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RESEARCH ARTICLE

REAL TIME PCR FOR GENOTYPING OF HEPATITIS C VIRUS: EVALUATION AND APPLICATION

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ABSTRACT

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. It is associated with the development of end-stage liver disease and hepatocellular carcinoma. Studies have shown that patients infected with different genotypes of HCV may respond to different antiviral therapy differently and thus HCV genotype information is very important in helping physicians to better managing their patients. The aim of the study was to evaluate a real time PCR HCV genotyping assay against a nested RT PCR. The results indicated that real time PCR is feasible, easier and more sensitive than the nested RT PCR. A total of 96 samples were genotyped by both methods. Both methods detected HCV genotype 1 in 32 samples, genotype 4 in 39 samples, HCV enotype 3 in 19 samples, HCV genotype 2 were detected by nested RT PCR in 2 samples and 6 samples by real time PCR. The results showed that 100% sensitivity of HCV genotypes was observed with real time PCR technique and only 95.8% sensitivity of HCV genotypes was observed with nested PCR technique. The most commonly detected genotypes in 96 positive HCV plasma samples was genotype G4 (40.6 %), followed by genotype G1 (33.3%) with the predominant subtype 1a (21.8%) and subtype 1b (11.5%). The genotype 2 was detected in 6% of patients, HCV genotype 3 was detected in 19.8% of the tested plasma samples. Real time PCR seems to be reliable diagnostic method for genotyping of HCV isolates.

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INTRODUCTION

Since identified as non-A non-B hepatitis, by Choo and coworkers in the late 1980s (Choo *et al*., 1989; Wasley and Alter 2000), hepatitis C virus (HCV) continues to be a major disease burden on the world with an estimated worldwide prevalence of about 3% and affecting 170 million people worldwide (WHO, 1999). HCV infection is a common cause of chronic viral hepatitis which often leads to end-stage liver disease and/or hepatocellular carcinoma (Mc Omish *et al.,* 1993). Progression to chronic disease occurs in the majority of HCV-infected persons, and infection with the virus has become the main indication for liver transplantation (Georg *et al.,* 2001). HCV is a member of the Hepacivirus of the flaviviridae family (Robertson *et al.,* 1998). The virus is positive-sense, single strand RNA of a proximately 9.6 Kb which codes for a polyprotein with a single open reading frame (ORF) of 3008-3033 amino acid (Spinsanti *et al* 2008. Tanaka *et al.,* 1995). HCV is divided into multiple genotypes with several subtypes.

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These genotypes can differ up to 30% from each other in nucleotide sequence. All genotypes can lead to progressive liver disease, but they have been shown to have varying clinical significance depending and different duration of treatment. For example, patient with HCV genotype 1 may benefit from a longer course of therapy and genotypes 2 and 3 are more likely to respond to combination interferon-ribavirin therapy (Mohsen, 2001; Thomson *et al.,* 2005). The gold standard for HCV genotyping is nucleotide sequencing followed by phylogenetic analysis (Chan, *et al.,* 1992). Other molecular methods for HCV genotyping rely on amplification of HCV RNA, followed by either re-amplification with type-specific primers (Spinsanti, *et al.,* 2008 and Seeff, 997), hybridization with type-specific probes (Spinsanti *et al.,* 2008) or restriction fragment length polymorphism (RFLP) analysis (Larke *et al.,* 2002).

MATERIALS AND METHODS

Ninety six HCV infected patients diagnosed by ELISA attending Saint James Medical Laboratory (Tripoli, Libya)

were involved in this study. EDTA whole blood samples were collected and plasma were obtained and stored at -80oC.

Extraction of HCV- RNA

RNA extraction was performed using the SV total RNA isolation kit as described by the manufacturer (Promega USA). Briefly, a total of 175 µl of RNA lysis buffer containing β-mercaptoethanol (BME) was added into 1.5 ml microcentrifuge tube and 175µl of plasma was added. This was mixed by inversion for 3-4 min and then 350µl RNA Dilution Buffer (RDA) was added, mixed by inversion for 3-4 min, and heated in Dry heat block, at 65ºC for 5 min. The mixture was centrifuged at 14,000 rpm for 10 min and the cleared lysate was transferred to a new microcentrifuge tube. A total of 200µl of ethanol (96%) was added to the sample, and mixed by pipetting. The mixture was transferred to spin basket assembly and centrifuged for 1 min and elute was discarded. A total of 600µl of RNA Wash Solution (RWA) was added to the mixture and centrifuged at 14,000 rpm for 1 min and elute discarded, another 250µl RWA was added to the mixture and centrifuged at 14,000 rpm for 2 min and the elute was discarded. The spin basket was then transferred to the elution tube, where 100µl nuclease free water was added and centrifuged at 14,000 rpm for 1 min. The RNA was stored -70ºC until further processing.

HCV genotyping Nested RT PCR

All samples were tested for HCV genotyping using RT-nested PCR and real-time PCR. The component of the first round PCR contains 20µl of Access Quick Master,0.2µM of AC2 and SC2 primers, AMV reverse transcriptase and nuclease free water. The mixture was placed in 0.2 ml tube and 5µl of RNA template was added to the tube, finally the mixture was placed in the thermocycler (Palm cycler, Corbett Research, Australia) and subjected to thermo cycling parameter. In the second round, 3 reaction mixtures (23µl) were prepared. All contain, Go Taq green mix, nuclease free water and primer S7 (0.2µM).In addition PCR mix 1 contains G1b (0.2µM), G2a (0.2µM) and G2b (0.2µM); PCR mix II contains G3a (0.2µM) and G4 (0.2µM); PCR mix III contains G1a (0.2µM) and G3b (0.2µM); 2µl of the first round PCR products was added to each reaction. The reaction was subjected to 30 cycles of denaturation at 94^{o}C for 40 sec, annealing at 55^{o}C for 30 sec, and elongation at 72^{o}C for 40 sec. PCR products were analyzed by 3% agarose gel electrophoresis.

Agarose gel electrophoresis

Agarose gels were prepared by adding 1.5 g of agarose to 5ml of TAE buffer and 45ml of distilled water. The mixture was dissolved by boiling and casted in dedicated electrophoresis tank trays. PCR products from negative controls, positive controls, clinical samples and 50 bp DNA ladder were loaded into the gel wells. The run was performed at 100 volt for 40 min. Finally, the gel was stained in ethidium bromide and photographed for band size analysis.

Real-time PCR for the detection of HCV genotype

For genotyping of HCV isolates, a total of 4 PCR reaction mixtures were prepared. Each mixture contains RT-PCR mix 25 µl (1X), primer F (0.4µM), primer R (0.4µM) and 14 µl Rnase free water. PCR reaction 1, 2, 3, and 4 contained (0.1µM) probes G1, G2, G3 and G4, respectively, followed by 5 µl of RNA template into each reaction. All reaction tubes were loaded into the Rotor-Gene 3000 for amplification. Thermo cycling conditions included reverse transcription at 50°C for 30 min and initial activation at 95°C for 15 min followed by 45 cycles of denaturation 94°C for 15 sec; annealing and extension 60°C for 60 sec. Fluorescence was measured at 60°C on FAM and JOE channel.

Statistical analysis

Statistical analysis of the results using ANOVA, ($P \leq 0.05$) showed that a highly significant difference was found with F. value of (15.51044**) between the number of patients infected with four genotypes and subtypes of HCV. Highly significant difference with F value of (9.427836**), between the number of infected cases with different HCV genotypes over the five different age groups. No significant difference between the infection rate in the two genders with F value of 1.794237.

RESULTS

All of the 96 HCV RNA positive samples were analyzed by the HCV genotyping nested RT PCR. All, but 4, samples were genotyped. As demonstrated in figure 1, a PCR product of 208 bp were seen for isolates of HCV Genotype 1a; 234 bp for genotype 1b; 139 bp for G2a; 337 bp for G2b; 232 bp for G3a; and 99 bp for G4. Among the 92 samples genotype, 21 samples were G1a (21.8%); 11 samples were G1b (11.5%); 1 sample was G2a (1.041%); 1 sample was G2b (1.041%); 19 samples were G3a (19.8%); and 39 samples were G4 (40.6%). Figure 1 and table 1.

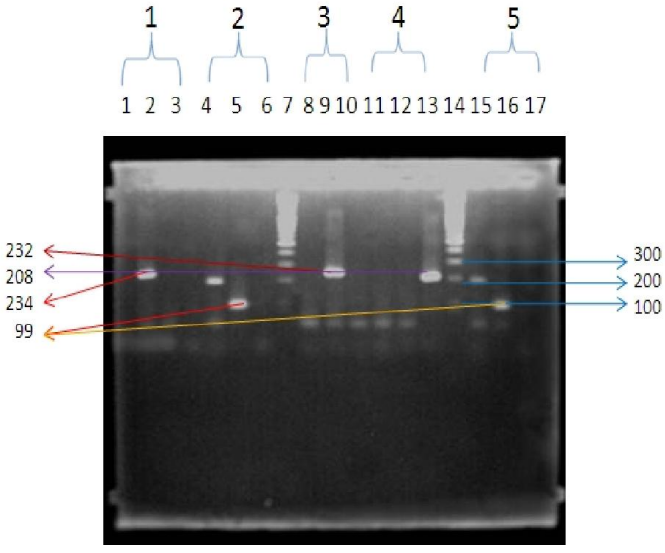

Figure 1. Typical agarose gel electrophoresis showing patterns of PCR products from different HCV-genotypes determined by the Nested PCR method

Panels 1, 2, 3, 4, 5 showing result of HCV in 5 samples. Panel 1= shows band of 234 bp in mix I for subtype 1b. Panel 2 and 5= shows band of 99 bp in mix II for genotype 4. Panel 3=

shows band of 232 bp in mix II for subtype 3a. Panel 4= shows band of 208 bp in mix III for subtype 1a.

Table 1. PCR mixtures of HCV genotyping nested RT PCR

Primers	Genotype						
	1a	1b	2a	2b	3a	3b	4
Mix I	-	-	-	-	-	-	-
G1b	-	234 bp	-	-	-	-	-
G2a	-	-	139 bp	-	-	-	-
G2b	-	-	-	337 bp	-	-	-
Mix II	-	-	-	-	-	-	-
G3a	-	-	-	-	232 bp	-	-
G4	-	-	-	-	-	-	99 bp
Mix III	-	-	-	-	-	-	-
G1a	208 bp	-	-	-	-	-	-
G3b	-	-	-	-	-	176 bp	-

HCV genotyping Real-time PCR

All of the 96 HCV RNA positive samples were analyzed by the HCV genotyping real-time PCR. Figures 2 and 3 represent the typical result. All of the 96 samples including the 4 unclassifiable samples by the nested RT PCR were genotyped.

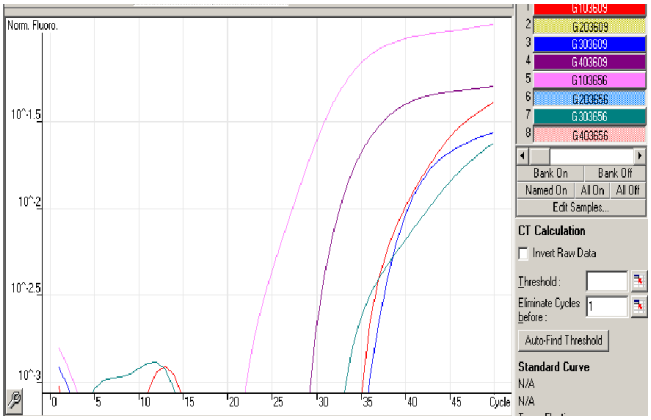

Figure 2. Different genotypes of HCV in the current plasma samples under study. Which are G1 =red, G1=pink, G2=blue, G3=green and G4=violet

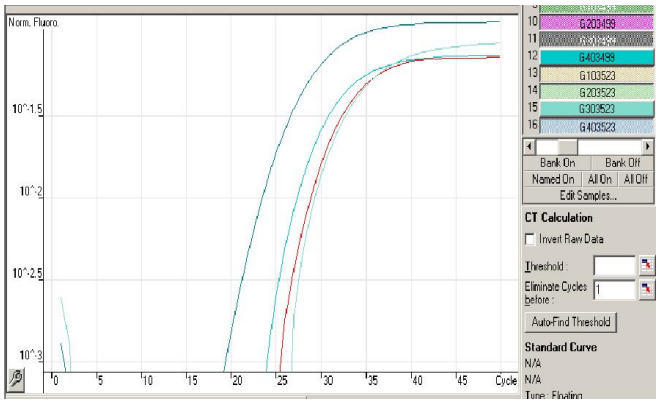

Figure 3. Different genotypes of HCV in the current plasma samples under study. Which are G1 =red, G3=light green, G3=more light green and G4=dark

Hepatitis C genotypes distribution in the study population

From total of 96 patients, 55 were males (57.3 %) and 41 were females (42.7%). The distribution of patients in the study according to their age and their age range was from 5 to 84 years old (mean age 39.5 ± 14). The present of majority patients aged between 35-49 years old was (39.1%), followed by the age group 20-34 years old (33.3%).

Table 2. Frequency of Genotype Nested RT PCR and Real time PCR

Genotype	Nested RT PCR		Real time PCR	
	No.	%	No.	%
G1	G1a=21 G1b=11	21.8 11.5	32	33.3
G2	G2a=1 G2b=1	1.041 1.041	6	6.25
G3	G3a=19	19.8	19	19.8
G4	39	40.8	39	40.8
G5 and G6	0	0	0	**0**
Unfeasible	4	4.16	0	**0**
Total	96	%95.84	96	**%100**

Using both the nested PCR and the real time PCRs the genotypes detected were G1 (G1a and G1b), G2 (G2a and G2b), G3 (G3a), and G4. The most commonly detected genotypes was G4 (40.6 %) n = 39, followed by G1 (33.3%) n = 32 with the predominant subtype 1a (21.8%) n = 21 and subtype 1b (11.5%) n = 11, followed by subtype 3a (19.8%) n = 19 and G2 was detected in six patients (6.25%).

DISCUSSION

HCV infection is one of the most important *Flaviviridae* infections with significant clinical problems throughout the world in humans and it is responsible for the second most common cause of viral hepatitis (Leiveven *et al.,* 2004). The viral genome of HCV is RNA and the virus genetically is very unstable and mutates rapidly. This means that the virus can quickly become resistant to anti-viral agents making treatment more difficult. Additionaly, with rapid mutation making an effective vaccine will also be a challenge. Data obtained from different parts of the world have focused on the increasing interest of HCV genotyping by mass screening as it is useful to answer the epidemiological questions and development of vaccine against HCV. Furthermore, it has been shown to be beneficial to facilitate therapeutic decisions and strategies (McHutchison *et al.,* 1999). It has been also demonstrated that the severity of the disease, its progression and response to therapy may vary according to the genotype (Nausbaum *et al.,* 1995). To date more than 11 genotypes and 70 subtypes of HCV are distributed worldwide (at least 11 major genotypes of HCV, each having multiple subtypes). Substantial regional differences appear to exist in the distribution of HCV genotypes. Thus, knowledge on the distribution of various genotypes in Libya is essential for its prognostic implications in chronic hepatitis C infection (Hana Elasifer *et al.,* 2010). HCV strain is usually determined by PCR amplification of genome followed by sequencing or by line-probe assays (Zein, N. N. 1996).

Genotypes 1, 2, and 3 are the most frequently encountered genotypes worldwide. However, significant differences are noticed when subtype distribution is investigated (Silini, 1995). The present study the frequency of various genotypes of HCV in Libya was observed. All the provided serum samples

tested were HCV-RNA positive by qualitative PCR, except six serum samples showing negative HCV RNA test and were excluded from genotyping. The remaining 96 HCV-RNA positive were genotyped by both nested RT PCR and real time PCR. In the current study, the most commonly detected genotypes in the whole group were G4 (40.6 %). This result agrees with that obtained by many other reports in Arabic countries where G4 predominates. In Egypt, demonstrated the predominance of G4 (Ray *et al.,* 2000). Another study performed by Fakeeh and Zaki in Jeddah, in Saudia Arabia about the prevalence and common genotypes among ethnic groups and they concluded that G4 is common (Fakeeh *et al.,* 1999). In Gazza Strip, Shemer-Avni *et al*. reported that HCV-G4 predominates (Shemer-Avni, *et al.,* 1998). In Lebanon a study conducted by Rami *et al*., in Libanese thalassemics and the results indicated the predominance of HCV-G4 (Ramia *et al.,* 2002). Similar conclusion obtained by other authors in Syria, from hemodialysis patients (Abdulkarim *et al.,* 1998) and in Kuwait (Pasca *et al*., 2001). However, the result of our study is quite different from that obtained and based on only one known study in Jordan in hemodialysis patients, where is the dominant genotype is G1, followed by G4 (Bdour, 2002).

Also different conclusion from Tunisia was obtained, where, subtype 1b is largely the dominant subtype, followed by 1a, 2a, 2b, 3a and 4 (Djebbi *et al.,* 2003). In Morocco the situation is quite different from our study, where higher circulation of G2 was demonstrated (Benani *et al.,* 1997). Although G4 is generally uncommon in Western countries, varying prevalence's have been reported in Southern Europe. During the past two years it has become evident that the number of patients infected with HCV G4 in Europe and the USA, and most recently in Southern India, is rising. This emerging pattern might reflect changing immigration patterns in those areas (Lyra *et al.,* 2004). The results of the current study showed that 100% sensitivity of HCV genotypes was observed with real time PCR technique and only 95.8% sensitivity of HCV genotypes was observed with Nested RT PCR technique. However, despite of the fact that Nested RT PCR technique is quite laborious and less sensitive than real time PCR, it has advantage of its capability to detect sub genotypes of HCV.

Conclusion

The present study proved that the Real-time PCR is more sensitive in detection of HCV genotypes than Nested RT PCR although it has better ability to detect subgenotypes of HCV. Moreover, high specificity identification of HCV genotypes can be accomplished by using both Nested RT PCR and Real-time PCR techniques. Finally, as it has been noticed in details in the result the most common genotype of HCV in Libya is G4 followed by G1.

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