cases are studied for quantification. The acceleration of nucleotide switch in the HCV genome resulted in the diversification and evolution into the different genotypes. Differences among the HCV genotypes in geographic distributions provide an insight in epidemiologic markers which can be used in detecting the source of HCV infection within a population and for further prognosis. HCV genotype 3 was most frequently detected with viral load >500 IU/ml in 65.625 of the patients considered for this study. It also contributes to the development of steatosis i.e. fatty acid liver disease and insulin resistance. They directly contribute to the development and progression of HCV disease including cirrhosis and liver cancer. There is also a risk for liver failure and a faster rate of fibrosis progression. Another genotype 1a is also very common in distribution and its prevalence in HCC patients was significantly higher than in chronic hepatitis and liver cirrhosis patients. Also, genotype 1b infection is still considered as a significant risk factor. The miRNA are also being studied to be used as potent molecular biomarkers. The miRNA contributes in the regulation of lipid homeostasis and live diseases progression (Ghosh et al., 2020). As miR-122 are highly expressed in the liver, the elevated levels of miR-155 and miR-122 and decreased levels of miR-16 and miR-199a are related to the pathogenesis of HCV-mediated HCC (Dhayat et al., 2015).

CONCLUSION

Hepatitis causes inflammation in the liver. There are so many reasons like the use of drugs, alcohol, etc., which causes inflammation in the liver tissue. HCV infection is caused by body fluids and a blood transfusion from the already infected patient. HCV is an enveloped ssRNA that causes a discrepancy in the liver all around the world. Due to the late diagnosis, the mortality and morbidity rate is high due to the HCV or HCC. To prevent this reliable methods and approaches are needed to be present to early diagnose the HCV infection to improve the survival rates and sufficiently start the viable treatment. The molecular diagnosis using RT-PCR provides the most trusted data and the results obtained are widely accepted. No other method as easily accessible as RT-PCR is available in the world. It is easy, feasible, reliable and mostly precise which diagnoses the presence of HCV infection. The genotypic investigation can also be studied by RT-PCR. The distribution of HCV genotypes varies according to geographical regions. Among all the genotypes, Genotype 1-3 is most widely distributed throughout the world. Even from the above three, Genotype 3 was found to be the most prevalent in the current study. The HCV genotype 3 is one of the most replicating virus which is known to damage the hepatic cells and liver tissue, thus requires the proper line of treatment after precise diagnosis. There is a regimen available for treating the infection by the use of Sofosbuvir or Ribavirin in the duration of 24 weeks.

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