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Hepatitis C Genotypes in Libya: Correlation with Patients' Characteristics, Level of Viremia, and Degree of Liver Fibrosis

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ABSTRACT

Objectives: Our study sought to determine the distribution of hepatitis C virus (HCV)-genotypes among patients attending two tertiary care hospitals in Benghazi and Tripoli, Libya, and correlate this with patient's characteristics, viral load, and degree of fibrosis. ***Methods***: We conducted a retrospective study of 286 HCV-RNA positive Libyan patients referred from different health care facilities in east and west Libya for specific HCV treatment. HCV genotyping was carried out by gene amplification. Liver histology was graded by Metavir score according to the stage of fibrosis. ***Results:*** HCV genotypes 1, 2, 3, and 4 were found in 24.1%, 10.8%, 3.4%, and 61.5% of the patients, respectively. Genotype 4 was detected more frequently in patients from east Libya (Benghazi) compared to west Libya (Tripoli) (75.9% vs. 41.6%, $p = 0.245$). Genotype 1 was more frequent in patients from west Libya compared to east Libya (34.1% vs. 16.8%, $p = 0.657$). There was a significant correlation between HCV genotype distribution and viral load. Patients with genotype 4 exhibited a higher degree of liver fibrosis ($p < 0.001$). ***Conclusions:*** HCV genotype 4 is the predominant genotype in Libya followed by genotype 1. However, as we go from the east to the west of the country, genotype 1 increases. Genotype 4 was associated with higher level of viremia and higher stage of liver fibrosis. It is important to note that both genotypes 1 and 4 are associated with a poor response to pegylated interferon and ribavirin combination therapy. The findings emphasize the need to develop improved strategies in Libya for the successful treatment of HCV infection with novel newly available antiviral drugs.

Hepatitis C virus (HCV) is a single-stranded RNA virus with properties similar to those of Flavivirus.[1] An estimated 170–200 million people worldwide are chronically infected with HCV.[2] Chronic HCV infection is a leading cause of chronic liver diseases throughout the world.[3] Effective treatment for chronic HCV infection is currently available, which eradicates the virus in more than 90% of treated cases.[4] In Libya, a recent nationwide general population-based survey on more than 65 000 subjects showed that the overall seroprevalence of anti-HCV positivity is 1.3%.[5]

HCV genotypes are distributed differently depending on geography and etiology of infection. The incidence of HCV isolates found in clinical practice is of clinical significance in the treatment of HCV infected patients as different subtypes may respond unequally to pegylated interferon-alfa and ribavirin combination treatment,[6,7] and a lesser extent to some novel direct acting antivirals (DAAs).[8,9] Due to a marked heterogeneity, HCV has been classified into six major genotypes and more than 90 subtypes using molecular biology.[10] In Western Europe and the United States, genotype 1a and 1b are more common followed by genotypes 2 and 3.[11] The other genotypes are less frequently found in these countries but are common in other areas. For example, genotype 4 in Egypt, genotype 5 in South Africa, and genotype 6 in Southeast Asia.[11]

Genotyping has been used to study the modes of transmission of HCV infection and identify the source of infection.[12] Although persons infected with genotype 1, and to lesser extent genotype 4,

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respond less often to pegylated interferon-alfa and ribavirin combination treatment, HCV genotype should not be a deciding factor on whether or not to treat, particularly with the increasing availability of novel oral DAAs currently widely used in some parts of the world.[13] However, knowledge of the genotype is still important in terms of the response to pegylated interferon-alfa and ribavirin combination therapy, with a better response associated with genotypes 2 and 3 than genotypes 1 and 4. Moreover, with the novel DAAs therapy, some of treatment regimens and duration might differ on the basis of HCV genotypes.[14]

Data on the distribution of HCV genotypes in Libya are limited to only few reports published all from western regions (i.e., Tripoli city).[15–17] Our study aimed to determine the distribution of HCV genotypes in Libyan patients attending two tertiary care hospitals in east (Benghazi city) and west (Tripoli city) Libya and to correlate this with the age and gender of the patients, their source of infection, the level of HCV viremia, and liver histopathology findings.

METHODS

We conducted a retrospective study from January 2009 to June 2010. We recruited a total of 286 consecutive anti-HCV positive patients from the two main tertiary care hospitals in Benghazi and Tripoli (the two largest cities in Libya). HCV-RNA had been investigated, and HCV genotype assayed in all referral patients for potential specific antiviral therapy. The patient cohort comprised of 286 patients (166 patients and 120 patients recruited from the hepatitis clinics, Department of Infectious Diseases at Aljomhoria Teaching Hospital in Benghazi and the Tripoli Medical Center in Tripoli, respectively).

Patients' characteristics including age, gender, time of first exposure, mode of transmission, and other relevant clinical data were recorded.

Patients were eligible for inclusion in the study if they tested positive for anti-HCV antibody, a positive quantitative HCV-RNA viremia, and a recent liver histopathology result (i.e., performed within 12 months of inclusion in the study).

Patients were excluded from the study if they had coinfection with hepatitis B or HIV, concomitant metabolic liver disease, or concomitant autoimmune liver disease.

The study was approved by the research ethics committee of Al-Arab Medical University, Benghazi, Libya, and was conducted in accordance with the Helsinki Declaration and under the supervision of the National Center for Disease Control of Libya.

Antibodies against HCV were determined by third generation commercial enzyme-linked immunosorbent assay (ELISA) (INNO-LIA-HCV; Innogenetics, Belgium). The assay utilizes well-defined antigens derived from HCV immunodominant proteins from the core region, the E2 hypervariable region (HVR), the NS3 helicase regions and the NS4A, NS4B, and NS5A regions as described previously.[18] A 10 µL of the appropriate specimen or control (positive and negative) was added to 1 mL of sample diluent to each test trough; the samples were sealed and incubated overnight (16 hours). All the following steps were incubated at room temperature by placing the tray on a shaker. After washing, 1 mL of conjugate solution was added to each test trough (30-minute incubation) and washed. Then, substrate solution was added to each test trough and incubated for 30 minutes. After aspirating the liquid, 1 mL of stop solution was added to each trough and incubated for 10–30 minutes. Strips were removed from the test troughs and placed, membrane side up, on absorbent paper. As soon as the strips dried, the results were interpreted according to the manufacturer's instructions.

Determination of HCV-RNA levels was performed using the COBAS® TaqMan® HCV test, v 2.0, which utilizes the real-time polymerase chain reaction as described previously.[18,19] These were: i) manual for specimen preparation to extract the HCV-RNA, ii) automated reverse transcription of the target RNA to complementary DNA (cDNA), and iii) amplification of target cDNA using HCV specific complementary primers. This is followed by the simultaneous detection of cleaved dual fluorescent dye-labeled oligonucleotide probes that permit quantitation of the HCV target in the amplified product (amplicon).

HCV genotyping was performed using the Abbott® Real Time HCV assay v 2.0 tests, which consist of three reagent kits.[20] These were: 1) Abbott Real Time HCV Amplification reagent kit, 2) Abbott Real-Time HCV Control kit, and 3) Abbott Real-Time HCV Calibrator (Abbott Molecular Inc., Des Plaines, IL). The HCV genotype reagent kits were provided as two separated kits, the HCV

amplification reagent Kit and the HCV genotype II control kit. The HCV amplification reagent kit comprised the internal control and the amplification reagent pack A, B, and C. The internal control reagent provided two vials of 1.2 mL of each. This consists of less than 0.01% non-infectious armored RNA with internal control sequences in negative human plasma. In the case of the amplification reagent pack, pack A contains the primer that designed to amplify all HCV isolates, and pack B is designed to amplify the NS5B region of genotype 1b.

Frozen samples were left to thaw at room temperature and were vortexed for 2/3 seconds to ensure good mixing. The dry heat block used throughout this assay was set at 50 °C and 75 °C for both 7.5 mL and 1.5 mL tubes, respectively. Reagents used throughout this assay were the assay controls, the internal control, and the amplification reagents. After preparation of the working solution (*m*Lysis buffer) by mixing the two vials of the internal control, each sample was individually tested with the three HCV genotype II amplification reagent packs. Each of the reagent packs (i.e. A, B, and C) were deployed to support the analysis of up to 24 samples. A volume of 2 000 µL of the internal control solution was mixed gently with one bottle of *m*Lysis buffer. The sample was prepared by adding 100 µL of the *m*Microparticles to each 12 sample for 75 mm tube diameter and the *m*Lysis buffer using a multichannel pipette. Tubes were gently mixed by inverting the solution. A 2.4 mL volume of the *m*Lysis buffer containing internal control was transferred to each lysis tube to which 500 µL of the serum from samples (i.e., specimens, calibrators, and controls) were added. Lysis tubes were then placed in the 50 °C heating block for 20 minutes. Tubes were placed on the magnetic stand for the total removal of fluid and the *m*Wash 2. The master mix tubes were prepared using the DNAase- and RNAase-free tubes.

The Abbott 96-well optical reaction plate was placed in a Strata Cooler 96 stored as indicated in the Strata Cooler 96 manual (Abbott real time HCV genotype II kit manual). A 40 µL aliquot of master mix A, B, and C was transferred to the Master Mix tube as described in the preparation manual. Sample and controls eluted volume (20 µL) were transferred to the Abbott 96 Deep-Well Plate and placed into the reaction vessels (subsystem carrier). The carrier racks containing the Abbott *m*sample preparation system reagents and the Abbott 96 Deep-Well Plate were loaded on the Abbott *m*2000rt Analyzer® and the Abbott® Real Time assay was initiated. The assay covers all known six HCV genotypes.[20]

Ultrasound guided liver biopsy was performed to determine the degree of liver pathologic grade. Specimens were cut and stained with hematoxylin and eosin, and pathologic changes were graded by Metavir score system according to the degree of periportal necrosis, portal and lobular inflammation, and fibrosis.[21,22] Fibrosis was graded on a 5-point scale from 0 to 4 (i.e., from F0 = normal liver histology to F4 = cirrhosis) as described by Bedossa et al.[21]

Data were analyzed in IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA). Results were expressed as frequency, mean and standard deviation (SD). Chi-square test and Fisher's exact test were used to determine the differences in categorical variables and with *t*-test for normally distributed continuous variables. Results were considered as statistically significant if the *p*-value was less than 0.050.

RESULTS

A total of 286 patients with chronic HCV infection were studied, there were 138 male and 148 female. The mean age was 45.0±13.8 years. Table 1 shows the detailed distribution of HCV genotypes. HCV genotypes 1, 2, 3, and 4 were found in 24.1%, 10.8%, 3.4%, and 61.5% of patients, respectively. Genotypes 5 and 6 were not detected. The distribution of HCV genotypes was not homogeneous in both referral centers. Genotype 4 was detected with greater frequency in patients from east Libya compared to patients from west Libya (75.9% vs. 41.6%, p = 0.245). In contrast, genotype 1 was detected more frequently in patients from west Libya compared to patients from east Libya (34.1% vs. 16.8%, p = 0.657). Furthermore, genotype 2 was distributed more in the west than the east of the country (17.5% vs. 6.0%, p = 0.805). Although there was a trend for a clear difference in the comparisons of the frequency of these genotypes, there was no statistically significant difference.

Table 2 shows the distribution of HCV genotypes among patients in the two hospitals according to gender, age, and mode of transmission. There was no significant difference in the age and gender distribution between the two groups. Parenteral transmission accounted for the majority of HCV

Table 1: Distribution of hepatitis C genotypes among Libyan patients in two tertiary hospitals in Benghazi and Tripoli.

Genotypes	Benghazi n = 166	Tripoli n = 120	Total n = 286	*p*-value*
Genotype 1				0.657
1 (unclassified)	13 (7.8)	21 (17.5)	34 (11.8)	
1a	4 (2.4)	8 (6.7)	12 (4.1)	
1b	11 (6.6)	12 (10.0)	23 (8.0)	
Total	28 (16.8)	41 (34.1)	69 (24.1)	
Genotype 2				0.805
2 (unclassified)	7 (4.2)	12 (10.0)	19 (6.6)	
2a	1 (0.6)	3 (2.5)	4 (1.3)	
2b	1 (0.6)	1 (0.8)	2 (0.6)	
2c	0	2 (1.6)	2 (0.6)	
2a/c	1 (0.6)	3 (2.5)	4 (1.3)	
Total	10 (6.0)	21 (17.5)	31 (10.8)	
Genotype 3				0.657
3 (unclassified)	1 (0.6)	5 (4.1)	6 (2.0)	
3a	1 (0.6)	3 (2.5)	4 (1.3)	
Total	2 (1.2)	8 (6.6)	10 (3.4)	
Genotype 4				0.245
4 (unclassified)	103 (62.0)	35 (29.1)	138 (48.2)	
4a	8 (4.8)	5 (4.1)	13 (4.5)	
4c	4 (2.4)	1 (0.8)	5 (1.7)	
4c/d	11 (6.6)	9 (7.5)	20 (6.9)	
Total	126 (75.9)	50 (41.6)	176 (61.5)	

**Chi-square was used to determine the statistical differences in frequency, and all comparisons were based on the distribution of total genotypes between the two groups. Data presented as n(%).*

Table 2: Distribution of hepatitis C virus (HCV) genotypes among patients in two tertiary hospitals in Benghazi and Tripoli according to gender, age, and risk factors.

Characteristics	Benghazi n = 166	Tripoli n = 120	Total n = 286	*p*-value*
Gender				0.474
Male	77 (46.3)	61 (50.8)	138 (48.2)	
Female	89 (53.6)	59 (49.1)	148 (51.7)	
Age, years				
Mean ± SD	46.9 ± 12.2	44.2 ± 14.7	45.0 ± 13.8	
< 60	149 (89.7)	105 (87.5)	254 (88.8)	0.091
≥ 60	17 (10.2)	15 (12.5)	32 (11.1)	0.572
Risk factors for HCV infection**				
Previous surgical procedures	52 (30.9)	40 (27.0)	92 (29.1)	0.798
Transfusion of blood or blood product	51 (30.3)	39 (26.3)	90 (28.4)	0.797
Dental surgical therapy	14 (8.3)	9 (6.0)	23 (7.2)	0.830
Intravenous drug abuse	8 (4.7)	21 (14.1)	29 (9.1)	< 0.006
Extramarital sex	8 (4.7)	10 (6.7)	18 (5.6)	0.324
Tattooing and/or piercing	7 (4.1)	6 (4.0)	13 (4.1)	0.779
Family history of hepatitis	6 (3.5)	4 (2.7)	10 (3.1)	1.000
Unknown	22 (13.0)	19 (12.8)	41 (12.9)	0.608

**t-test was used to determine the statistical differences between the means±standard deviation (SD) and Fisher's exact test between the frequencies.*
***More than one risk factor could present in one patient.*
Data presented as n(%) unless otherwise stated.

Table 3: Distribution of gender, age, risk factors, viral load, and histological findings among patients according to hepatitis C (HCV) genotype.

Characteristics	Genotype 4 n = 176	All other genotypes n = 110	*p*-value*
Gender			
Male (n = 138)	78 (44.3)	60 (54.4)	0.089
Female (n = 148)	98 (55.6)	50 (45.4)	
Age, mean ± SD, years	53.8 ± 14.7	42.6 ± 10.7	< 0.001
Duration of infection, mean ± SD, years	14.9 ± 5.4	9.1 ± 7.7	< 0.001
Risk factors for HCV infection*†			
Previous surgical procedures	64 (32.4)	28 (23.5)	0.051
Transfusion of blood or blood product	54 (27.4)	36 (30.2)	0.795
Dental surgical therapy	16 (8.1)	7 (5.8)	0.505
Extramarital sex	8 (4.0)	10 (8.4)	0.142
Intravenous drug abuse	10 (5.0)	19 (15.9)	0.002
Tattooing and/or piercing	7 (3.5)	6 (5.0)	0.574
Family history of hepatitis	7 (3.7)	3 (2.5)	0.745
Unknown	31 (15.7)	10 (8.4)	0.056
Total**	197 (100.0)	119 (100.0)	
HCV-RNA level, mean ± SD, Log copies/mL	5.8 ± 0.8	5.4 ± 1.3	0.001
Stage of fibrosis (F)			
0 (F0)	6 (3.4)	10 (9.0)	
1 (F1)	40 (22.7)	30 (27.2)	
2 (F2)	88 (50.0)	54 (49.0)	
3 (F3)	30 (17.0)	12 (10.9)	
4 (F4)	12 (6.8)	4 (3.6)	
Mean ± SD	2.0 ± 0.8	1.0 ± 0.4	< 0.001

Data presented as n(%) unless otherwise stated.
**t-test was used to determine the statistical differences between the means±standard deviation (SD) and Fisher's exact test between the frequencies.*
†More than one risk factor could present in one patient.

infection in both groups. The main source of HCV infection was blood or blood products transfusion (30.3% and 26.3% in Benghazi and Tripoli, respectively, $p = 0.797$), and previous exposure to surgical procedures (30.9% and 27.0% in Benghazi and Tripoli, respectively, $p = 0.798$). Patients with intravenous drug abuse were more frequent in Tripoli than Benghazi (4.7% vs. 14.1%, $p < 0.001$).

Table 3 shows the distribution of age, gender, risk factors, viral load, and histological findings according to HCV genotype type 4 versus non-genotype 4. In contrast to other genotypes, patients with genotype 4 were older ($p < 0.001$) and had a longer duration of HCV infection ($p < 0.001$), and had a higher level of HCV-RNA viremia ($p = 0.001$) than those with non-genotype 4.

Stage 0 or 1 fibrosis was observed in 30.0% (86/286) patients and stage 2 to 4 fibrosis in 69.9% (200/286) patients. An analysis of the histopathological alterations according to viral genotype showed a higher mean degree of fibrosis among patients infected with genotype 4 ($p < 0.001$) [Table 3].

DISCUSSION

We examined the distribution of HCV genotypes among Libyan patients with chronic hepatitis C infection attending two main tertiary care hospitals in the eastern and western parts of Libya. We also investigated the association between the distribution of HCV genotypes and demographic characteristics, potential risk factors, level of HCV viremia, and liver histopathology.

A predominance of HCV genotype 4 followed by genotype 1 was found in patients both in east and west Libya. The frequency of genotype 4 was higher in the east than the west of the country. The differential prevalence of HCV genotypes appears

to be linked to the geographic areas of origin. Libya is a Mediterranean country located in North Africa between Egypt in the east and Tunisia and Algeria in the west. Genotype 4 is the predominant genotype in Egypt accounting for more than 85% of HCV cases.[23,24] Genotype 1 is the most frequent genotype in Tunisia[25] and Algeria.[6] This geographical distribution of HCV genotypes may explain the spectrum of HCV genotypes we observed.

A recent systematic review and meta-analysis exposed that the distribution of HCV genotypes in Middle Eastern countries varies geographically.[26] The distribution follows two main patterns. HCV genotype 1 is mainly predominant in non-Arab Middle Eastern countries such as Turkey (82%) and Iran (55%) and is similar in Europe and North America. In Arab Middle Eastern countries, HCV genotype 4 is the largely predominant genotype (e.g., Egypt (86%), Palestine (67%), Yemen (64%), Qatar (64%), Iraq (64%), Syria (57%), Saudi Arabia (56%), and Kuwait (45%)).[26] Our study, as well as the previous studies from Libya, found a similar genotype pattern (genotype 4) to those observed in these countries.

We found a significant difference in the mean viral load between patients infected with genotype 4 and genotypes other than 4. Our finding is consistent with other similar reports.[27,28] This could be explained by the efficient replication of genotype 4. However, it differs from previous studies reporting that the mean viral load is not significantly different between the genotypes 1, 2, 3, and 4.[29,30]

We observed an association between histopathological changes and HCV genotype. Our findings indicate a significant risk of high-staged liver fibrosis with increased histological activity in patients infected with genotype 4 and genotype 1. Similar results have been reported in studies conducted in other countries.[31–33]

Blood transfusion and previous surgical procedures were the main modes of HCV transmission in our cohort. This is not surprising since the obligatory analysis of donated blood for anti-HCV antibodies in Libya began in 1998. Similar findings were reported in the Middle East,[34] Eastern Europe,[35] central Africa,[36] and South America.[37] Intravenous drug use, tattooing, and body piercing were not significantly associated with HCV infection in Libya, contrary to data from other countries.[38,39] Whether this is due to the small number of participants in the present study remains to be elucidated.

The importance of HCV genotyping has considerably increased in the last decade. It has been used to study the worldwide molecular epidemiology of HCV infection, and to trace sources of HCV infection in high-risk groups such as drug abusers and blood products recipients. Genotyping has also been used to study relationships between the type/subtypes and clinical status, pathogenesis, and/or disease outcome. Determination of an individual's HCV genotype is an important issue for the management of HCV infection.[40] Although the impact of HCV genotypes on the long-term outcome of HCV infection is still controversial, it is well established that different HCV genotypes are associated with different response rates to the combination treatment of chronic HCV infection with pegylated-interferon-alpha and ribavirin.[3,6] This regimen is still used as a standard therapy in low-income countries.[41,42] Patients infected with genotypes 1 and 4 have lower response rate to this regimen compared to patients infected with genotypes 2 and 3.[43,44]

A better understanding of the HCV structure, genome organization and genotyping have facilitated efforts to advance the efficacy, tolerability, and safety of HCV treatment. This has led to the development of multiple drugs targeting specific steps within the HCV life cycle, called DAAs. These are molecules that target specific nonstructural proteins of the virus and resulting in disruption of viral replication and infection. There are four classes of DAAs, which are defined by their mechanism of action and therapeutic target. The four classes are nonstructural proteins 3/4A (NS3/4A) protease inhibitors, NS5B nucleoside polymerase inhibitors, NS5B non-nucleoside polymerase inhibitors, and NS5A inhibitors.[45] Regimens that use DAAs significantly improve sustained virologic response rates above 95% in patients infected with all HCV genotypes (1 to 4), and often, also allow shorter treatment durations. Sofosbuvir is an oral NS5B polymerase inhibitor which was Food and Drug Administration (FDA) approved for HCV genotypes 1, 2, 3, and 4. The combination of ledipasvir/sofosbuvir is the first oral regimen without pegylated-interferon and ribavirin approved by the FDA for HCV.[46] Daclatasvir, an NS5A inhibitor, was FDA approved in July 2015 for use with sofosbuvir for chronic HCV genotype 3 in

treatment-naive or treatment-experienced patients.[47] The combination of ombitasvir/paritaprevir/ritonavir was also FDA approved in July 2015 for the treatment of genotype 4 without cirrhosis,[48] and for use with dasabuvir for the treatment of genotype 1 including patients with compensated cirrhosis.[49] It is worth mentioning that the access to these novel oral DAAs is limited in low-resource countries, including some countries with a high prevalence rate of HCV.

Our study had some limitations, including the relatively small number of participants and missing epidemiological evidence about their age at the time of encountering the HCV infection.

CONCLUSION

Our study demonstrated that HCV genotype 4 is the most predominate in Libya followed by genotype 1. However, as we go from the east to the west of the country, genotype 1 increases at the expense of genotype 4. Genotype 4 was associated with higher level of viremia and higher stage of liver fibrosis. It is important to note that both genotypes 1 and 4 are associated with a poor response to pegylated-interferon and ribavirin combination therapy. Our findings emphasize the need to develop improved strategies in Libya for the successful treatment of HCV infection with the newly available antiviral therapies.

Disclosure
The authors declared no conflicts of interest. No funding was received for this study.

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