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Research article

DISTRIBUTION PATTERN OF HCV GENOTYPES AND ITS SIGNIFICANCE WITH VIRAL LOAD

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ABSTRACT

Background &Aim: Hepatitis C virus (HCV) is the most important causative agent of hepatitis infection and leading high risk for progression of liver cirrhosis and hepato-cellular carcinoma. HCV Genotyping and quantification of RNA in infected patients is compulsory for designing the remedial strategies. Therefore, the current study is intended to find out the distribution pattern of HCV genotypes in HCV infected patients and their significance with the viral load. **Materials** &**Method**s: There are one hundred fifteen HCV infected patients RNA samples were included in this study. HCV genotypes was analyzed by linear array the Roche method after that through viral load measurement was analyzed by Cobas Amplicor Roche. **Results:** The genotype 4 was observed in 78/115 (67, 82%) patients followed by genotype 1 was observed in 21/115 (18.26%) patients; genotype 2 was observed in 9/115 (7.82%) patients; genotype 3 was observed in 7/115 (6.08%) patients and genotypes 5& 6 were not detected among one hundred fifteen patients. Genotype 4 was found to be the most predominant and significantly higher viral load as compared to genotypes 1, 2&3. **Conclusion:** The current study identified that HCV genotype 4 and then genotype1 accounted for approximately 87% of the HCV infection in Sirt region of Libya. Genotype 4 was associated with more harshness of liver diseases as compared to other genotypes. This may be due to more capable viral replication of genotype 4 than other types and this study might be continue for antiviral therapy among Libyan population in feature.

Keywords: Genotypes-hepatitis C virus-RT-PCR viral load-linear array

INTRODUCTION

Hepatitis C virus is a hepatotropic virus of the family Flaviviridae and genus Hepacivirus having single stranded RNA of positive polarity as genomic material. There are different types of genotypes have been identified along with hepatitis C virus isolate from different part of the world. At present, there are six main groups of sequence variants have been distinguished subsequent to genotypes 1-6 and every genotypes having a number of more closely related subtypes(a,b,c etc)[1].

Infectivity of Hepatitis C virus is the important root of chronic liver diseases worldwide[2]. Chronic stage of hepatitis C is one of the foremost causes of liver cirrhosis and final-stage liver disease, resulting in liver malfunction, hepatocellular carcinoma, liver transplantation and early death. Gratitude to antiviral therapy, there are about 50% of patients with progressive hepatitis can be cured if the infection is diagnosed in right time and management is available[3]. Hepatitis C virus infection (HCV) are epidemic with a universal incidence of up to 3%[4,5].There are 130-170 million people infected with hepatitis C virus (HCV), and 2.3 to 4.7million new infections per year have been reported by WHO[5].HCV chronic infection is well known after exposure of six months and there is an extremely low down of natural clearance. Most of the HCV infected patients with chronic diseases are asymptomatic or contain only mild nonspecific symptoms as long as cirrhosis is not present and most frequent complaint is tiredness, vomiting, weakness, muscle pain, and joint pain and weight loss[6]. Chronic liver diseases (CLD) consist of ranges of diseases such as chronic hepatitis, liver cirrhosis, and hepato cellular carcinoma[7]. It is responsible for over 1.4 million deaths yearly and is distinguished by everlasting inflammatory processes that predispose to liver cancer[5]. The liver cirrhosis can be present at the time of diagnosis or may extend for the duration of 5 to 10 years. On the other hand, the survival of healthy HCV carriers showing patiently normal serum alanine transaminase (ALT) values and least changes in liver histology has been reported. For the reason that, these patients are thought to have better long time prognosis, the ability to distinguish which patients take an indolent course or ultimately extend Hepato cellular carcinoma is an essential medical problem[8.]

In that condition, observing the rate of progression from chronic hepatitis to cirrhosis and hepatocellular carcinoma is most beneficial with advanced clinical and laboratory technique. Latest molecular technology like quantitative real time polymerase chain reaction allow to measure the hepatitis C virus (HCV) RNA as viral load and it has become pivotal role in the assessment of patients with chronic hepatitis C infection[9]. Quantification of viral load is well established in HCV patients with chronic hepatitis C and the response to alpha interferon therapy is associated with serum HCV RNA levels before the disease management[10, 11, 12]. A very low viral load (< 2x 10^6 RNA copies/ml) is a strong predictor of a continual response to therapy. In addition, the latest studies have publicized that the period of combination therapy may be customized according to pretreatment viral load and HCV genotypes, advocated a longer duration of therapy in patients with a high viral load (>2x10^6 RNA copies/ml) who are infected with HCV genotype 1.Hence, the assessment of viral load is helpful for monitoring antiviral therapy. Therefore, the measurement of serum HCV RNA levels must to be specific, correct and reproducible, and identical in order to present an accurate estimation of treatment response and comparisons between the clinical issues[13, 14, 15.]The present study was decided to investigate the distribution of pattern of HCV genotypes in patients with chronic infection and their importance with viral load.

MATERIALS AND METHODS

Patients: This prospective study was included forty four females (44) and seventy one (71) male patients. A total of one hundred fifteen patients (115) have been randomly selected with clinical history of hepatitis C virus infection, who attended the medical outpatient department and wards of Ibn Sina Teaching Hospital, a tertiary care hospital in Sirt Region of Libya, during the year of 2010 to 2011. The ethics panel and internal review board of the organization approved the procedure. Informed consent was obtained from individual patients.

Serum Collection: Five milliliter blood sample was collected from each patient. Serum sample

were separated immediately (within1hour) to prevent viral RNA degradation and each serum sample was then dispensed into screw capped vials and stored at –80°C. This sample was utilized for detection of anti-HCV antibodies; HCV-RNA detection and subsequent genotyping analysis. Statistical analysis was performed using Microsoft office excel2007.

The Cobas Amplicor HCV test v. 2.0 is based on Five Major Processes

HCV RNA Extraction: HCV RNA was extracted[16] by adding 400µl of working lyses, 100µl control and 100µl serum sample; incubated at 60°C for 10 minutes, added 500µl of 100% isopropyl alcohol; allowed for room temperature; centrifuged at 15000rpm for 15 minutes, removed supernatant as much as possible; added 1 ml of 70% Ethanol for extracting thread like nucleic acid, centrifuged for 5 minutes, and supernatant aspirate without any residual ethanol and diluted the pellets in 1ml of dilution buffer for further PCR amplification and genotypes analysis.

Reverse Transcription: Reverse transcription of the target RNA to generate complementary DNA (cDNA). The processed specimens are added to the amplify reaction mixture in amplification tube (A-tubes) in which both reverse transcription and PCR amplification occur. The downstream or antisense primer is biotinylated at the 5`end, the upstream or sense primer is not biotinylated. The reaction mixture is heated to allow the downstream primer to anneal specifically to the HCV target RNA and the HCV internal control target RNA. In the presence of Mn+2 and excess deoxynucleotide triphosphates (dNTPs).

Target Amplification: PCR amplification of target cDNA using HCV specific complementary primers as well as amplification of internal control. The HCV internal control has been added in the Cobas Amplicor HCV test, v 2.0 to permit the identification of processed specimens containing substance that may interfere with PCR amplification. The HCV quantitation standard is a non infectious 351 nucleotide in vitro transcribed RNA molecules with primer binding regions identical to those of the HCV target sequence and it generates a product of the same length and base composition as the HCV target RNA. These features were selected to ensure equivalent amplification of the HCV internal control and the HCV target RNA.

Hybridization: Hybridization of the amplified products to oligonucleotide probes specific to the target. Following PCR amplification, the analyzer automatically adds denaturation solution to the A-tubes to chemically denature the HCV amplicon and the HCV internal control amplicon to form single-stranded DNA. An oligonucleotide probe specific for HCV amplicon or HCV internal control is added to the individual. The biotin labeled HCV and HCV internal control amplicon are hybridized to the target-specific oligonucleotide probes bound to the magnetic particles. This hybridization of amplicon to the target-specific probes increases the overall specificity of the test.

Detection: Detection of the probe bound amplified products. Following the hybridization reaction, the Cobas Amplicor analyzer washes the unbound material and then adds Avidin-horseradish peroxidase conjugate which binds the biotin-labeled amplicon hybridized to the target-specific oligonucleotide probes, then washing step occurs. After that, a substrate solution containing hydrogen peroxide and tetra-methyl-benzidine (TMB) is added to form a colored complex measured at wave length 660 nm by Cobas Amplicor photometer.

Genotypes Assay: Hybridization Reaction: Following PCR amplification, the Cobas Amplicor analyzer automatically adds 100 µl denaturation solutions to the A-tubes to chemically denature the HCV amplicon and the HCV internal control amplicon to form single strand DNA. Added 100µl denatured amplicon manually to the proper well of the typing tray that contains 4ml of hybridization buffer and

reserved a single linear array HCV genotyping strip in tray, which was coated with a series of oligonucleotide probe specific for various HCV genotypes (Roche Molecular System, USA).The biotin-labeled HCV amplicon will hybridize to the genotypes-specific oligonucleotide probe only if the amplicon contains the matching sequence of the genotype-specific probe.

Genotypes Detection: After the genotypes hybridization reaction was completed, the linear array HCV genotyping strip was washed several times to remove any unbound amplicon.Streptavidin-Horseradish Peroxidase Conjugate is then added to the genotypes linear array strip and its binds to the biotin-labeled amplicon hybridized to the genotype specific oligonucleotide probe on the genotypes strip. The genotypes strip was washed to remove any unbound streptavidin-horseradish peroxidase conjugate and then a substrate solution containing hydrogen peroxide and 3, 3, 5, 5,-tetramethylbenzidine (TMB) were added to each genotypes strip. The streptavidin-horseradish peroxidase catalyses-the oxidation of TMB to form a blue colored complex, which precipitates at the probe positions where hybridization occurs. The linear array HCV genotyping strip has then interpreted visually by comparing the pattern of positive (blue) and faint bands to a reference table of genotypes patterns (Fig.1).

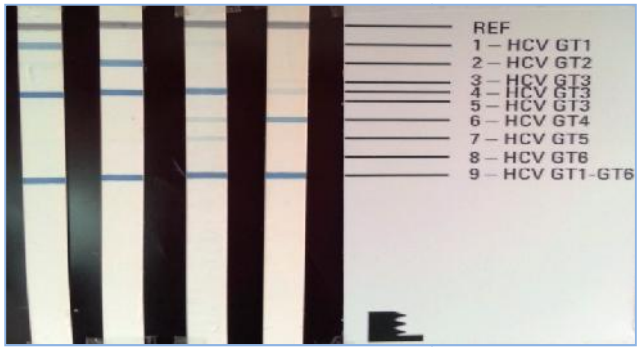

Fig:1 HCV Genotypes pattern-Linear Array
Strip1-GT1; Strip 2-GT2; Strip3 GT3; Strip 4 GT4 , Strip 5; reference table (from left to right side)

RESULTS

The study has been designated to test one hundred fifteen patient samples (confirmed cases by PCR) with 15 kits control. All the cases and the controls have been distributed in accordance to different factors in addition to the molecular study. All HCV RNA viral load samples were subjected to genotypes analysis. The study discovered the presence of HCV genotypes 1, 2, 3, & 4 by HCV linear array method. The HCV genotype 4 was observed in 78/115(67, 82%) patients. Of these, 49 male patients were showed high viral load (1.14×10^5 to 9.81×10^7), 29 female patients were showed moderate viral load (3.24×10^2 to 3.31×10^4).Genotype 1 was observed in 21/115(18.26%) patients. Of these, 14 male patients were showed high viral load (1.47×10^5 to 5.60×10^6), 7 female patients were showed moderate viral load (5.38×10^3 to 5.72×10^4).Genotype 2 was observed in 9/115(7.82%) patients. Of these, 4 male patients were showed a high viral load (1.50×10^5 to 1.21×10^6), 5 female patients were shown moderate viral load (2.74×10^3 to 4.66×10^3).Genotype 3 was observed in 7/115(6.08%) patients. Of these, 4 male patients were showed high viral load (6.24×10^5 to 2.06×10^6), 3female patients were shown moderate viral load ((2.74×10^3 to 4.66×10^3) (Fig.2).

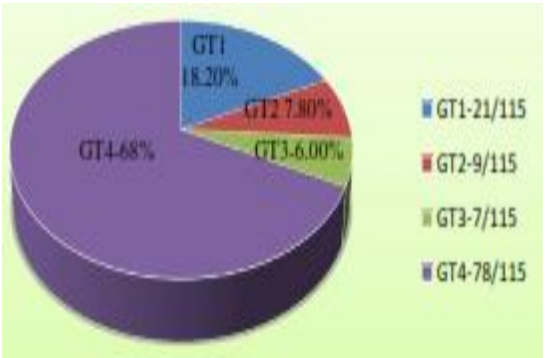

Fig: 2 Genotypes Pattern of Libyan Population

HCV RNA quantification was carried out in all 115 positive patients and it was compared between the four genotypes. The genotypes 4 and then genotype 1 having high viral load and then

it was significantly higher than average viral load of the HCV patients infected with 2 and 3 genotypes (Table-1).The study results highlighted that the 49 male HCV genotype 4 patients had severed chronic hepatitis and 29 female HCV genotype patients were confirmed as acute and some chronic hepatitis cases according to the viral load. However, HCV genotype 1, genotype 2 and genotype 3 patients were detected and there were chronic and acute cases also. These results have been shown relationship with earlier study that HCV genotype 4 was more predominant in the Middle East and Egypt where it account for >80% all chronic hepatitis cases (>34 million people)[17].

Table. 1: HCV RNA viral load-Genotypes

Viral Load(IU/mL)	Genotype1 (18.26%) 14male+7female (21)	Genotype2 (7.82%) 4male+5female (9)	Genotype3 (6.0%) 4male+3female (7)	Genotype4 (68%) 49male+29female (78)
High viral load	**$1.47x10^5$to $5.60x10^6$**	**$1.50X10^5$to $1.21x10^6$**	**$6.24x10^5$ to $2.06x10^6$**	**$1.14x10^5$ to $9.81x10^{7*}$**
Low viral load	**$5.38x10^3$ to 5.72 $x10^4$**	**$2.74x10^3$to 4.66x 10^3**	**$5.0x10^3$ to $1.80x10^4$**	**$3.24x10^3$ to 3.31x 10^{4*}**

DISCUSSION

HCV genotypes distribution varies according to the geographical region. It has been isolated and reported in different part of country. Genotypes 1-3 are generally dispersed all over the world[18-19] .Genotype 1a is widespread in North and South America, Europe, and Australia[20].Genotype 1b is prevalent in North America and Europe[21], and is also found in parts of Asia . Genotype 2 is present in most developed countries[22], although it is extremely lower than genotype 1.Genotype 3a is more common in specific risk group like intravenous drug user (IVDU) [23]. Genotype 4a is a single types circulating in all over the Middle East, Egypt and South Africa with subtype 5a[24].Genotypes 6 was mostly found in South-East Asia[25].

Current data showed that genotype 4 (67.82%) to be the most predominant genotype circulating in patients with chronic hepatitis C. The present studies confirmed the results of previous review (35.7%) from Libya, which have conclude that genotype 4 is the most predominant genotype in Libya[26]. Likewise in Egypt 4a is predominant[27], 4e, 4c, and 4d common in Sudan[28], types 4 in Saudi Arabia[29], and in Dubai and Qatar[30] is most predominant genotypes 4, 3 and 1.This analysis finding the distribution pattern of genotype appear to be comparable to the genotypes pattern which was reported from Middle East[24, 17] but different from other countries like Asia, Europa, America, where genotype 1 is predominant circulating in their population [20,21]. The present studies were not able to isolate even single genotypes of 5 and 6 from any infected patients that understood to be absent in Libya, and it is the most prevalent HCV genotypes in South Africa and South-East Asia, respectively[24,25] and it may be missing or very rare in the other part of the world.

HCV genotype is the toughest prediction matter for persistent virological response as patients with the dissimilar HCV genotype act in response in a different way to antiviral treatment. For this reason, as well as quantification of HCV RNA and genotyping has become gradually more significant in a routine diagnostic laboratory for decision making in antiviral treatment. Measurement of viral load seems to be a precious prognostic sign for the result of antiviral therapy since ALT levels do not usually enlighten the disease activity. Indeed, patients with elevated viral load present a poor response to interferon

therapy than those with very low-level viral copy. The possibility of a reversion after termination of therapy is higher in patients with high HCV RNA viral load prior to therapy .The relationship between HCV genotypes and viral load residue debatable; in a number of studies have high titer viral load was associated with highly developed liver stage[31]though others found no relationship with either, viraemia, histology or aminotransferase enzymes activity[32-33].In current study, the HCV RNA viral load (1.14×10^5 to 9.81×10^7) in patients with genotype 4 was significantly higher than persons with genotypes 1, genotype 2and genotype 3. This may be due to more capable of viral replication of genotype 4 as compared to the others genotypes in this region. The only restriction of this study is the detection of a limited number of samples with untypable genotypes. All of the samples were HCV RNA positive, had a standard viral load and thus might be genotyped by sequencing method to choose the accurate genotype, therefore, we were not able to sequence these samples due to lack of sequencing facility in our center.

CONCLUSION

The present study adorned that genotype 4 is the predominant circulating genotype in the Sirt Region of Libya and then by genotype1. However, the harshness of liver disease was more in genotype 4 and then 1 as assessed by elevated viral load.HCV genotypes, regular viral load and management of antiviral response could provide information for supervision of personalized treatment in feature among the Libyan population.

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