Partial sequencing analysis of the \textit{NS5B} region confirmed the predominance of hepatitis C virus genotype 1 infection in Jeddah, Saudi Arabia

Sahar EL Hadad\textsuperscript{1,2,*}, Hesa Al-Hamdan\textsuperscript{1}, Sabah Linjawi\textsuperscript{1}

\textsuperscript{1} Department of Biological Science, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia, \textsuperscript{2} Research Center of Genetic Engineering and Bioinformatics, VACSERA, Cairo, Egypt

* Saharelhadad@hotmail.com

Abstract

Chronic hepatitis C virus (HCV) infection and its progression are major health problems that many countries including Saudi Arabia are facing. Determination of HCV genotypes and subgenotypes is critical for epidemiological and clinical analysis and aids in the determination of the ideal treatment strategy that needs to be followed and the expected therapy response. Although HCV infection has been identified as the second most predominant type of hepatitis in Saudi Arabia, little is known about the molecular epidemiology and genetic variability of HCV circulating in the Jeddah province of Saudi Arabia. The aim of this study was to determine the dominance of various HCV genotypes and subgenotypes circulating in Jeddah using partial sequencing of the \textit{NS5B} region. To the best of our knowledge, this is the first study of its kind in Saudi Arabia. To characterize HCV genotypes and subgenotypes, serum samples from 56 patients with chronic HCV infection were collected and subjected to partial \textit{NS5B} gene amplification and sequence analysis. Phylogenetic analysis of the \textit{NS5B} partial sequences revealed that HCV/1 was the predominant genotype (73%), followed by HCV/4 (24.49%) and HCV/3 (2.04%). Moreover, pairwise analysis also confirmed these results based on the average specific nucleotide distance identity: $\pm 0.112$, $\pm 0.112$, and $\pm 0.179$ for HCV/1, HCV/4, and HCV/3, respectively, without any interference between genotypes. Notably, the phylogenetic tree of the HCV/1 subgenotypes revealed that all the isolates (100%) from the present study belonged to the HCV/1a subgenotype. Our findings also revealed similarities in the nucleotide sequences between HCV circulating in Saudi Arabia and those circulating in countries such as Morocco, Egypt, Canada, India, Pakistan, and France. These results indicated that determination of HCV genotypes and subgenotypes based on partial sequence analysis of the \textit{NS5B} region is accurate and reliable for HCV sub-type determination.
Introduction

Hepatitis C virus (HCV) is an important human pathogen, which is estimated to infect 130–170 million people worldwide. HCV infection leads to chronic hepatitis (CHC), which in turn leads to liver steatosis, fibrosis, liver cirrhosis (LC), and hepatocellular carcinoma (HCC) [1–2]. HCV is a member of the Hepacivirus genus of the Flaviviridae family of viruses. The HCV genome is small and enveloped with one single-stranded positive-sense RNA molecule of approximately 9600 bp, structured in a coding region that contains one large open reading frame (ORF) flanked by non-translated regions (NTR) at the 5' and 3' ends, which encodes a polyprotein precursor of about 3,000 amino acids. The precursor is cleaved into at least 10 different proteins comprising the structural proteins, core, E1, E2, and p7, as well as the non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig 1) [3–7].

Previously, HCV was classified into six major genotypes [9]; however, recently, seven major genotypes and numerous subtypes have been reported [10]. HCV genotypes 1 and 2 are primarily predominant in West Africa, 3 in South Asia, 4 in Central Africa and the Middle East, 5 in Southern Africa, and 6 in South East Asia [11–15]. To date, only one infection caused by HCV genotype 7 has been reported, where the virus was isolated in Canada from a Central African immigrant [16]. HCV infection has been defined as a reportable disease in Saudi Arabia since 1990 [17]. However, the prevalence of HCV in Saudi Arabia varies between different provinces; according to a report by the Saudi Government produced upon monitoring HCV incidence among 13 Saudi Arabian administrative provinces from 1995 to 2006, the highest prevalence is observed in western provinces such as Al-Baha and Jeddah (0.32%), and the lowest prevalence is in southern provinces such as Jizan (0.016%) [18]. Descriptions of HCV epidemiology in the Kingdom of Saudi Arabia (KSA) have led to HCV infection being considered a major public health problem in the KSA, especially among hemodialysis patients and intravenous drug users (IDU) [19–21]. A total of 437,292 official reports of HCV infections among persons living in the KSA have been submitted to the WHO, which gives an estimated prevalence of about 1.8% overall [22–23]. Several previous studies conducted in Saudi Arabia have proven that HCV genotypes 4, 1 [24–26], and 2 are the most prevalent genotypes, followed by genotype 3.

Definition of the HCV genotype is now a part of the pre-treatment process for patient management. It is also useful for inspecting outbreaks of infections and for understanding the epidemiology and biological features of this virus. Several methods targeting different regions of the HCV genome have been used for assessing HCV genotypes. The most accurate method so far is to sequence an appropriate coding region that is divergent enough to allow the discrimination of the genotypes and subgenotypes [27–28]. Many genotyping methods targeting
different regions of the HCV genome, such as 5’NTR, the core (C), Envelope (E1), and 5’ non-coding region (NS5B) have been used in previous studies [9]. Since the 5’NTR is one of the most highly conserved regions of the HCV genome, it has historically been used for virus detection and is now one of the best-characterized regions. For practical reasons, the 5’NTR has also been chosen as the target for various genotyping methods including the InnoLipa HCV II test [29–30], sequencing [31], and the duplex mobility assay [32]. In this study, we analyzed the sequence of the NS5B region to determine the HCV genotypes and subgenotypes of 49 isolates collected from chronic HCV patients in the Jeddah province between 2014 and 2015. All the samples had already been genotyped based on 5’NTR analysis.

The current study was performed to determine the genotypes and subgenotypes using phylogenetic analyses of HCV strains collected from chronically infected patients in Saudi Arabia to establish a simple, accurate, and a reliable genotyping system for HCV for use in diagnosis. To the best of our knowledge, this is the first study determining HCV subtypes based on analysis of the nucleotide sequence of the NS5B region in the Jeddah province of Saudi Arabia.

Materials and methods

Patient samples

Serum samples were collected from 56 patients with chronic HCV infection from Saudi Arabia; S1 Table contains the data for all the patients. The patients were randomly selected based on availability from different hospitals in Jeddah; a review of the medical records confirmed that all the samples were positive for anti-HCV antibodies and that none of the patients had co-infection with HIV or HBV. The samples were divided into aliquots and stored at −70˚C until use.

The study protocol was reviewed and approved by the Deanship of Scientific Research Ethical Committee of King Abdulaziz University and the King Abdulaziz Hospital Ethical Committee. Written informed consent was obtained from all the patients after full explanation of the purpose of the study.

HCV-RNA extraction and HCV-NS5B amplification

HCV RNA was obtained from all the serum samples using the Mini Elute Viral Extraction Kit (QIAGEN, Inc., Valencia, CA, USA) according to the manufacturer’s instructions. The resultant HCV RNA samples were stored at −70˚C until use [33]. The presence of the HCV-NS5B gene was determined by nested PCR using the One-step RT-PCR Master Mix kit and the Hot start Taq plus PCR Master Mix Kit (QIAGEN) using the primers shown in Table 1. The first round of RT-PCR amplification was performed according to the manufacturer’s instructions using 10 μL HCV-RNA and 50 pmol each of primers NS5BOS1 and NS5BOAS2. The cycling

---

Table 1. Primers used for partial HCV-NS5B amplification and their nucleotide positions.

| Primer Code | Primer Sequence | Primer Length | Nucleotide Position |
|-------------|----------------|---------------|---------------------|
| NS5BOS1     | 5’TGGGGTTCCTCAGATACCC-3’ | 21            | 8337–8357           |
| NS5BOAS2    | 5’CCTGGTCATAGCCTCCGGAGA-3’ | 21            | 8729–8708           |
| NS5BIS1     | 5’GATACCGCTGCCCTGGGCAA-3’ | 20            | 8350–8370           |
| NS5BIAS2    | 5’CCTCCGGAGGAGATGTCAG-3’ | 19            | 8717–8698           |

Nucleotide position numbering is based on the HCV reference sequence contained in ~9600 bp, which was retrieved from DDBJ/EMBL/GenBank (Accession number: JF735132).

https://doi.org/10.1371/journal.pone.0178225.t001
conditions were as follows: a reverse transcription step for 30 min at 50˚C, 15 min at 95˚C, fol-
lowed by 35 cycles of denaturing for 1 min at 95˚C, annealing for 45 s at 59˚C, and an elon-
gation step for 1 min at 72˚C, with a final extension period of 10 min at 72˚C. Nested PCR using
the NS5BIS1 and NS5BIAS2 primers was performed on 10 μL of the samples negative for PCR
products in the first round of amplification. The second round of amplification was performed
with an initial 5 min preheating step at 95˚C, followed by 35 cycles of denaturing for 30 s at
95˚C, annealing for 30 s at 55˚C, and elongation for 1 min at 72˚C, with a final extension
period of 10 min at 72˚C. All the PCR contamination precautions were taken to ensure speci-
cicity of the reaction [16, 34–35].

Interpretation of HCV-NS5B partial nucleotide sequences
Purified nested PCR products were sequenced in both directions using a Big Dye Terminator
v3.1 Cycle Sequencing kit (Applied Biosystems (ABI), Foster City, CA, USA). The ABI Prism
310 genetic analyzer was used for electrophoresis and data collection per the manufacturer’s
protocol. All the sequences were assembled using SeqMan II software (DNASTar Inc.,
Madison, WI, USA) and multiple alignments with the reference sequences of
HCV-NS5B
genotypes and subgenotypes (1–6) were confirmed using CLUSTALW software [36].

Phylogenetic analysis
Phylogenetic analysis of the amplified regions from each sample, corresponding to the partial
NS5B gene, was performed to analyze the nucleotide heterogeneity of the isolates with the ref-
erence HCV genotypes/subtypes retrieved from the DDBJ/EMBL/GenBank databases (S2
Table). Phylogenetic trees were constructed by MEGA 6 software using the neighbor-joining
method based on the Tamura-Nei model of evolutionary distance, and the topology was evalu-
ated by bootstrap analysis (1,000 replicates) [36–40].

Nucleotide sequence submission
The base sequence data as well as partial sequence data reported in this study have been sub-
mitted to DDBJ/EMBL/GenBank under the accession numbers KX774463, (KX810070-
KX810071), (KX810077-KX810086) and (KX784096-KX78129) (S3 Table).

Results
Amplification of the HCV-NS5B region using nested PCR
Nested RT-PCR confirmed the presence of the HCV-NS5B gene in 49 (87.5%) of the 56 anti-
HCV positive samples. The remaining seven (12.5%) samples did not show the presence of
HCV-NS5B even after the second round of PCR amplification, which may have been due to
low viral load. All the positive PCR products were of the expected size of approximately 400–
500 bp, which included almost the partial NS5B genomic region (Fig 2).

Identification of HCV genotypes based on the partial nucleotide
sequence of the NS5B gene
The phylogenetic tree based on the ~400-bp NS5B partial gene from each sample and genes
from the six reference HCV strains revealed three distinct clusters comparable to the six HCV
genotypes. Thirty-six of the 49 (73.47%) isolates (2A, A4, A5, 6A, A7, A8, A9, A10, A11, A12,
A13, A14, A15, A16, A17, A18, A19, A20, A22, A23, 24A, 25A, 26A, 30A, 31A, 34A, 36A, No-
5, WE-5, C, L-5, O-5, 3R, E-5, G-5, and S-5) seemed to be more closely related to HCV geno-
type 1 based on nucleotide distance identity (mean: ±0.112), and HCV genotype 4 was verified
in 12 (24.49%) isolates (D-5, Mo-5, P-5, 1, 1R, 29A, F-5, 35A, A3, 32A, 28A, and 33A); 1 (2.04%) isolate (A21) seemed to be related to HCV genotype 3 (Fig 3 and S4 Table), and the nucleotide distance (means: ±0.112 and ±0.179) verified this relationship. No samples were identified as HCV genotypes 2, 5, or 6 (Fig 3 and S4 Table).

Identification of HCV subgenotypes based on the partial nucleotide sequence of the NS5B gene

Partial NS5B reference sequences of 25 HCV/1 subgenotype strains, including the 36 isolates from the present study, were used to generate a phylogenetic tree. The reference sequences were retrieved from the DDBJ/EMBL/GenBank databases along with their accession numbers and country of origin for identification. All the 25 isolates were grouped into clusters that represented the seven subgenotypes of HCV/1 (1a, 1b, 1c, 1d, 1e, 1l, and 1k). An exclusive subset of the 36 HCV/1 isolates (2A, A4, A5, 6A, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A17, A18, A19, A20, A22, A23, 24A, 25A, 26A, 30A, 31A, 34A, 36A, No-5, WE-5, C, L-5, O-5, 3R, E-5, G-5, and S-5) (100%) was observed to be closest to the reported HCV/1a strains, which included HCV/1a reference strains isolated from Pakistan, France, and Canada (Fig 4). Another subgenotype phylogenetic tree was constructed for the 14 NS5B sequences of HCV/3 subgenotype strains retrieved from the DDBJ/EMBL/GenBank databases and the isolate from the present study. The isolate A21 from the present study was grouped with branches and seemed to be more closely related to the HCV/3a subgenotype. This identification was verified using HCV/3a reference strains from different countries such as Pakistan, Brazil, Malaysia, and Canada (Fig 5).

A specific phylogenetic tree of the 12 remaining isolates corresponding to the HCV/4 genotype revealed that 10 (83.33%) of these 12 isolates (D-5, MO-5, P-5, 29A, F-5, 35A, A3, 32A, 28A and 33A) were related to the 4a subgenotype, and this result was verified using HCV/4a references strains from Egypt and France. The other two (16.67%) isolates (1R and 1) were defined as the HCV/4t subgenotype, but they assembled with reference strains isolated from Canada (Fig 6).

Discussion

HCV has drawn attention for the unique geographic distribution of its six characteristic genotypes. Genotyping of HCV is routinely performed in many laboratories for providing counseling regarding treatment, classification, and epidemiology to monitor the distribution of virus strains and to identify risk factors for transmission and investigate contamination between
individuals. Accurate subtype determination is necessary for epidemiology and risk factor of transmission between individual. [41–43]. In addition, the study of viral diversity provides a better understanding of the origins and dynamics of viral infections. Genetic variants of HCV are known to be widely spread around the world. Genotypes 1, 2, and 3 are found in all countries [44]. Comprehensive data on the distribution of HCV genotypes in Middle Eastern countries has recently been published [45], which indicates that HCV genotype 1 is largely predominant in some countries such as Turkey (82%), Cyprus (68%), and Iran (55%) [46–47].

Fig 3. Phylogenetic tree based on six ~400-bp partial NS5B reference sequences representing all HCV genotypes (1–6). The phylogenetic tree was constructed using the neighbor-joining method (MEGA 6 software), and the reference sequences were retrieved from the DDBJ/EMBL/GenBank databases. All the reference isolates are indicated with their accession numbers and with closed blue circles. In addition, the 49 isolates from Saudi Arabia patients in this study whose nucleotide sequences were determined in the present study are shown. Thirty-six of the isolates clustered with HCV/1 and are represented by red branches, 12 isolates were grouped as HCV/4 and are represented by dark blue branches, and one isolate was grouped as HCV/3 and is represented by light blue branches. Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 replications.

https://doi.org/10.1371/journal.pone.0178225.g003
Fig 4. Phylogenetic tree for HCV/1 NS5B gene sequences constructed using the neighbor-joining method (MEGA 6 Software). Tree shows the phylogenetic relationship of 25 HCV-NS5B reference sequences and 36 HCV-NS5B sequences from this study. The reference HCV/1 subgenotype isolates are indicated by different colored branches along with their accession numbers and countries of origin. The 36 isolates from the present study are indicated by closed red triangles. Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 replications.

https://doi.org/10.1371/journal.pone.0178225.g004
whereas HCV genotype 4 is predominant in Egypt, Saudi Arabia, Bahrain, and United Arab Emirates [24, 19, 25, 45, 48–52].

Analysis of the HCV genotype within a population is a useful epidemiological tool for study of the evolution of HCV infection in different geographical regions. HCV genotyping is also important because it provides information with regard to strain variations and potential association with disease severity [53]. Saudi Arabia has been classified as a country with an intermediate prevalence of HCV based on surveys conducted using samples from blood donors [54]. Despite the reported declines in HCV prevalence in the KSA over the last 10 years, HCV is still considered a major public health problem in the country, especially among hemodialysis patients and intravenous drug users [20, 55]. HCV has been found to be transmitted in the KSA through many routes including surgical operations, intravenous abuse [56], and sexual routes [57–58]. Currently, the characterization of HCV epidemiology in the KSA relies heavily on HCV seroprevalence studies, which are typically transversal studies and are conducted in select populations such as blood donors and hemodialysis patients [19–20].

![Phylogenetic tree of HCV/3 NS5B gene sequences constructed using the neighbor-joining method (MEGA 6 Software).](https://doi.org/10.1371/journal.pone.0178225.g005)
analysis of the 49 current isolates demonstrated that HCV genotype 1 is the most dominant HCV genotype (73.47%) among Saudi patients with chronic HCV using HCV-NS5B sequencing analysis. This result contrasts with previous observations that postulated that HCV genotype 4 appeared to be predominant in Middle Eastern countries, including Saudi Arabia; these reports were based on HCV-5'UTR sequencing analysis [48–52].

Fig 6. Phylogenetic tree of HCV/4 NS5B gene sequences constructed using the neighbor-joining method (MEGA 6 Software). Tree shows the phylogenetic relationship of 18 HCV-NS5B reference sequences and 12 HCV-NS5B sequences from the present study. Reference HCV/4 subgenotype isolates are indicated by different colored branches along with their accession numbers and countries of origin. The 12 isolates from the present study are indicated by closed red triangles. Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 replications.

https://doi.org/10.1371/journal.pone.0178225.g006
The choice of the genome region to be analyzed for identification of HCV genotypes and subgenotypes is crucial [59]. The \textit{NS5B} region is highly informative in phylogenetic analysis and has received the most attention for the characterization of HCV isolates worldwide [16, 27, 60–61]. With regard to \textit{NS5B} nucleotide sequence genotypes, in the present phylogenetic investigation of the partial \textit{HCV-NS5B} nucleotide sequence, we succeeded in genotyping 100% of the amplified samples. \textit{HCV-NS5B} phylogenetic analysis revealed that the most prevalent genotypes were HCV/1 (73.47%), HCV/4 (24.49%), and HCV/3 (2.04%). Using pairwise distance analysis of the \textit{NS5B} nucleotide sequence, we also succeeded in matching 100% of the current (amplified) isolates with their specific HCV genotypes using phylogenetic trees. Pairwise analysis of the isolates from the present study belonging to the HCV/1, HCV/4, and HCV/3 genotypes revealed average specific nucleotide distance identities of ±0.112, ±0.112, and ±0.179, respectively, without any interference between genotypes, which confirmed the outcome of our phylogenetic analyses. Many previous studies have indicated that the degree of accuracy of sequence variation of \textit{NS5B} correlates well with HCV subtype definition, which relies on the highly informative character of specific \textit{NS5B} motifs; in contrast, most classification errors caused by the use of the 5’NTR region are related to poor discriminating power and to the absence of target motifs specific for some subtypes. Therefore, we recommend that sequence analysis of the \textit{NS5B} region be used rather than 5’NTR analysis for epidemiologic studies in the future [62–67].

In the current study, phylogenetic trees for different \textit{NS5B} subtypes were constructed to estimate the precision of \textit{NS5B-HCV} subtyping. The phylogenetic trees for the \textit{HCV-NS5B} subtypes succeeded in classifying 100% of the HCV isolates. The common subtypes were 1a (73.47%), 4a (20.41%), 4t (4.08%), and 3a (2.04%). \textit{NS5B} phylogenetic analysis of HCV/1 subtypes showed the grouping of all the strains in one branch, which confirmed that the common subtype of HCV genotype 1 among the isolates from the present study was 1a. This result contradicts that obtained using the 5’NTR phylogenetic trees generated in the present study as well as in other previous studies [19,25, 68]. One reason for this difference could be that the classification of isolates under the 1a subtype in the present study was mostly based on comparison with reference strains from Canada, Pakistan, and France.

Next, with regard to HCV/4 subtypes, the phylogenetic trees revealed that the common subtypes of HCV genotype 4 were 4a (83.33%) and 4t (16.67%). Although the strains from the current study defined as subtype 4a were generally similar to the reference strains isolated from France and Egypt, these strains defined as subtype 4a were clustered in a unique branch, which suggested that the patients in France and Egypt from whom the reference strains were isolated acquired their infection in their own country [69–70]. The isolates from the present study defined as subtype 4t were associated with reference strains from Canada.

With respect to the HCV/3 subtypes, although phylogenetic trees showed only one isolate from the present study as genotype 3, \textit{HCV-NS5B} nucleotide sequence analysis successfully classified it as 3a subtype. The isolate from the present study with subtype 3a was found to be closely related to reference strains from Canada, Malaysia, and Pakistan.

HCV is a leading etiology of hepatocellular carcinoma (HCC). In Japan, Egypt, Saudi Arabia, southern Europe, and the USA, HCV is the main risk factor for HCC. Serological markers of HCV infection have thus been found in 71%, 80% 73%, and 39.5% of HCC cases from Egypt, Japan [71], the USA [72], and Saudi Arabia [73], respectively. These studies in agree with our data, which illustrated the most common HCV subtype is 1a (73.47%) and HCC developed in patients with either genotype 2a HCV or genotype 1b HCV [74]. Additionally, the current data will facilitate the clinical deployment of new protease inhibitors, which have significantly improved treatment outcome in patients with HCV genotype 1; HCV genotypes 1 and 4 were previously considered to be the most difficult to treat [75].
Conclusion

To the best of our knowledge, this study is the first comprehensive research to address genotype and subgenotype analysis of HCV in the Jeddah province of Saudi Arabia using nucleotide sequencing. In this study, we aimed to determine the genotype and subgenotype for HCV isolates circulating in the Jeddah province through sequence analysis of the NS5B gene in a set of 49 HCV strains isolated from patients with chronic HCV infections from Saudi Arabia. Our results showed that the most common HCV genotypes circulating in Jeddah were genotypes 1, 4, and 3 (in that order), and some of the HCV isolates from the present study showed sequence similarity with some international HCV strains upon comparison with HCV reference sequences from Egypt, Morocco, Canada, Japan, India, and France.

Moreover, our study confirmed that HCV-NS5B gene sequence analysis provides precise genotype and subtype identification and an accurate epidemiological representation of circulating viral strains. The pairwise distance analysis also confirmed 100% of the results.

Although the molecular aspects of HCV infection were obtained through testing a small population in this study, the outcome confirmed the genotypes and subtypes of HCV that were circulating in Jeddah, Saudi Arabia. However, further studies with more samples need to be conducted to investigate if a shift in genotypes has occurred. Expanding the current study will allow us to follow changes in subtype distribution and to identify recombinants or even new subtypes or genotypes. Thorough insertion diagnostic methods that can assign viral genotype and subtype classifications are therefore greatly desired. Moreover, such methods will perform better when sequence variation from the NTR is eliminated and protein-coding regions such as NS5B are used instead. Indeed, further research that expands to cover all the regions of KSA is required to support the findings of the current study.

Supporting information

S1 Table. Characteristics of the 56 patients with chronic HCV infection enrolled in the present study. (DOCX)

S2 Table. Complete genomes of HCV genotypes and NS5B reference sequences of subgenotypes retrieved from DDBJ/EMBL/GenBank databases. (DOCX)

S3 Table. The current HCV samples codes and their accession numbers in the GenBank. (DOCX)

S4 Table. Pairwise distances between the partial nucleotide sequence of the NS5B gene of HCV genotypes (1–6) and those of the 49 isolates from the present study generated using MEGA 6 software. (DOCX)

Acknowledgments

The authors are grateful to the King Abdulaziz city for science and technology (KACST), Jeddah, KSA, for their technical and financial support as well as to Dr. Nezar Redhwan for providing us the laboratory for practical studies.

Author Contributions

Conceptualization: SE.
Data curation: SE HAH SL.
Formal analysis: SE.
Funding acquisition: SE HAH.
Methodology: SE HAH.
Resources: HAH.
Software: SE.
Supervision: SE SL.
Writing – original draft: SE HAH SL.
Writing – review & editing: SE HAH.

References
1. Alter MJ. Epidemiology of hepatitis C virus infection. World Journal of Gastroenterology. 2007; 13: 2436–2441. https://doi.org/10.3748/wjg.v13.i17.2436 PMID: 17552026
2. Bartenschlager R, Penin F, Lohmann V, Andre P. Assembly of infectious hepatitis C virus particles. Trends Microbiology. 2011; 19: 95–103.
3. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nature Reviews Microbiology. 2007; 5: 453–463. https://doi.org/10.1038/nrmicro1645 PMID: 17487147
4. Simons JN, Leary TP, Dawson GJ, Muerhoff AS, Schlauder GG, Desai SM, et al. Isolation of novel virus-like sequences associated with human hepatitis. Nature medicine. 1995; 1: 564–569. PMID: 7585124
5. Ohba K, Mizokami M, Lau JY, Orito E, Ikeo K, Gojobono T. Evolutionary relationship of hepatitis C, pesti-, flavi-, plantviruses, and newly discovered GB hepatitis agents. FEBS Letters. 1996; 378: 232–234. PMID: 8557107
6. Fauquen CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. Virus Taxonomy:VIIIth Report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press; 2005.
7. Knip DM, Howley PM. Fields Virology. United States of America: Lippincott Williams & Wilkins; 2007.
8. Lloyd AR, Jagger E, Post JJ, Crooks LA, Rawlinson W, Hahn Y, et al. Host and viral factors in the immunopathogenesis of primary hepatitis C virus infection. Immunology and Cell Biology. 2006; 85: 24–32. https://doi.org/10.1038/sj.icb.7100010 PMID: 17130897
9. Bukh J, Miller RH, Purcell RH. Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Seminars in Liver Disease. 1995; 15: 41–63. https://doi.org/10.1055/s-2007-1007262 PMID: 7597443
10. Zhou S, Cella E, Zhou W, Kong WH, Liu MQ, Liu PL, et al. Population dynamics of hepatitis C virus subtypes in injecting drug users on methadone maintenance treatment in China associated with economic and health reform. Journal of Viral Hepatitis. 2017; doi: 10.1111/jvh.12677.
11. Fooke BC, Spooner LM, Belliveau PP. Bocceprevir a protease inhibitor for the treatment of chronic Hepatitis C. Annals of Pharmacotherapy. 2011; 45: 1085–1093. https://doi.org/10.1345/aph.1P744 PMID: 21828346
12. Ashfaq UA, Khan SN, Nawaz Z, Riazuddin S. In-vitro model systems to study Hepatitis C Virus. Genetic Vaccines Therapy. 2011; 9: 7. https://doi.org/10.1186/1479-0565-9-7 PMID: 21466709
13. Ali S, Ali I, Azam s, Ahmad B. Frequency distribution of HCV genotypes among chronic Hepatitis C patients of Khyber Pakhtunkhwa. Virology Journal. 2011; 8: 193. https://doi.org/10.1186/1743-422X-8-193 PMID: 21521506
14. Kamal SM, Nasse AI. Hepatitis C genotype 4: What we know and what we don’t yet know. Hepatology. 2008; 47: 1371–1383. https://doi.org/10.1002/hep.22127 PMID: 18240152
15. Chao DT, Abe K, Nguyen MH. Systematic review: Epidemiology of Hepatitis C genotype 6 and its management. Alimentary Pharmacology and Therapeutics. 2011; 34: 286–296. https://doi.org/10.1111/j.1365-2036.2011.04714.x PMID: 21623850
16. Murphy DG, Willems B, Deschenes M, Hilzenrat N, Mousseau R, Sabbah S. Use of sequence analysis of the NS5B region for routine genotyping of hepatitis C virus with reference to C/E1 and S’ untranslated region sequences. Journal of Clinical Microbiology. 2007; 45: 1102–1112. https://doi.org/10.1128/JCM.02366-06 PMID: 17287328
17. Al-Tawfiq JA, Anani A. Profile of viral hepatitis A, B, and C in a Saudi Arabian hospital. Medical Science Monitor. 2008; 14: 52–56.

18. Madani TA. Hepatitis C virus infections reported over 11 years of surveillance in Saudi Arabia. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2009; 103: 132–136. https://doi.org/10.1016/j.trstmh.2008.08.001 PMID: 18789464

19. Shobokshi OA, Serebour FE, Skakni LI. Hepatitis C genotypes/subtypes among chronic hepatitis patients in Saudi Arabia. Saudi Medical Journal. 2003; 24: 87–91.

20. Karkar A. Hepatitis C in dialysis units: The Saudi experience. Hemodialysis International. 2007; 11: 354–367. https://doi.org/10.1011/j.1542-4758.2007.00192.x PMID: 17576302

21. Memish ZA, Knawy BA, El-Saied A. Incidence trends of viral hepatitis A, B, and C seropositivity over eight years of surveillance in Saudi Arabia. International Journal of Infectious Diseases. 2010; 14: 115–120.

22. WHO. (2009) The growing threats of hepatitis B and C in the Eastern Mediterranean Region: a call for action. Access date, April 10, 2013, from: http://applications.emro.who.int/docs/EM_RC56_3_en.pdf.

23. Lavanchy D. Evolving epidemiology of hepatitis C virus. Clinical Microbiology and Infection. 2010; 17: 107–115.

24. Al Faleh FZ, Huraib S, Sbeih F, Al Karawi M, Al-Mofleh LA, Sougiiyah M, et al. Hepatitis C virus genotypes in patients with chronic liver disease and haemodialysis patients from Saudi Arabia. Journal of Viral Hepatitis. 1995; 2: 293–296. PMID: 8732175

25. Shobokshi OA, Serebour FE, Skakni L, Al-Saffy YH, Ahdal MN. Hepatitis C genotypes and subtypes in Saudi Arabia. Journal of Medical Virology. 1999; 58: 44–48. PMID: 10235444

26. Osoba AO, Ibrahim M, Abdelaal MA, AlMowallad A, Al Shareef B, Hussein BA. Hepatitis C virus genotyping by polymerase chain reaction and DNA enzyme immunoassay among Saudi patients in the Western Province Saudi Arabia. Annals of Saudi Medicine. 2000; 20: 394–397. PMID: 12764639

27. Chen Z, Weck KE. Hepatitis C virus genotyping: interrogation of the 5 untranslated region cannot accurately distinguish genotypes 1a and 1b. Journal of Clinical Microbiology. 2002; 40: 3127–3134. https://doi.org/10.1128/JCM.40.9.3127-3134.2002 PMID: 12025422

28. Weck KE. Hepatitis C virus genotyping: interrogation of the 5 untranslated region cannot accurately distinguish genotypes 1a and 1b. Journal of Clinical Microbiology. 2002; 40: 3127–3134. https://doi.org/10.1128/JCM.40.9.3127-3134.2002 PMID: 12025422

29. Stuyver L, Wyseur A, van Arnhem W, Hernandez F, Maertens G. Second-generation line probe assay for hepatitis C virus genotyping. Journal of Clinical Microbiology. 1996; 34: 2259–2266. PMID: 8862595

30. Stuyver L, Wyseur A, van Arnhem W, Lune L, Laurenski JM, et al. Hepatitis C genotype by means of 5-UR/core line probe assays and molecular analysis of untypeable samples. Virus Research. 1995; 38: 137–157. PMID: 8578655

31. Germer JJ, Majewski DW, Rosser M, Thompson A, Mitchell PS, Smith TF, et al. Evaluation of the TRUGENE HCV SNC genotyping kit with the new GeneLibrary module 3.1.2 for genotyping of hepatitis C virus from clinical specimens. Journal of Clinical Microbiology. 2003; 41: 4855–4857. https://doi.org/10.1128/JCM.41.10.4855-4857.2003 PMID: 14532242

32. White PA, Zhai X, Carter I, Zhao Y, Rawlinson WD. Simplified hepatitis C virus genotyping by heteroduplex mobility analysis. Journal of Clinical Microbiology. 2000; 38: 477–482. PMID: 10655331

33. McElhinney LM, Marston DA, Freuling CM, Cragg W, Stankov S, Lalosevic D, et al. Molecular diversity and evolutionary history of rabies virus strains circulating in the Balkans. Journal of General Virology. 2011; 92: 2171–2180. https://doi.org/10.1099/vir.0.032748-0 PMID: 21632560

34. ELHadad SR, Alakilli SY, Ramadan HA, Baeshen NA. Novel mutations of hepatitis B virus surface antigen genotype D among chronic Egyptian patients. African Journal of Microbiology Research. 2013; 7: 814–824.

35. Fakhr AE, Pourkheiran MR, Maes P, Atta AH, Marei A, Azab M, et al. Hepatitis C Virus NS5B Sequence-Based Genotyping Analysis of Patients From the Sharkia Governorate, Egypt. Hepatitis Monthly. 2013; 13: 1–5.

36. EL Hadad S, Alakilli S, Rabah S, Sabir J. Sequence analysis of sub-genotype D hepatitis B surface antigens isolated from Jeddah, Saudi Arabia. Saudi Journal of Biological Sciences. 2016; doi.org/10.1016/j.sjbs.2016.03.003.

37. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution. 2013; 30: 2725–2729. https://doi.org/10.1093/molbev/ms31222

38. Lu L, Wang M, Xia W, Tian L, Xu R, Li C, et al. Migration patterns of hepatitis C virus in China characterized for five major subtypes based on samples from 411 volunteer blood donors from 17 provinces and
39. Gul A, Zahid N, Ahmed J, Zahir F, Khan I, Ali L. Molecular characterization of Hepatitis C virus 3a in Peshawar. BMC infectious Disease. 2016; 16: 163.

40. Qiu P, Stevens R, Wei B, Lahser F, Howe A, Klappenbach J, et al. HCV Genotyping from NGS Short Reads and Its Application in Genotype Detection from HCV Mixed Infected Plasma. PLOS ONE. 2015; 10: e0122082. doi: 10.1371/journal.pone.0122082. PMID: 25830316

41. Liu CJ, Kao JH, Shau WY, Chen PJ, Lai M, Chen D. Naturally occurring hepatitis B surface gene variants in chronic hepatitis B virus infection: Correlation with viral serotypes and clinical stages of liver disease. Journal of Medical Virology. 2002; 68: 50–59. https://doi.org/10.1002/jmv.10169 PMID: 12210430

42. Kao JH, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology. 2003; 124: 327–334. https://doi.org/10.1053/gast.2003.50053 PMID: 12785176

43. Cantaloube JF, Laperche S, Gallian P, Bouchardieu F, Lamballerie X, deMicco P. Analysis of the 5’ Noncoding Region versus the NS5b Region in Genotyping Hepatitis C Virus Isolates from Blood Donors in France. Journal of Clinical Microbiology. 2006; 44: 2051–2056. https://doi.org/10.1128/JCM.02463-05 PMID: 16757597

44. Mellor J, Holmes EC, Jarvis LM, Yap PL. The International HCV Collaborative Study Group. Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. Journal of General Virology. 1995; 76: 2493–2507. https://doi.org/10.1099/0022-1317-76-10-2493 PMID: 7595353

45. Das BR, Kundu B, Khandapkar R. Geographical distribution of hepatitis C virus genotypes in India. The Indian Journal of Pathology and Microbiology. 2002; 45: 323–328. PMID: 12785176

46. Ramia S, Eid-Fares J. Distribution of hepatitis C virus genotypes in the Middle East. International Journal of Infectious Diseases. 2006; 10: 272–277. https://doi.org/10.1016/j.ijid.2005.07.008 PMID: 16564719

47. Khodabandehloo M, Roshani D. Prevalence of hepatitis C virus genotypes in Iranian patients: a systematic review and metaanalysis. Hepatitis Monthly. 2014; 14: 2295–22928.

48. Bdour S. Hepatitis C virus infection in Jordanian haemodialysis units, serological diagnosis and genotyping. Journal of Medical Microbiology. 2002; 51: 700–704. https://doi.org/10.1099/0022-1317-51-8-700 PMID: 12171303

49. Osaba AO. Hepatitis C virus genotypes in Saudi Arabia. Saudi Medical Journal. 2002; 23: 7–12. PMID: 11938356

50. Al-Ahdal NM, Rezeig AM, Kessie G. Genotyping of Hepatitis C Virus Isolates from Saudi Patients by analysis of Sequences from PCR-Amplified Core Region of the Virus Genome. Annals of Saudi Medicine. 1997; 17: 201–204.

51. Boriskin YS, Bakir TM, Al-Aska AI, Booth JC. Is hepatitis C virus genotype 4 predominant in Saudi Arabia? New Microbiologica. 1999; 22: 173–180. PMID: 10423734

52. Fakeeh M, Zaki AM. Hepatitis C: Prevalence and common genotypes among ethnic groups in the Jad-dah, Saudi Arabia. The American Society of Tropical Medicine and Hygiene. 1999; 61: 889–892.

53. Garcia-Montalvo BM, Galguera-Colorado PL. Distribution of hepatitis C virus genotypes, risk factors and liver disease in patients from Yucatan, Mexico. Annals of Hepatology. 2008; 7: 345–349. PMID: 19034234

54. Aljarbou AN. Current Prevalence of HBV and HCV Seropositivity: The Initiative for Attentiveness and Deterrence of Viral Hepatitis in the Qassim Region of Saudi Arabia. Antivirals & Antiretrovirals Journals. 2012; 4: 075–079.

55. Abdo A, Sanail FM, AlFaleh FZ. Epidemiology of viral hepatitis in Saudi Arabia: Are we off the hook? The Saudi Journal of Gastroenterology. 2012; 18: 349–357. https://doi.org/10.4103/1319-3767.103425 PMID: 23150019

56. Poustchi H, Sepanlou SG, Esmaill S, Mehrabi N, Ansarymoghadam A. Hepatocellular Carcinoma in the World and the Middle East. Middle East Journal of Digestive Diseases. 2010; 2: 31–41. PMID: 25197510

57. Alter MJ. Community acquired viral hepatitis B and C in the US. Gut. 1993; 34: 17–19.

58. Sandres-Saune K, Deny P, Pasquier C, Thibault V, Duverlie G, Izopet J. Determining hepatitis C genotype by analyzing the sequence of the NS5B. Journal of Virological Methods. 2003; 109: 187–193. PMID: 12711062

59. Stuyver L, van Arnhem W, Wyseur A, Hernandez F, Delaporte E, Maertens G. Classification of hepatitis C viruses based on phylogenetic analysis of the envelope 1 and nonstructural 5B regions and
identification of five additional subtypes. Proceedings of the National Academy of Sciences. 1994; 91: 10134–10138.

60. Tokita H, Okamoto H, Iizuka H, Kishimoto J, Tsuda F, Miyakawa Y, et al. The entire nucleotide sequences of three hepatitis C virus isolates in genetic groups 7–9 and comparison with those in the other eight genetic groups. Journal of General Virology. 1998; 79: 1847–1857. https://doi.org/10.1099/0022-1317-79-8-1847 PMID: 9714232

61. Tokita H, Okamoto H, Iizuka H, Kishimoto J, Tsuda F, Lesmana L, et al. Hepatitis C virus variants from Jakarta, Indonesia classifiable into novel genotypes in the second (2e and 2f), tenth (10a) and eleventh (11a) genetic groups. Journal of General Virology. 1996; 77: 293–301. https://doi.org/10.1099/0022-1317-77-2-293 PMID: 8627233

62. Robertson N, Myers G, Howard C, Brettin T, Bukh J, Gaschen T, et al. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. Archives of Virology. 1998; 143: 2493–2503. PMID: 9930205

63. Morice Y, Roulot D, Grando V, Stirnemann J, Gault E, Jeantlis V, et al. Phylogenetic analyses confirm the high prevalence of hepatitis C virus (HCV) type 4 in the Seine-Saint-Denis district (France) and indicate seven different HCV-4 subtypes linked to two different epidemiological patterns. Journal of General Virology. 2001; 82:1001–1012. https://doi.org/10.1099/0022-1317-82-5-1001 PMID: 11297675

64. Martial J, Morice Y, Abel S, Cabie A, Rat C, Lombard F, et al. Hepatitis C virus (HCV) genotypes in the Caribbean island of Martinique: evidence for a large radiation of HCV-2 and for a recent introduction from Europe of HCV-4. Journal of Clinical Microbiology. 2004; 42:784–781. https://doi.org/10.1128/JCM.42.2.784-781.2004 PMID: 14766854

65. Cantaloube JF, Gallian PH, Attoui PB, iagin P, Micco PD, Lamallerie X. Genotype distribution and molecular epidemiology of hepatitis C virus in blood donors from Southeast France. Journal of Clinical Microbiology. 2005; 43:3624–3629. https://doi.org/10.1128/JCM.43.8.3624-3629.2005 PMID: 16081888

66. Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology. 2005; 42: 962–973. https://doi.org/10.1002/hep.20819 PMID: 16149085

67. Baclig MO, Chan VF, Ramos JDA, Gopez-Cervantes J, Natividad F. Correlation of the 5′untranslated region (5′UTR) and non-structural 5B (NS5B) nucleotide sequences in hepatitis C virus subtyping. International Journal of Molecular Epidemiology and Genetics. 2010; 1: 236–244. PMID: 21537395

68. Darwish MA, Raouf TA, Rushdy P, Constantine N, rai MR, Edelman R. Risk factors associated with a high seroprevalence of hepatitis C virus infection in Egyptian blood donors. American Journal of Tropical Medicine and Hygiene. 1993; 49: 440–447. PMID: 7692754

69. Hibbs RG, Corwin AL, Hassan NF, Kamei M, Raddad L. The epidemiology of antibody to hepatitis C in Egypt. Journal of Infectious Diseases. 1993; 168: 789–790. PMID: 8394867

70. Rahman El-Zayadi A, Abaze H, Shawky S, Mohamed MK, Selim OE, et al. Prevalence and epidemiological features of hepatocellular carcinoma in Egypt-a single center experience. Hepatology Research. 2001; 19:170–179. PMID: 11164741

71. El-Serag HB. Hepatocellular carcinoma and hepatitis C in the United States. Hepatology. 2002; 36:74–83.

72. Miyakawa Y, Iino S. Toward prevention of hepatocellular carcinoma developing in chronic hepatitis C. Journal of Gastroenterology and Hepatology. 2001; 16:711–714. PMID: 11446875

73. Akbar HO, Al Ghamdi A, Qattan F, Fallatah HI, Al Rumani M. Chronic hepatitis C in saudi arabia: three years local experience in a university hospital. Hepatitis Monthly. 2012; 10.5812/hepatmon.6178.

74. Yotsuyanagi H, Koike K, Yasuda K, Moriya K, Hino K, Kurokawa K, et al. Hepatitis C virus genotypes and development of hepatocellular carcinoma. Cancer. 1995; 76: 1352–1355. PMID: 8620408

75. Pockros J. New direct-acting antivirals in the development for hepatitis C virus infection. Therap Adv Gastroenterol. 2010; 3(3): 191–202. https://doi.org/10.1177/1756283X10363055 PMID: 21180601