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Genotypes of hepatitis C virus circulating in Tunisia

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SUMMARY

Hepatitis C virus (HCV) isolates from 93 patients living in Tunisia, including 16 haemophiliacs, were genotyped by INNO LiPA and partial sequencing of the 5′ untranslated region of the viral genome. In non-haemophiliacs, subtype 1b was largely predominant (79%), types 1a, 2a, 2b, 3a and 4a occurred much less frequently at 5, 7, 3, 3 and 1% of cases, respectively. In the group of haemophiliacs, a co-dominance between subtypes 1a and 1b was noticed (38%). Type distribution of HCV in Tunisia differs from that reported in other countries of the Mediterranean and Middle East regions. Genotyping results in respect of clinical status, age, and genotyping methods, are discussed.

INTRODUCTION

Hepatitis C virus (HCV) is highly prevalent worldwide, infecting 3% of the world's population: about 170 million people are chronic carriers and more than one million new cases are reported annually [1, 2]. After acute infection, chronic hepatitis develops in 80% of cases and may lead to cirrhosis in 20% of patients [3] and/or hepatocellular carcinoma [4]. Although HCV infection is extensive throughout the world, its distribution shows wide geographical heterogeneity. Countries are classified as low, intermediate or highly endemic when prevalences are below 0·5%, around 1% and over 2%, respectively. Prevalences over 10% are reported in some countries in Africa and the Middle East, such as Cameroon, Guinea and Egypt [5–7].

The different regions of the HCV genome show extensive sequence variability allowing classification of the different isolates into types and subtypes [8]. The most widely used classification was proposed by Simmonds [9]; it identifies six genotypes numbered 1–6, in the order of their discovery, and several subtypes assigned lowercase letters, in alphabetic order, according to the order of their discovery. These genotypes have distinct geographical distributions throughout the world. Genotypes 1, 2 and 3 are distributed worldwide; genotype 4 was mainly found in North Africa, the Middle East and Central Africa. Genotypes 5 and 6 are exclusively detected in South Africa and Southwest Asia, respectively [10, 11].

In Tunisia, systematic nationwide screening of blood donors for HCV antibodies was introduced in 1994. Prevalence of HCV infection in the general population ranges from 0·4 to 0·7% [12, 13]. The circulating HCV genotypes are not yet well defined. In this paper, sera with detectable HCV RNA from Tunisian individuals were investigated for HCV genotypes and data are compared with those reported from other countries, especially in the Middle East and the Mediterranean region.

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Table 1. *Prevalences of HCV genotypes*

Patient category	Total	Age (years) (mean ± S.D.)	HCV genotypes, no. of samples (%)						
			1a	1b	2a	2b	3a	4a	Mixed infections
Haemophiliacs	16	22 ± 18	6 (38)	6 (38)	1 (6)				3 (19)
Other patients	77	43 ± 25	4 (5)	61 (79)	5 (7)	2 (3)	2 (3)	1 (1)	2 (3)
Group 1: Normal ALT	24	38 ± 11	1 (4)	14 (58)*	5 (21)		1 (4)	1 (4)	2 (8)
Group 2: NAL† < ALT level < 2 times NAL	29	40 ± 22	2 (7)	24 (83)*	0	2 (7)	1 (3)	0	0
Group 3: 2 times > NAL	24	47 ± 5	1 (4)	23 (96)*	0	0	0	0	0

* Group 1/Group 2 ($P = 0{\cdot}097$); Group 1/Group 3 ($P = 0{\cdot}006$); Group 1/Group 2 and 3 ($P = 0{\cdot}006$).
† NAL; normal ALT level.

METHODS

Ninety-three anti-HCV positive Tunisian individuals were included, of which, 16 were haemophiliac, 43 suffered from chronic hepatitis C (based on clinical echographical and histological evidence associated with repeatedly elevated serum ALT levels and were investigated as part of a therapeutical protocol with interferon), 11 were blood donors (detected HCV positive during the systematic screening process), and 23 were diagnosed as HCV positive during investigation for other health problems [hemodialysis ($n = 3$), thalassaemia ($n = 2$), lichen planus ($n = 4$), vasculitis ($n = 1$), Waldenström disease ($n = 1$), nephrotic syndrome ($n = 1$), unknown ($n = 11$)]. The studied population included 58 males and 35 females who were aged 13–76 years, 45 were under 40, 38 between 40 and 60 and 10 were over 60 years of age. All sera were re-confirmed anti-HCV positive by a third generation commercial ELISA kit (Murex Biotech anti-HCV, Version 4.0) and had detectable HCV RNA by PCR amplification of the 5′ untranslated region (5′ UTR) of the viral genome [10]. ALT levels were tested on the serum sample referred to our laboratory for HCV genotyping, using the Enzyline ALAT/ASAT monoreactive kit from BioMérieux. Genotyping was performed using two molecular methods targeting the 5′ UTR. The first one consisted of a reverse transcription followed by a nested PCR and sequencing of the 250 pb amplicon (nucleotides −279 to −29) [14]; the sequences were then aligned with the HCV 1a prototype sequence [8] and the genotype was determined according to Smith et al. [15], on the basis of the presence or not of genotype-specific mutations. The second method used the commercial second-generation line probe assay from Innogenetics (INNO LiPA HCV): after nested PCR, HCV genotypes were determined by hybridization of the amplification products with genotype-specific probes immobilized as parallel lines on membrane strips [16]. Sixty-one sera were tested by partial sequencing, 59 by INNO LiPA and 27 were tested by both methods. Sequences from 32 isolates have been submitted to Genbank and can be retrieved under accession numbers AF463461 to AF463491. Statistical analysis used software SPSS (Release 10.0.1) and Pearson χ^2 test was performed.

RESULTS

The distribution of HCV genotypes in the studied population is shown in Table 1. Among the non-haemophiliac patients, genotype 1b was largely predominant (79%). Genotypes 1a, 2a, 2b, 3a and 4a were detected at lower levels, 5, 7, 3, 3 and 1%, respectively. Among haemophiliacs, subtypes 1a and 1b were equally prevalent (38%), followed by subtype 2a (6%). The prevalence of subtype 1a was significantly higher in haemophiliacs than in the other patients ($P < 10^3$).

Non-haemophiliac patients were divided into three groups according to their ALT levels (Table 1): Group 1 included those with normal ALT ($n = 24$), Group 2 those with moderately elevated ALT levels ($n = 29$) and Group 3 those with ALT levels twice upper limit values ($n = 24$). Most blood donors (9 of 11) were in the group of those with normal ALT levels, patients with chronic hepatitis had elevated ALT levels excepted two patients who were receiving interferon therapy. Genotype 1b was significantly more prevalent among patients with elevated ALT levels (83% in Group 2, 96% in Group 3), than in patients with normal ALT (58%) ($P = 0{\cdot}006$).

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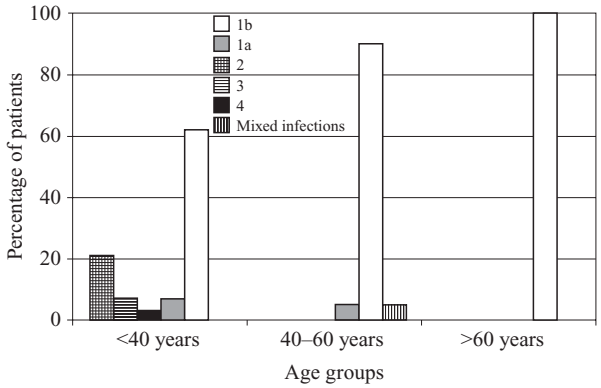

Fig. 1. HCV genotype distribution in blood donors and patients with chronic hepatitis, according to age groups. Genotypes indicated are those revealed by sequencing for sera tested exclusively by this method and those revealed by INNO LiPA for sera tested exclusively by this assay and those tested by both sequencing and INNO LiPA.

The proportion of patients with elevated ALT increased with age: 38% in patients under 40, 84% in patients aged 40–60 and 100% in patients over 60 years of age. More specifically, patients with ALT values more than twice the upper limit values were more frequent in patients aged over 60 (80%) than in patients aged 40–60 and those aged under 40, 37 and 7% respectively. The association of the disease severity, as assessed by ALT values, with advanced ages was statistically significantly ($P < 10^3$) in all cases.

The frequency of subtype 1b increased with age: it was the only genotype detected among patients over 60; its prevalence was 90 and 62% in patients aged 40–60 years and those under 40, respectively (Fig. 1). Statistical analysis showed significant association between infection with genotype 1b and advanced ages ($P = 0{\cdot}005$).

Therefore, disease severity was significantly affected by both age and genotype; and given the dependence of these two latter factors, no multivariate analysis could be carried out in order to assess separately the contribution of age and genotype on disease severity. An age stratified study, showed non significant association between ALT values and genotype ($P = 0{\cdot}355$ for ages under 40; $P = 0{\cdot}47$ for ages between 40 and 60).

Genotype identification could be accomplished for all samples tested by sequence analysis. By contrast, the INNO LiPA commercial test failed to characterize the genotype of 4 out of 59 samples (7%): one sample did not amplify with the kit's primers and three PCR products did not hybridize with any of the genotype-specific probes. Among the 27 sera typed by both methods, five samples yielded discordant results. Although a single genotype was detected by sequence analysis, the INNO LiPA identified one or two additional genotypes, strongly suggesting mixed infections. These sera were found to contain HCV genotypes 1a, 1b, 1b, 1b and 2 by direct sequencing, while INNO LiPA identified mixtures of genotypes corresponding to (1a + 1b), (1b + 3a), (1b + 3a), (1b + 2 + 4) and (2 + 3a), respectively.

DISCUSSION

In this study, HCV genotypes were assessed in Tunisian patients with known anti-HCV positivity. Excluding haemophiliacs who have a particular mode of infection, genotype 1b was largely predominant (79%), types 1a, 2a, 2b, 3a and 4 occurred much less frequently at 5, 7, 3, 3 and 1% of cases, respectively. This distribution differs from what has been reported in other countries of the Mediterranean region and the Middle East. It contrasts with data reported from Egypt where genotype 4 is quasi-exclusive (91%) and genotype 1 never exceeded 10% in the reported distributions for HCV genotypes [17, 18]. The distribution is also different from other Mediterranean countries especially those from the north of the Mediterranean Sea: in France and Italy, lower prevalences of subtype 1b were found (27–49% and 33–60%, respectively) [19–24]. In Morocco, subtype 1b, although the most prevalent, had lower prevalences (47–48%) with higher circulation of genotype 2 (29–37%) as compared to Tunisia [25, 26]. The high prevalence of the subtype 1b in Tunisia is reminiscent of the Japanese situation where it accounts for 73% of circulating genotypes, followed by genotype 2 (20%), subtype 1a accounting for only 2% in this country [27].

In haemophiliacs, genotype distribution was remarkably different with a co-domination of subtypes 1a and 1b (38%). The high frequency, among this group, of subtype 1a, most frequently detected in the United States and Europe, could be explained by the fact that all blood derivates used for Tunisian haemophiliacs are imported from Europe.

In respect of disease severity, previous studies reported high prevalence of subtype 1b among patients with chronic hepatitis. This subtype may be more associated with severe clinical forms of HCV infection, as compared to the other genotypes [11, 23]. Other authors reported similar hepatic damage with various HCV genotypes and then refuted this association.

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They considered subtype 1b as the oldest genotype and suggested that its association, in some reports, to severe forms of liver diseases, more likely reflects longer evolution of HCV infection than a more aggressive virus variant [11, 28]. In our study, when age groups were considered separately the association between elevated ALT values and genotype 1b was not significant, suggesting the role of age, rather than genotype, on disease severity.

In this report, two genotyping methods were used: sequencing in the 5′ UTR region of the genome and the INNO LiPA commercial kit from Innogenetics. In most of samples tested with the two methods (22 out of 27), concordant results were recorded. For the remaining five samples, while sequencing detected a single genotype, INNO LiPA identified one or two supplemental genotypes. As suggested by previous studies [29, 30], our results confirm that direct sequencing more likely identifies the dominant genotype, and that genotypes or subtypes present at lower levels in mixed infections are generally missed. The recommended procedure to improve detection of mixed infections is to clone PCR products before sequencing, however, this enhances the cost and the labour intensiveness of the method. Other techniques, more suitable for the detection of multiple infections, were recently described, such HTA (heteroduplex tracking assay) and S-PSMEA (semi-automated primer-specific and mispair extension analysis); they revealed the large underestimation of the real proportion of mixed infections by the usual genotyping methods [30, 31].

In conclusion, we have investigated in this study the molecular characteristics of HCV genotypes circulating in Tunisia and demonstrated that subtype 1b is largely predominant in the country. These results strikingly contrast with those reported in other countries of the region, especially Egypt where genotype 4 is predominant. Subtype 1b was reported to be associated to more severe forms of liver disease and lower response to interferon therapy [28], its predominance in Tunisia further emphasizes the public health problem caused by viral hepatitis in this country also endemic for hepatitis B [13].

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