HEPATITIS C VIRUS GENOTYPING IN SULAIMANI GOVERNORATE

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Abstract

To determine the HCV genotypes among HCV-infected patients in Sulaimani governorate Kurdistan region- Iraq, 76 blood samples were collected (60 samples were positive and 16 samples were negative for anti-HCV antibody) from different groups the samples comprised: 21 (27.63%) hemodialysis patients, 38 (50%) thalassemia patients, 4 (5.26%) cancer patients, 3 (3.95%) gastritis patients, 1 (1.32%) hemophilia patient and 9 (11.84%) blood donors. RT- nested PCR analysis showed that 35 (46.05%) were positive and 41 (53.95%) were negative for HCV-RNA. Amplification of 5'UTR of HCV-RNA positive samples were done by RT- nested PCR; the amplified product was purified and sequenced in IBL laboratory in Vienna. The sequenced samples were BLAST on NCBI online website. The alignment sequence and phylogenetic analysis used CLUSTAL W online software version 1.83 of 24 samples showed that genotype 1 was the predominant genotype among HCV infected patients in Sulaimani governorate, with an overall of (87.5%) patients. Genotype 2 was found in (8.3%), and genotype 4 was seen in (4.2%) patients.

The study aimed to detect the HCV- RNA in the blood samples by RT-nested PCR analysis and sequencing in order to determine the HCV genotypes in Sulaimani governorate-Kurdistan-Iraq.

Keyewords: HCV genotyping, HCV geographycial distribution, HCV PCR-sequencing

Introduction

The hepatitis C virus (HCV) is the main causative pathogen of post-transfusion hepatitis (Gallego and Varani, 2002). The virus establishes a persistent infection in the liver, leading to the development of chronic

hepatitis, liver cirrhosis and hepatocellular carcinomas (Yokota *et al.*, 2003). More than 75% of HCV infections become chronic and up to 20–30% progress to cirrhosis (Ardalan *et al*., 2004). According to the estimations of the World Health Organization, approximately 170 million people, 3% of the world population, are HCV positive with 3 to 4 million de novo infections each year (Pan *et al.,* 2007). HCV, first identified in 1989 (Choo *et al.,* 1989), is an enveloped virus classified in the *Hepacivirus* genus of the family *Flaviviridae* (Lindenbach *et al.*, 2007). HCV has a single-stranded, positive-sense RNA genome which contains two short untranslated region at each end (5'UTR and 3'UTR) and a single open reading frame that is translated to yield the viral proteins in the form of a polyprotein (Carrat *et al*., 2004). HCV demonstrates a high degree of sequence variation throughout its genome (Zein, 2000). Based mainly on phylogenetic analyses, all HCV isolates are grouped into six major HCV genotypes comprising numerous, more closely related subtypes (Robertson *et al*., 1998). Knowledge about HCV genotypes that infects people is important and affects the dosage and duration of the antiviral therapy. Patients infected with genotype 2 or 3 showed better response than those infected with genotype 1 or 4 (Carrat *et al.*,2004).

Materials and Methods

Sampling. Blood samples were collected between July/2009-October/2009 in Sulaimani governorate-Kurdistan-Iraq. Seventy six blood samples were collected from different groups all samples were screened for anti-HCV antibody by enzyme linked immunosorbent assay (ELISA) technique. The samples were collected from different ages, ranging from (2 - 80) year old. The serum was directly separated and each specimen was divided into two aliquot in labeled screw caped vials and frozen at (– 40°C) until used for performing the tests: one for HCV RNA detection and the other for sequencing.

HCV RNA detection and RT-PCR.

HCV RNA extracted from anti-HCV positive and anti-HCV negative sera. Five µl of extracted RNA was put in 95°C for 1 minute then placed on ice, single step cDNA synthesis and first round PCR was done according to the STRP^{Tm} Hepatitis C Virus detection kit- Cinnagen.

Three µl from the first round PCR product (RT-PCR) was used for the second round PCR (nested PCR) which gives (216 bp) product, the mixture put in thermal cycler run 35 cycles with the following parameters: 93°C – 40 seconds, 60°C – 40 seconds, 72°C – 40 seconds. The PCR products were analyzed on 2% agarose gel.

Amplification of HCV 5'UTR by RT nested PCR.

cDNA was synthesized at 45°C – 60 minute using 1µl of outer anti-sense primer according to RevertAid^{Tm} First Strand cDNA Synthesis kit-Fermantas. First round PCR reaction was performed in a volume of 25µl. By using outer sense (5'-CGTTAGTATGAGTGTCGTGC-3') and outer anti-sense (5'-GGTGCACGGTCTACGAGACCT-3') primers. The samples were placed in a thermal cycler. The samples were preheated at 94˚C for 3 minute followed by 35 cycles, each consisting of denaturing for 30 seconds at 94˚C, annealing for 40 seconds at 59.4˚C, and extension for 30 seconds at 72˚C with the final elongation at 72˚C for 10 minutes. For the amplification of 341bp 5'UTR nested PCR was analyzed using inner sense (5'-AGTGTCGTGCAGCCTCCAGG-3') and inner anti-sense (5'-GCAAGCACCCTATCAGGCAGT-3') primers. The samples were preheated at 94˚C for 3 minutes followed by 35 cycles, each consisting of denaturing for 30 seconds at 94˚C, annealing for 40 seconds at 61.5˚C, and extension for 30 seconds at 72˚C with the final elongation at 72˚C for 10 minutes.

Sequencing PCR of 5'UTR and phylogenetic analysis.

The amplified product was purified and sequenced in IBL laboratory in Vienna. The sequenced samples were BLAST on NCBI online website. The alignment sequence and phylogenetic analysis used CLUSTAL W online software version 1.83. The accession numbers of the prototype genotype sequences used to compare the 5'UTR sequences were as follows: genotype 1, AJ006322, EU164941, EU256033, EU862829, AF009606, AF011751, M62321, D10749. genotype 2, AB030907, AF177036. genotype 3, D17763. genotype 4, Y11604, AY766601. genotype 5, Y13184. genotype 6, D84262.

Statistical analysis. Statistical analysis was performed using free SPSS version 16 software, by applying the statistical analysis (Chi-square) test. P value less than 0.05 was considered as statistically significant.

Results.

The results of screening the 76 blood RT-PCR analysis showed that 35 (46.05%) samples were positive and 41 (53.95%) were negative for HCV-RNA, and statistical analysis showed significant differences between both techniques (Table 1). Two hundred and sixteen (216)bp band of the positive control was used as a detector for the HCV-RNA. The gel electrophoresis analysis, represent RT- nested PCR products for detected HCV in gastritis, hemophilia and cancer samples were clarified in (Fig.1). On the basis of sequencing and phylogenetic analysis, the 24 Sulaimani samples were classified as follows: genotype 1 in 21 patients (87.5%), genotype 2 in 2

patients (8.3%) and one patient with genotype 4 (4.2%) (Table 2). Phylogenetic tree of 24 Sulaimani HCV samples and some close reference HCV strains published in Gene Bank by using CLUSTAL W online software as shown in (Fig. 2).

Table 1: Positive and negative results for both ELISA and RT-PCR

Group	Total No. Examined	ELISA		RT-PCR	
		positive	negative	positive	negative
Thalassemia	38	34	4	14	24
Hemodialysis	21	16	5	11	10
Cancer	4	4	0	4	0
Gastritis	3	3	0	2	1
Hemophilia	1	1	0	1	0
Blood donor	9	2	7	3	6
Total	76	60 (78.95) **%**	16 (21.05 **) %**	35 (46.05) **%**	41 (53.95) **%**
P value	0.002				

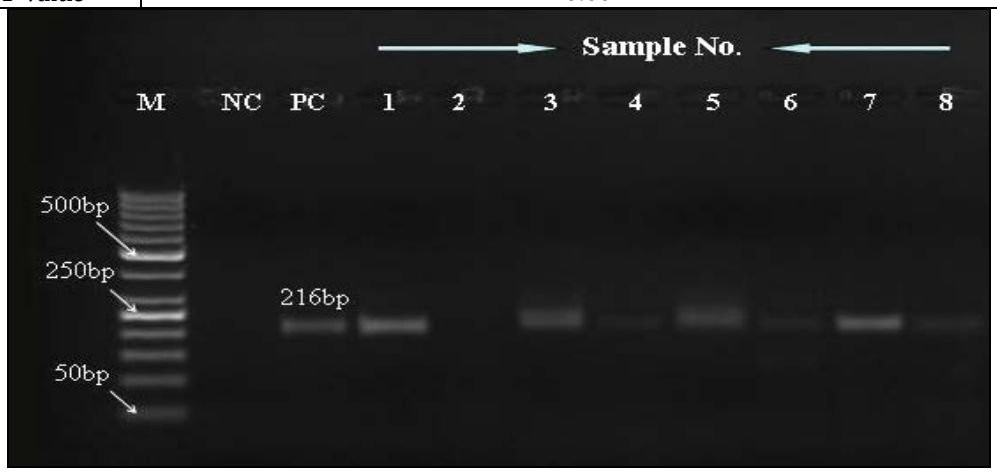

Figure 1: Gel electrophoresis for RT-nested PCR product from patients with gastritis, hemophilia and cancer samples

M: 50bp DNA ladder, **NC:** negative control, **PC:** positive control**, samples 1- 8**: represent samples

Table 2: The number and percentage of HCV genotypes in different patient groups in Sulaimani governorate

Group	Sequenced No.	No. of samples assigned to the following genotypes		
		Genotype 1	Genotype 2	Genotype 4
Thalassemia	8 (33.3%)	8	0	0
Hemodialysis	8 (33.3%)	7	1	0
Cancer	4(16.7%)	3	1	0
Gastiritis	1 (4.2%)	1	0	0
Hemophilia	1 (4.2%)	0	0	1

Blood donor	2 (8.3%)	2	0	0
Total	**24**	**21 (87.5%)**	**2 (8.3%)**	**1 (4.2%)**

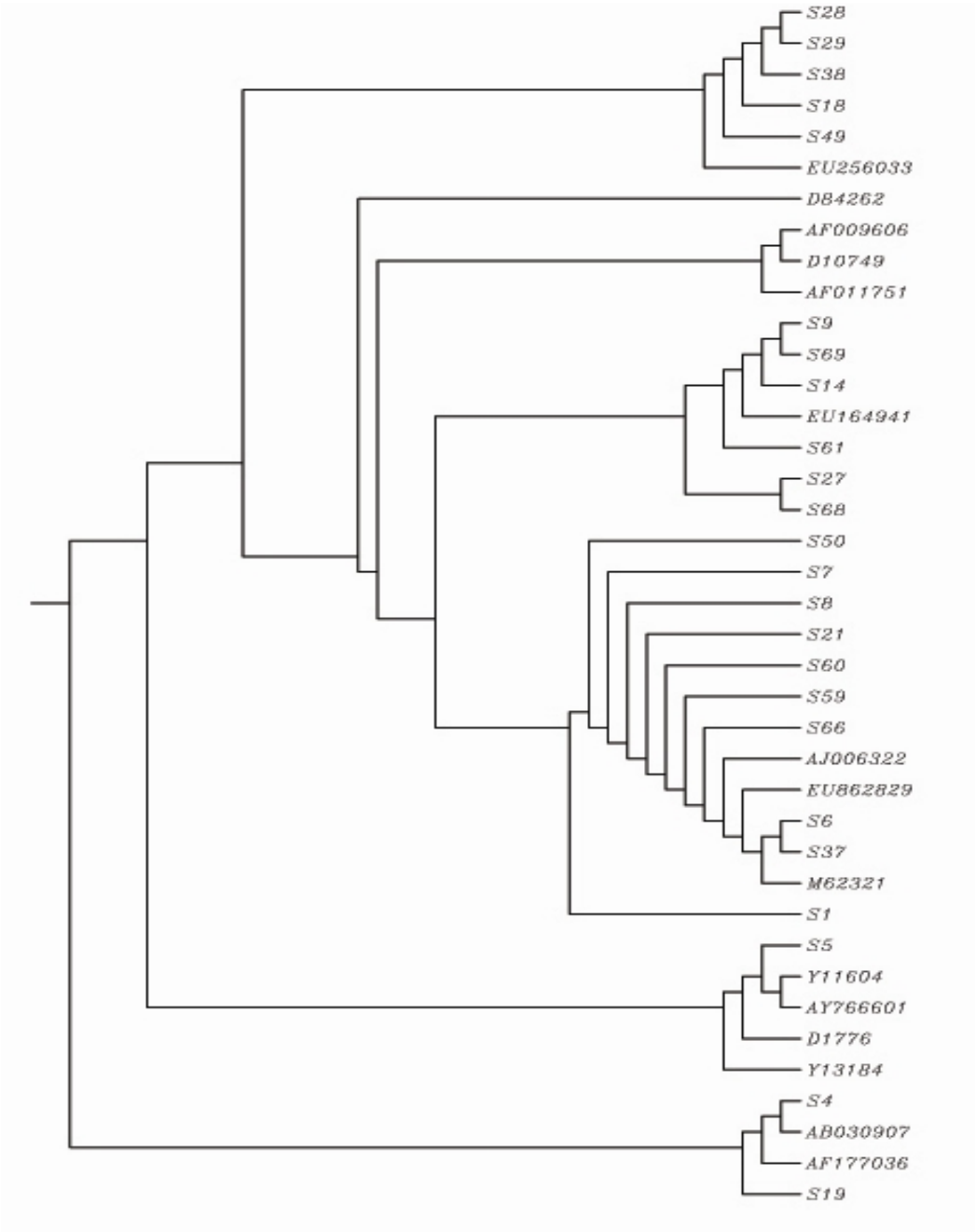

Figure 2: phylogenetic tree of HCV 5'UTR of 24 Sulaimani HCV samples with reference HCV strains published in Gene Bank. S: represent Sulaimani HCV samples.

Discussion

The ultimate goal of this investigation is to determine the genotype of HCV in Sulaimani governorate. The importance was to provide treatment of HCV patients. This study is considered to be the first study on the genotyping of HCV in Sulaimani governorate, Kurdistan region of Iraq among different patient groups. A total of 76 serum samples were investigated for HCV- RNA, followed by genotyping, out of those, 35 (46.05%) sera displayed HCV-RNA positive results and 41 (53.95%) sera demonstrated negative HCV-RNA (Table 1). The results were in agreement with those obtained by Hama, (2009) who observed that not all anti-HCV positive samples showed positive results for HCV-RNA (63.7%) in Sulaimani. Two (12.50%) of the 16 negative anti-HCV samples displayed HCV-RNA positive results. The obtained results were relatively in agreement with the results of Al- Kubaisy *et al.,* (2006) who found that 2 (16.7%) samples were positive for HCV-RNA from 12 anti-HCV negative sera. The presence of specific antibodies against HCV and absence of HCV-RNA is a common finding and may be related to one of the following causes: a) there is a cross reaction with antibodies different from anti-HCV (Ren *et al.,* 2005), b) the initial level of viraemia may be low and below detectable levels for PCR assay at the time of sampling (Kanistanon *et al.,* 1997; Mison *et al.,* 1997), or c) patients might have been cured of HCV infection at the time of sampling (Simmonds, 1997). HCV has high genomic variability and at least six different genotypes and an increasing number of subtypes have been reported (Simmonds, 1999). Genotypes 1, 2 and 3 are predominantly found in Europe, Japan, Brazil, Australia and the United States (Busek and Oliveria, 2003). HCV genotyping is important because it provides information as to strain variation and potential association with disease severity. In addition, it is of epidemiologic value because it sheds light on whether prevalent HCV strains are similar to that endemic in a certain region, such as here in the Middle East (Kabir *et al.,* 2006). Although, the 5'UTR contains multiple genotype-specific sequences distributed over small variable regions, which provide accurate genotyping information for HCV genotypes but is not able to completely differentiate between all subtypes (Chen and Weck, 2002; Noppornpanth *et al.,* 2006). In the present study the HCV genotypes were determined by sequencing the 5'UTR and comparing it with reference sequences of known genotypes. A similar assay was used for HCV genotyping by Campiotto *et al.,* (2005), and Idrees *et al.,* (2009). Results of the sequence analysis of 5'UTR region demonstrated that genotype 1, 2 and 4 were detected in 21 (87.5%), 2 (8.3%), and 1 (4.2%) samples respectively as shown in (Table 2). Genotype 1 was found as a predominant genotype among studied patients in Sulaimani governorate. These results were in accordance with the predominance of genotype 1

observed in most countries worldwide (Viazov *et al.,* 1994; Simmonds, 1995). Genotype 1 representing 77% in China, 82% in Spain, 90% in Brazil (Holland *et al.,* 1996), 86.92% in Germany (Feucht *et al.,* 1996), 85% in the United States (O'Brien *et al.,* 1997), and 82% in Bulgarian patients (Anotonov, 2010). Genotype 1 was the predominated in Turkey and Israel as well (Chamberlain *et al.,* 1997; Bozdayi *et al.,* 2004), and based on a study done in Jordan, genotype 1 was the dominant genotype (73.3%) (Bdour, 2002). The pattern of observed genotypes are similar to those reported by Jia-Qing *et al.,* (2006) from Hong Kong but it was different from other Middle East countries such as Republic of Yemen, Kuwait, Iraq, and Saudi Arabia, where genotype 4 was the most prevalent (Ohno *et al.,* 1996). Although genotype 4 was almost entirely reported from the Middle East and western countries (Mellor *et al.,* 1995), this genotype was uncommon in Sulaimani governorate according to the results of this study. Data from the present work differ from those published by Al-Kubaisy *et al.,* (2006) who observed that HCV genotype 4 (35.4%) was more predominant than other genotypes among Iraqi thalassemia patients, also differ from the results of Al- Kubaisy, (1998) who demonstrated that the common HCV- genotypes circulating among Iraqi pregnant women were 4, 1, 1a, 1b and 3a, in decreasing order. As mentioned, the present study indicated that in Sulaimani governorate, HCV genotype 1 was predominant. This result was not surprising because Kabir *et al.,* (2006) and Somi *et al.,* (2008) demonstrated that HCV genotype 1 was the most prevalent genotype in Iran, a neighboring country to the governorate. This can be attributed to the fact that many patients get treatment in the Iranian hospitals, familiar relationships, also due to the mass-fled and the immigration between the province and Iran, and afterwards the return of many inhabitants of Sulaimani who lived in Iran for along time. Another important finding in this study was the detection of genotype 2 for the first time in Iraq from two patients one of them coming back from Europe. This genotype was found in many countries like, Spain (Esteban *et al.,* 1996), Japan (Lee *et al.,* 2001; Mukaide *et al.,* 2005), Italy (Cicciarello *et al.,* 1997;Simpore *et al.,* 2004), Brazil (Campiotto *et al.,* 2005), and Mexico (Sosa-Jurado *et al.,* 2010). The presence of this genotype in Sulaimani governorate may reflect the growing communications with these countries. Another important finding in the present study was the detection of genotype 4 only in one hemophilic patient who had a history of treatment in Baghdad. This result was in a concordance with the results of Al-Kubaisy, (1998) and Al-Kubaisy *et al.,* (2006) they reported predominate of genotype 4 among pregnant women and thalassemia patients in Iraq respectively. Besides, in a study, the predominance of HCV genotype 4 is reported in the Arab world, and most other countries in the Middle East (Matar *et al.,* 1996). It is also worth mentioning that genotype 4 was the

second major genotype existing in Iran (Idrees *et al.,* 2009). Multiple sequence alignment of Sulaimani HCV samples with genotype prototype sequences selected from the Gene Bank (Fig 2) the 24 Sulaimani HCV samples can be classified on the basis of phylogenetic analysis of the 5'UTR, with reference isolates and Sulaimani HCV samples grouped according to the close relatedness to reference genotype isolates. The occurrence of many variants is not surprising because such variations have also been reported from neighboring countries. The possibility of identifying more and more variants cannot be ruled out in the present situation of high prevalence of hepatitis C in Iraq. In the present study, the HCV viral samples didn't classify to subtype level due to the limitations of information on differentiation between different HCV subtypes by 5'UTR analysis. In some isolates, only one or two minor nucleotide changes distinguish subtypes, e.g., an adenine to guanine substitution between subtypes 1a and 1b. The relative failure in subtyping the genotype 2 samples at the 5'UTR by LiPA methodology has already been reported (Stuyver *et al.,* 1995). Furthermore this method was unable to detect more than one genotype if present in the patient (Idrees *et al.,* 2009). Nevertheless, classifying HCV at the genotype level has been shown to be sufficient for clinical prognosis and treatment orientation (Campiotto *et al.,* 2005).

Conclusion

We conclude that (i) The 5'UTR sequence analysis was sufficient for the routine genotyping of HCV in clinical settings. (i i) The predominant HCV genotype circulating among HCV- infected patients in Sulaimani governorate, Kurdistan region of Iraq was genotype 1.

References:

Gallego J; and Varani G. 2002. The hepatitis C virus internal ribosome-entry site: a new target for antiviral research. *Biochemical Society Transactions*; 30 (part 2):140-146.

Yokota T; Sakamoto N; Enomoto N; Tanabe Y; Miyagishi M; Maekawa S; Yi L; Kurosaki M; Taira K; Watanabe M; and Mizusawa H. 2003. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO reports*; 4: 602–608.

Zein N. N. 2000. Clinical Significance of Hepatitis C Virus Genotypes. *Clin Microbiol Rev*; 13(2): 223-235.

Carrat F; Bani-Sadr F; Pol S; Rosenthal E; Lunel-Fabiani F; Benzekri A; Morand P; Goujard C; Pialoux G; Piroth L; Salmon-Ceron D; Degott C; Cacoub P; Perronne C; ANRS HCO2 RIBAVIC Study Team. 2004. Pegylated interferon alfa-2b vs standard interferon alfa-2b plus ribavirin for

chronic hepatitis C in HIV-infected patients: a randomized controlled trial. *JAMA*; 292(23): 2839-2848.
Ardalan F. A; Osquei M. RF; Toosi M. N; and Irvanloo G. 2004. Synergic effect of chronic hepatitis C infection and beta thalassemia major with marked hepatic iron overload on liver fibrosis: a retrospective cross-sectional study. *BMC Gastroenterology*; 4: 1- 4.
Choo Q. L; Kuo G; Weiner A. J; Overby L. R; Bradley D. W; and Houghton M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A non-B viral hepatitis genome. *Science*; 244(4902): 359-362.
Lindenbach B. D; Thiel H. J; and Rice C. M. 2007. Flaviviridae: the virus and their replication . Fields Virology. eds Knipe DM. and Howley PM. (Lippincott Williams & Wilkins Philadelphia) 5th Ed 1: pp.1101-1152.
Pan Q. W; Henry S. D; Scholte B. J; Tilanus H. W; Janssen H. LA; and van der Laan L. JW. 2007. New therapeutic opportunities for Hepatitis C based on small RNA. *World J Gastroenterol*; 13(33): 4431-4436.
Robertson B; Myers G; Howard C; Brettin T; Bukh J; Gaschen B; Gojobori T; Maertens G; Mizokami M; Nainan O; Netesov S; Nishioka K; Shin-I T; Simmonds P; Smith D; Stuyver L; and Weiner A. 1998. Classification nomenclature and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. *VDN*; 143(12): 2493-2503.
Al-Kubaisy W. A; Al-Naiband K. T; and Habib M. 2006. Seroprevalence of hepatitis C virus specific antibodies among Iraqi children with thalassaemia. *Eastern Mediterranean Health Journal*; 12: 204-210.
Hama S. A. 2009. Prevalence of Hepatitis B C and D infections among Different Risk Groups in Sulaimani Governorate. Ph.D. Thesis. University of Sulaimani.
Kanistanon D; Neelamek M; Dharakul T; and Songsivilai S. 1997. Genotypic Distribution of Hepatitis C Virus in Different Regions of Thailand. *Journal of Clinical Microbiology*; 35(7): 1772–1776.
Mison L. M; Yong I. F; ODonghue M; Cowley N; Thorlton N; and Hyland C. A. 1997. Prevalence of hepatitis C virus and genotype distribution in an Australian volunteer blood donor population. *Transfusion*; 37(1): 73–78.
Ren F. R; Lv Q. S; Zhuang H; Li J. J; Gong X. Y; Gao G. J; Liu C. L; Wang J. X; Yao F. Z; Zheng Y. R; Zhu F; Tiemuer M; Bai X; and Shan H. 2005. Significance of the signal-to-cutoff ratios of antihepatitis C virus enzyme immunoassays in screening of Chinese blood donors. *Transfusion*; 45(11): 1816-1822.
Simmonds P. 1997. Clinical prevalence of hepatitis C virus genotypes. *Gut*; 40: 291–293.
Simmonds P. 1999. Viral heterogeneity of the hepatitis C virus. *J Hepatol*; 31(suppl 1): 54–60.

Busek S; and Oliveria G. 2003. Molecular epidemiology of the hepatitis C virus in Brazil. *Genet Mol Res*; 2: 117-23.
Kabir A; Alavian S-M; and Keyvani H. 2006. Distribution of hepatitis C virus genotypes in patients infected by different sources and its correlation with clinical and virological parameters: a preliminary study. *Comparative Hepatology*; 5(4): 1-6.
Chen Z; and Weck K. E. 2002. Hepatitis C virus genotyping: interrogation of the 5' untranslated region cannot accurately distinguish genotypes 1a and 1b. *J Clin Microbiol* ; 40: 3127-3134.
Noppornpanth S; Sablon E; De Nys K; Lien T. X; Brouwer J; Brussel M. V; Smits S. L; Poovorawan Y; Osterhaus A. D; and Haagmans B. L. 2006. Genotyping hepatitis C viruses from Southeast Asia by a novel line probe assay that simultaneously detects core and 5' untranslated regions. *J Clin Microbiol*; 44(11): 3969-3974.
Campiotto S; Pinho J. R. R; Carrilho F. J; Da Silva L. C; Souto F. J. D; Spinelli V; Pereira L. M. M. B; Coelho H. S. M; Silva A. O; Fonseca J. C; Rosa H; Lacet C. M. C; and Bernardini A. P. 2005. Geographic distribution of hepatitis C virus genotypes in Brazil. *Brazilian Journal of Medical and Biological Research*; 38: 41-49.
Idrees M; Butt. S; Awan Z; Aftab M; Khubaib B; Rehman I; Akram M; Manzoor S; Akbar H; Rafiqe S; and Riazuddin S. 2009. Nucleotide identity and variability among different Pakistani hepatitis C virus isolates. *Virology Journal*; 6(130): 1-6.
Anotonov A. K. 2010. Diagnostic and treatment of chronic HCV infection in Bulgaia past and present. *Clujul Medical*; 1: 13-15.
Feucht H. H; Zollner B; Schroter M; Hoyer A; Sterneck M; and Polywka S. 1996. Distribution of Genotypes and Response to Alpha-Interferon in Patients with Hepatitis C Virus Infection in Germany. *Eur. J. Clin. Microbiol. Dis*; 15(2): 128-132.
Holland P. V; Barrera J. M; Ercilla M. J; Yoshiga C. F. T; Wang Y; Deolim G. A. B; Betlach B; Kuramoto K; and Okamoto H. 1996. Genotyping hepatitis C virus isolates from Spain Brazil China and Macau by a simplified PCR method. *J. Clin. Microbiol*; 34(10): 2372-2378.
Simmonds P. 1995. Variability of hepatitis C virus. *Hepatology*; 21(2): 570-583.
O'Brien C. B; Henzel B. S; Wolfe L; Gutekunst K; and Moonka D. 1997. cDNA sequencing of the 5' non coding region (5'NCR) to determine hepatitis C genotypes in patients with chronic hepatitis C. *Dig. Dis. Sci*; 42(5): 1087-1093.
Viazov S; Zibert A; Ramakrishnan K; Widell A; Cavicchini A; Schreier E; and Roggendorf M. 1994. Typing of hepatitis C virus isolates by DNA enzyme immunoassay. *Virol Methods*; 48(1): 81-91.

Bdour S. 2002. Hepatitis C virus infection in Jordanian hemodialysis units: serological diagnosis and genotyping. *J Med Microbiol*; 51: 200-4.

Bozdayi A. M; Aslan N; Bozdayi G; Turkyilmaz A. R; Sengezer T; Wend U; Erkan O; Aydemir F; Zakirhodjaev S; Orucov S; Bozkaya H; Gerlich W; Karayalcin S; Yurdaym C; and Uzunalimoglu O. 2004. Molecular epidemiology of hepatitis B C and D viruses in Turkish patients. *Arch Virol* ; 149: 2115-2129.

Chamberlain R; Adams N; Saeed A; Simmonds P; and Elliot R. 1997.Complete nucleotide sequence of a type 4 hepatitis C virus variant: The predominant genotype in the Middle East. *J Gen Virol*; 78: 1341-7.

Jia-Qing H; Ge Fan Z; Ching L; Man Fung Y; and Benjamin C. Y. 2006. Hepatitis B or Hepatitis C Viral Infection and Genotypes –Which Subgroup Is More Prone To The Development Of Hepatocellular Carcinoma? A Systematic Review. Health and Health Services Research Fund Final Report 01030421. p.1-33.

Mellor J; Holmes E. C; Jarvis L. M; Yap P. L; Simmonds P; and The International HCV Collaborative Study Group. 1995. Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: Implications for virus classification. *J Gen Virol*; 76(10): 2493-2507.

Ohno T; Mizokami M; Saleh M. G; Orito E; Ohba K. I; Wu R. R; Koide T; Tibbs C. J; Nouri-Aria K. T; Tokudome S; and Williams R. 1996. Usefulness and limitation of phylogenetic analysis for hepatitis C virus core region: Application to isolates from Egyptian and Yemeni patients. *Arch Virol*; 141(6): 1101-1113.

Al-Kubaisy W. A. 1998. Epidemiologic and genotypic distribution of hepatitis C in Iraqi pregnant women. Ph.D thesis.

Somi M. H; Keivani H; Ardalan M. R; Farhang S; and Pouri A. A. 2008. Hepatitis C virus Genotypes in Patients with End-Stage Renal Disease in East Azerbaijan Iran. *Saudi J Kidney Dis Transpl*; 19(3): 461-465

Esteban J. I; Jordigomez J; Martell M. M; Cabot B; Quer J; Camps J; Gonzalez A; Otero T; Moya A; Esteban R; and Guardia J. 1996. Transmission of hepatitis c virus by a cardiac surgeon. *N Engl J Med*; 334(9): 555-560.

Cicciarello S; Borgia G; Crowell J; Ciampi R; Cerini R; Orlando R; Mainolfi M; Reynaud L; Milano M; and Piazza M. 1997. Prevalence of hepatitis C virus genotypes in southern Italy. *European Journal of Epidemiology*; 13(1): 49–54.

Lee Y. S; Yoon S. K; Chung E. S; Bae S. H; Choi J. Y; Han J. Y; Chung K. W; Sun H. S; Kim B. S; and Kim B. K. 2001. The Relationship of Histologic Activity to Serum ALT HCV genotype and HCV RNA titers in Chronic Hepatitis C. *J Korean Med Sci*; 16(5): 585-591.

Mukaide M; Tanaka Y; Kakuda H; Fujiwara K; Kurbanov F; Orito E; Yoshioka K; Fujise K; Harada S; Kozaki T; Takemura K; Hikiji K; and Mizokami M. 2005. New combination test for hepatitis C virus genotype and viral load determination using Amplicor GT HCV MONITOR test v2.0. *World J* Gastroenterol; 11(4): 469-475.

Simpore J; Ilboudo D; Samandoulougou A; Guardo P; Castronovo P; and Musumeci S. 2004. HCV and HIV Co-Infection in Pregnant Women Attending St. Camille Medical Centre in Ouagadougou (Burkina Faso). *Journal of Medical Virology*; 9999: 1–5.

Sosa-Jurado F; Santos-Lopez G; Guzman-Flores B; Ruiz-Conde J. I; Melendez-Mena D; Vargas-Maldonado M. T; Martinez-Laguna Y; Contreras-Mioni L; Vallejo-Ruiz V. and Reyes-Leyva J. 2010. Hepatitis C virus infection in blood donors from the state of Puebla Mexico. *Virology Journal*; 7:1-16.

Matar G. M; Sharara H. M; Abdelnour G. E; and Abdelnour A. M. 1996. Genotyping of hepatitis C virus isolates from Lebanese hemodialysis patients by reverse transcription-PCR and restriction length polymorphism analysis of 5'noncoding region. *J. Clin. Microbiol*; 34(10): 2623- 2624.

Stuyver L; Wyseur A; van Arnhem W; Lunel F; Laurent-Puig P; Pawlotsky J; Kleter B; Bassit L; Nkengasong J; van Doorn L; and Maertens G. 1995. Hepatitis C virus genotyping by means of 5'-UR/core line probe assays and molecular analysis of untypeable samples. *Virus Research*; 38(2-3): 137-157.