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HCV Genotype and Subtype Distribution of Patient Samples Tested at University of Texas Medical Branch in Galveston, Texas

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Abstract: Six genotypes of Hepatitis C Virus (HCV), each comprising multiple subtypes have been identified worldwide. Substantial regional differences appear to exist in the distribution of HCV genotypes and subtypes. The HCV genotype variability accounts for the responses to treatments and clinical outcomes and HCV genotyping is standard of care for viral hepatitis C management. The aim of this study was to determine the distribution of HCV geno/subtypes routinely generated in the Molecular Diagnostics Laboratory at the University of Texas Medical Branch (UTMB) in Galveston, Texas. Analysis of the HCV geno/subtype results from 1,318 patients between September 2007 and February 2009 revealed subtype 1a was the most frequent, identified in 828 (62.8%) specimens. Subtypes 3a, 1b, 2b, 4, 6 and 3b were identified in 163 (12.4%), 144 (10.9%), 110 (8.2%), 7 (0.5%), 2 (0.2%) and 1 (0.1%) samples, respectively. Cases of mixed infection were rarely identified (1.4%).

Key words: Hepatitis C virus, genotype, subtype, LiPA 2.0, patients, infection

INTRODUCTION

Hepatitis C Virus (HCV) is believed to infect 200-300 million people worldwide (about 3% of the world's population). It is estimated that in the United States 2.7-3.9 million people are chronically infected with HCV and 17,000 new HCV infections occur annually. Between 70-80% of people who contract HCV infection develop chronic infection. Chronic hepatitis C is closely associated with the development of cirrhosis and hepatocellular carcinoma and is the most common cause of adult liver transplantation in the United States and the world (Saito *et al*., 1990; Shuhart *et al*., 1997).

HCV is a member of the genus *Hepacivirus* belonging to the family Flaviviridae. The virus has a single-stranded RNA approximately 9,600 bp genome (Tang and Grise, 2009). A comparison of HCV genomic sequences from around the world revealed substantial heterogeneity of nucleotide sequences within several regions of the viral genome (Choo *et al*., 1991).

Phylogenetic analyses have shown that HCV strains can be classified into 6 genotypes (numbered 1-6) and a large number of subtypes within each genotype (Simmonds *et al*., 2005). HCV genotypes 1-3 appear to have a worldwide distribution but their relative prevalence varies from one geographic area to another. HCV genotype 1 is reported to be the most common in the United States (Nainan *et al*., 2006; Rustgi, 2007). Current treatment of HCV infection is a combination of pegylated interferon α and the antiviral drug ribavirin for a period of 24 and 48 weeks, depending on HCV genotype. Sustained cure rate (sustained viral response) of 75% or better occurs in patients with HCV genotypes 2 and 3 after 24 weeks of treatment whereas the rates are 50% in patients with HCV genotypes 1 and 6 given 48 weeks of treatment and 65% in patients with genotype 4 given 48 weeks of treatment, respectively. Therefore, HCV genotype is an important parameter for clinical management of HCV infection and HCV genotyping is routinely performed in clinical laboratories (Al-Olaby and Azzazy, 2011; Teoh *et al*., 2010). Genotype data that are routinely generated in clinical laboratories can also be used to examine genotype distribution and molecular epidemiology of HCV infection in the patient populations and geographical regions which clinics/laboratories serve.

Additionally, the new generation of HCV genotyping platforms can accurately provide subtype information (Verbeeck *et al*., 2008). Since HCV genotyping is part of routine patient care, linking particular geno/subtypes with other clinical information should help to recognize the potential impact of HCV geno/subtypes on patient prognosis. In this study we analyzed HCV genotyping results from 1,318 patients that were tested in the Molecular Diagnostics Laboratory in the Department of

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Pathology at the University of Texas Medical Branch (UTMB) in Galveston, Texas between September 2007 and February 2009.

MATERIALS AND METHODS

Specimens and RNA isolation: HCV genotyping results were generated in 1,318 patients referred to the Molecular Diagnostics Laboratory at UTMB between September 2007 and February 2009. The study was approved by the UTMB Institutional Review Board (IRB). RNA was extracted using QIAGEN MagAttract Virus Mini M48 kit (Qiagen, Valencia, CA, USA). Briefly, lysis of 200 μL of serum or plasma was carried out in the presence of protease and buffer AL provided in the QIAGEN kit. Lysates were thoroughly mixed with MagAttract suspension B to allow optimal adsorption of viral RNA to the silica surface of the magnetic particles. Viral RNA remained bound to the magnetic particles while contaminants were removed by a sequence of wash steps with buffers AW1, AW2 and ethanol. The viral RNA was eluted in 50 μL buffer AVE supplied by the kit.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): The reverse transcription of RNA into complementary DNA (cDNA) and the amplification of a 240 bp fragment of the 5'untranslated region (5'-UTR) and a 270 bp fragment of core region of HCV were performed simultaneously using VERSANT HCV Amplification 2.0 kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) as previously described (Verbeeck *et al*., 2008). The master mix was prepared using the supplied ENZ MIX with reverse transcriptase, HotStartTaq polymerase and uracil-N-glycosylase and the AMP MIX with two pairs of biotinylated primers, deoxynucleoside triphosphates, deoxyuridine triphosphates, magnesium chloride and RNasin. Reactions were performed in individual tubes containing 15 μL of master mix and 10 μL of the extracted RNA. The tubes were placed for 10 min at room temperature prior to amplification to allow degradation of any contaminating uracil containing DNA.

PCR was performed using GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster city, CA, USA) using the following thermal profile: reverse transcriptase at 50°C for 30 min; initial PCR activation at 95°C for 15 min; denaturation at 95°C for 30 sec; annealing at 50°C for 30 sec; extension at 72°C for 15 sec; repeated denaturation, annealing and extension 40 times and final extension at 72°C for 2 min.

Genotype analysis: The VERSANT HCV Genotype Line Probe 2.0 kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY) that detects both 5'-UTR and core regions of HCV was used to genotype and subtype HCV (Verbeeck *et al*., 2008). Probes with poly (T) tails, specific for the 5'-UTR and core region of the different HCV genotypes and subtypes were bound to nitrocellulose strips in parallel bands. About 21 bands including the conjugation control and amplification control 1, represented the 5'-UTR region; 4 bands, including amplification control 2, represented the core region. Biotinylated RT-PCR amplicons were hybridized to nitrocellulose strips. The hybridization assays were performed in an Autoblot 3000H apparatus (MedTec, Hillsborough, NC, USA). After hybridization, alkaline phosphatase-streptavidin conjugate was bound to the biotinylated amplicons in the hybrids. The substrate solution (5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium) reacted with the alkaline phosphatase-streptavidin complex and formed a purple/brown precipitate which resulted in a visible banding pattern on the strips.

Aligning the developed nitrocellulose assay strips with the LiPA Reading Card and comparing the band patterns with patterns listed on the LiPA 2.0 interpretation chart determined HCV genotypes and subtypes.

Statistical analysis: Adjusted Odds Ratios (OR) and the 95% Confidence Intervals (CI) for age group, gender and ethnicity were calculated using logistic regression in SUDAAN as described (Nainan *et al*., 2006). For the analysis of data related to HCV geno/subtypes and patient demographics there were sufficient numbers to calculate prevalence estimates for persons infected with genotypes 1-3 and subtypes 1a and 1b in Caucasians, African-American and Hispanics.

RESULTS AND DISCUSSION

Determination of HCV genotype is useful to assess the likelihood of response to antiviral therapy. Because of geographic clustering of distinct HCV genotypes, genotyping is also a useful tool for molecular epidemiology investigation of HCV in a given population (Choo *et al*., 1991). Researchers used the new generation line-probe assay (LiPA 2.0, Siemens Medical Solutions Diagnostics, Tarrytown, NY) for genotyping which utilizes sequences from the core region in addition to the 5'-UTR to identify genotype 6, subtypes c-l. The discrimination of genotype 6 subtypes from genotype 1 may be clinically relevant because genotype 6 may have a better sustained virologic response than genotype 1 (Hui *et al*., 2003). Moreover, the new generation line-probe assay improves the accuracy of the identification of

Table 1: Distribution of HCV geno/subtypes according to demographic characteristics

	Percentages of HCV geno/subtypes														
Characteristics	1a	1b	1	1a/1b	2	2a/2c	2b	2c	3	3a	3b	4	6	1/2	1/3
Age (years)															
1-19 (n = 3)	100.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20-39 (n = 277)	60.3	7.2	1.4	-	2.2	-	10.8	-	-	16.6	-	1.1	0.4	-	-
40-59 (n = 983)	63.4	11.8	1.6	0.1	1.0	0.9	7.5	0.1	0.7	11.5	0.1	0.4	0.1	0.6	0.1
≥60 (n = 55)	63.6	14.5	5.5	-	1.8	-	7.3	-	-	7.3	-	-	-	-	-
Gender															
Male (n = 1072)	63.1	11.0	1.9	0.1	1.0	0.7	7.6	0.1	0.6	12.7	-	0.6	0.2	0.6	0.1
Female (n = 246)	61.8	10.6	1.2	-	2.4	0.8	11.0	-	0.4	11.0	0.4	0.4	-	-	-
Ethnic group*															
Caucasian (n = 612)	59.5	7.8	1.5	-	2.0	1.1	11.6	0.2	0.3	14.5	0.2	0.3	-	0.8	0.2
African-America (n = 345)	70.7	21.4	2.6	-	0.3	-	2.3	-	0.6	1.4	-	0.6	-	-	-
Hispanic (n = 311)	63.3	6.1	1.3	0.3	1.0	0.6	7.4	-	0.3	18.6	-	0.6	-	0.3	-
Asian (n = 4)	-	-	-	-	-	-	-	-	-	50.0	-	-	50.0	-	-
Total (n = 1318)	62.8	10.9	1.8	0.1	1.3	0.7	8.2	0.1	0.5	12.4	0.1	0.5	0.2	0.5	0.1

*Ethnic information was available in 1272 patients

Table 2: Distribution of HCV geno/subtypes among different geographic regions

	HCV geno/subtype case number (%)						
Geographic region	1a	1b	2*	3a	4	Others	References
United States (n = 179)	104 (58.0)	38 (21.0)	27 (15.0)	8 (5.0)	2 (1.0)	0 (0.0)	Zein *et al.* (1996)
Northeast Mexico (n = 147)	42 (28.6)	55 (37.4)	20 (13.6)	15 (10.2)	2 (1.4)	13 (8.8)	Rivas-Estilla *et al.* (2008)
Mexico (n = 421)	75 (17.8)	169 (40.1)	97 (23.1)	22 (5.2)	2 (0.5)	56 (13.3)	Dehesa-Violante *et al.* (2007)
Turkey (n = 89)	17 (19.1)	67 (75.3)	3 (3.4)	0 (0.0)	2 (2.2)	0 (0.0)	Abacioglu *et al.* (1994)
Former Soviet Union (n = 131)	6 (5.0)	107 (82.0)	5 (5.0)	13 (10.0)	0 (0.0)	0 (0.0)	Viazov *et al.* (1997)
Iran (n = 156)	59 (37.8)	26 (16.7)	0 (0.0)	45 (28.8)	2 (1.3)	24 (15.4)	Kabir *et al.* (2006)
Southeast United States (texas) (n = 1318)	828 (62.8)	144 (10.9)	135 (10.2)	163 (12.4)	7 (0.5)	41 (3.1)	This study

*Included all subtypes of genotype 2

subtypes 1a and 1b which is limited by only using the 5'-UTR for analysis (Chen and Weck, 2002; Nadarajah *et al.*, 2007; Verbeeck *et al.*, 2008).

The most frequent HCV geno/subtype among the 1,318 patients tested at UTMB between September 2007 and February 2009 was 1a (62.8%), followed by 3a (12.4%), 1b (10.9%) and 2b (8.2%) (Table 1). About 7 cases of genotype 4 and 2 cases of genotype 6 as well as 19 cases of mixed geno/subtypes were identified (Table 1). HCV genotype 1a was the most prevalent in the patient population which is consistent with a study published in 1996 that involved 4 geographic regions in the United States (Midwest, Northeast, Southeast and West) where genotype 1a was the predominant in all 4 areas (Zein *et al.*, 1996). Zein *et al.* (1996) reported subtype 1b as the second most common, followed by 2b and 3a whereas we found that the second most common subtype was 3a followed by subtypes 1b and 2b (Table 1).

In Southern and Eastern Europe, subtype 1b has been reported to be the most common similar to Turkey and Russia (Table 2) (Abacioglu *et al.*, 1994; Dusheiko *et al.*, 1994; Viazov *et al.*, 1997). On the other hand, subtype 1a is more prevalent in Iran, closely followed by 3a and 1b, similar to the genotype distribution in India (Table 2) (Chowdhury *et al.*, 2003; Kabir *et al.*, 2006). Genotype analysis of HCV performed in the Mexican population revealed a predominance of subtype 1b (Table 2) (Dehesa-Violante *et al.*, 2007; Rivas-Estilla *et al.*, 2008). However, when we evaluated at the distribution of genotypes by ethnic group we found that among Hispanics a group composed of a high percentage of Mexican immigrants, subtype 1a was the most common similar to the general population of the United States (Table 1 and 2). The study revealed a male predominance in the tested patient population (63.1%). The majority of cases (63.4%) were between age 40 and 59 (Table 1). Caucasian, African-American, Hispanic and Asian patients constituted, respectively 48.1, 27.1, 24.5 and 0.3% of the test population (Table 1). The sample size and geno/subtype prevalence permitted further analysis of the relative odds of infection by genotypes 1-3 and subtypes 1a and 1b among Caucasians, African-American and Hispanics. This analysis revealed that in reference to Caucasians, African-American were 8.89 fold more likely to be infected with genotype 1 instead of genotypes 2-3 and 2.30 fold more likely to be infected with 1b instead of 1a (Table 3). It has been shown that African-American seem to have a lower sustained viral response to the combined treatment of interferon and ribavirin (Howell *et al.*, 2000; Muir *et al.*, 2004) which may correlate with the higher odds of infection with genotype 1 as well as subtype 1b in the African-American population.

Table 3: Relative odds of genotype 1 infection and relative odds of genotype 1b infection among patients infected with genotypes 1-3 according to demographic characteristics[1]

	Adjusted odds ratio (95% CI)[2]	
Characteristics	Genotype 1 vs. 2 and 3	Genotype 1b vs. 1a
Age (years)		
<40	Reference	Reference
≥40	1.52 (1.13-2.05)	1.60 (0.97-2.64)
Sex		
Male	1.19 (0.86-1.64)	0.98 (0.62-1.55)
Female	Reference	Reference
Ethnic group		
Caucasian	Reference	Reference
African-American	8.89 (5.22-15.10)	2.3 (1.55-3.42)
Hispanic	1.1 (0.82-1.49)	0.73 (0.42-1.28)

[1]Analysis included only Caucasians, Blacks and Hispanics. [2]Based on logistic regression analysis by using sample weights (Nainan *et al.*, 2006)

Several studies provided evidence that infection with HCV subtype 1b usually has a more aggressive clinical course and is most often associated with the development of hepatocellular carcinoma in non-cirrhotic liver and with unresponsiveness to interferon treatment (Dusheiko *et al.*, 1994; Kabir *et al.*, 2006; Teoh *et al.*, 2010).

CONCLUSION

In the study, researchers determined that the most frequent HCV subtype in the patient population was 1a, followed by 3a, 1b and 2b. To the knowledge, this is the 1st report of HCV geno/subtype distribution in this patient population and it could serve as a starting point for further studies in particular to confirm the role of ethnicity in the predominance of HCV subtypes and response to therapy and to evaluate distribution of HCV subtypes and route of infection among subpopulations.

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