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Correlation between HCV genotypes infection and AST, ALT, and AFP levels in Saudi Patients.

Sahar EL Hadad[1, 2]*, and Hesa Al-Hamdan[1].

[1]Department of Biological Science, Faculty of Science, King Abdulaziz University, Jeddah, KSA.
[2]Research center of Genetic Engineering and Bioinformatics, VACSERA, Cairo, Egypt.

ABSTRACT

Hepatitis C virus (HCV) has six genotypes that have been classified depending on the conserved region 5'UTR of the HCV genome. Our objective was to perform HCV genotypes based on 5'UTR gene sequence analysis, as well as establishing an association between these genotypes and the levels of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and alpha-fetoprotein (AFP) among 50 chronic HCV Saudi patients. The present HCV-5'UTR phylogenetic tree has verified the predominance of HCV/4 (60.7629%) followed by genotype 1 (36%) and genotype 3 (2%). ALT, AST, and AFP have elevated significantly in 38.8%, 55.55% and 11.11% of HCV/1 patients samples, 29.03%, 54.83%, and 19.35% of the patients infected with HCV/4, while patients infected with HCV/3 demonstrated a normal level of the certain liver markers. We concluded no significant association between HCV/1 and 4 in the levels of ALT, AST, and AFP in Saudi patients, in spite of the number of the HCV genotype 4 patients reported increasing in HCV biochemical markers were higher compared to those detected in patients suffering from HCV genotype 1. Sustained surveillance of hepatitis cases is required to further delineate the risk factors and to verify effective protection strategies.

Keywords: A alpha-fetoprotein; Alanine aminotransferase; Aspartate aminotransferase; HCV genotypes; Liver markers; HCV/1; HCV/4; HCV/3

Corresponding author

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INTRODUCTION

Regardless of reporting declines in HCV commonness over the final 10 years in Saudi Arabia [1], the disease signifies a chief public health problem in the country [2]. Decreasing in HCV prevalence among Saudi population in the absence of effective HCV vaccine could be related to the use of more specific screening assays for detection of HCV antibodies [3]. Upwards to 80% of patients infected with HCV develop chronic liver disease [4]. Patients with post-necrotic liver cirrhosis secondary to HCV are also capable of developing hepatocellular carcinoma with an annual rate of 1 to 4% [5]. HCV was found in 88 patients out of 223 patients with Hepatoma (39.5%) in the Western region [6]. Several serological studies in chronic HCV Saudi patients have proven that 59.6% had genotype 4, 25.1% had genotype 1, 8.3% had genotype 2 and 6.4% had genotype 3 [7, 8] announcing the predominance of HCV genotype 4 followed by genotype 1 [9].

Alanine aminotransferase (ALT) is a cytosolic enzyme expressed from the liver cells, and to a lesser extent in the marrow and skeletal muscles. ALT is the most frequently used hepatic serum biomarker in a variety of laboratories in vivo assays, significant elevations in ALT indicate release of ALT by hepatocytes. Aspartate aminotransferase (AST) is a mitochondrial enzyme, which is existing in great amounts in the liver cells., the marrow, kidney and in skeletal muscle [10]. ALT is the most frequently used hepatic serum biomarker in a variety of laboratories in vivo assays, significant elevations in ALT indicate release of ALT by hepatocytes. AST concentrations tend to mimic ALT activity with regard to liver impairment; however, AST is a less specific biomarker as high concentrations of the enzyme are found in a diversity of other tissues [11].Mutations in the serum prevalence of ALT and AST are indicative of the early levels of viral hepatitis, as comfortably as other liver insults or injuries [10].

In summation, the version of ALT and AST together may give specific information, increased ALT and AST are considered a hallmark of hepato-necrosis and liver damage [12].Association of liver damage with serum HCV-RNA viral load, ALT level, and HCV genotypes has been discussed in many previous studies and the results are quite different. The severity of liver damage was correlated to serum HCV-RNA viral load, which could be accelerated by high HCV load and fat degeneration[13] where both serum ALT and ASTwere associated with liver impairment [14]. In contrast, no correlations with HCV load and the severity of liver damage and the clinical features and ALT level [15], additionally no substantial correlation between HCV RNA levels, ALT and AST values in patients untreated with anti-viral therapies[16].

Alpha-fetoprotein (AFP), which is a tumor-associated fetal protein, has been used as a marker hepatocellular carcinoma (HCC) and other germ cell tumors [17].In addition, elevated serum AFP concentrations have been measured in patients with other non-cancerous diseases, including acute viral hepatitis, chronic active hepatitis and cirrhosis [18].Indeed, serum AFP levels were correlated with the severity of fibrosis/cirrhosis among patients with chronic hepatitis C [19], whereas an elevated serum AFP level was highly specific for the chronic HCV diagnosis [20]. This study aimed to investigate the distribution of HCV genotypes in Saudi Arabiachronic hepatitis C patients in particular Jeddahprovince using nucleotide sequence of the 5UTR-HCV region and evaluated the differences in HCV genotypes and ALT, AST and AFP among chronic hepatitis C patients in Saudi. It is wished that the results of our analysis can serve as a source for the selection of suitable cure protocols, and decision making for the prevention of chronic hepatitis C in Jeddah province as well as in other areas.

MATERIAL AND METHODS

Patients Samples Collection

A sum of 51 HCV RNA positive serum samples of chronic Saudi patients (male: 28, female: 23 ages: 22-75 y) was collected from Jeddah province and was subjected to this study. Serum samples were split into aliquots and stored in a refrigerator (-80°C) within 2hr after blood sampling. *Serum samples with Co-infection with other virus infection such as human immunodeficiency virus or hepatitis B virus were excluded.* All the patients were duly informed about the study, and the informed consents were approved by the Research Ethics Committee at King Abdulaziz University, Jeddah, Saudi Arabia.

Biochemical and immunological methods

HCV patients' blood samples were taken in the morning after 12-h overnight fast. All the patient's serum samples were measured at the laboratory of King Abdulaziz University Hospital. An automatic biochemical analyzer (Dade Lhrinq Inc. Newark. DE19714. USA) was employed for assessing liver function including Alanine Aminotransferase (ALT), and Aspartate Aminotransferase (AST). Normal outcomes from these examinations were distinguished as follows; ALT (5-55 U/L) and AST (5-34 U/L)[21].AFP was detected and quantified by standard radiometric assay methods (DiaSorin SA, Sallugia, Italy), and its normal limits considered was (0.0-15,0 ng/ml) [22].

HCV-5'UTRamplification, nucleotide sequencing, and phylogenetic analysis

HCV genotyping was accomplished using the 5" UTR gene sequencing. Viral RNA extraction using the Mini Elute Viral Extraction Kit (QIAGEN, Inc., Valencia, CA, USA) was executed according to the manufacturer's instructions. The resultant HCV RNA samples were subjected to One Step RT-PCR using One-step RT-PCR Master Mix kit (QIAGEN) and specific HCV-5'UTR primers UHCF 71-955'GAAAGCGTCTAGCCATGGCGTTAGT-3' and UHCR 295-314 5'CTCGCAAGCACCCTATCAGG-3'[23]. The PCR products were purified and sequenced in forward and reverse directions using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems (ABI), Foster City, CA, USA) 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-5UTR* genotypes (1-6) were confirmed using Megastate 6 software. MEGA 6 software using the neighbor-joining method based on the Tamura-Nei model of evolutionary distance were used for phylogenetic trees generation, and the topology was evaluated by bootstrap analysis 1,000 replicates[24-28]. The references HCV genotypes sequences retrieved from the DDBJ/EMBL**/**GenBank databases has been subjected to the phylogenetic analysis of the obtained nucleotide sequences samples.

Statistical analysis

Data was accomplished between HCV genotype 4 and HCV genotype 1 with respect to biochemical markers using Megastat software, and the one-way ANOVA parametric test. Statistical difference was assumed when $P<0.05$.

RESULTS

Amplification of HCV 5'UTR Gene

One step RT-PCR confirmed the presence of HCV-cDNA bands related to 50 out of 51 (98.07%) positive qRT-PCR while the total absences of HCV-cDNA were verified in one (2%) sample. The current HCV-cDNA band sizes were assessed by direct comparison with a 100 bp step ladder DNA marker ranged from 100-1200 bp where they were at the expected size of approximately 230 bp (Figure 1).

Sequencing Interpretation and Phylogenetic Analysis of HCV Genotypes Based on the Partial Nucleotide Sequence of 5'UTR Genes

Partial sequences of the HCV- 5'UTR gene interpreted successfully to all the 50 positive PCR products and assembled for the sequences verification. Phylogenetic tree analysis of HCV-5'UTR gene (230 bp) nucleotide sequence was performed including the 50 present isolates and 6 reference sequences of all HCV genotypes (1-6). The currently constructed tree included three main clusters; the first cluster grouped HCV genotype 1,2, and 6, the second cluster included HCV genotype 4 and 5, while the third one specific for HCV genotype 3. The present isolates grouped as follows; 31 out of the 50 (62%) isolates seemed to be more related to HCV/4 and verified with nucleotide distance identity average +/-0.015-0.022, while 18 (36%) present isolates more related to HCV/1and its nucleotide distance identity equal +/-0.03, followed by 1 (2%) isolate grouped with branch belonged to HCV/3 and its nucleotide distance identity equal +/-0.06, no samples were identified either as HCV genotype 2, 5 or 6 (Figure. 2).

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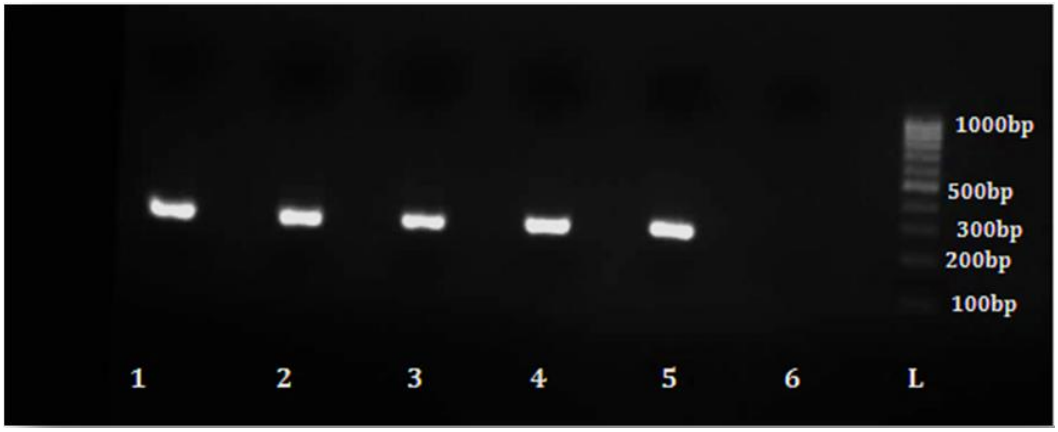

Figure 1: PCR amplification of HCV 5'UTR gene using One-step RT-PCR. Where (L) represented the 100 step DNA ladder marker that ranged from 100-1200 bp. Lane 1, 2, 3, 4 and 5 represented specific cDNA fragments of HCV-5'UTR gene which approximately ranged 230 bp; Lane 6 represented negative control sample (NCS) for RNA.

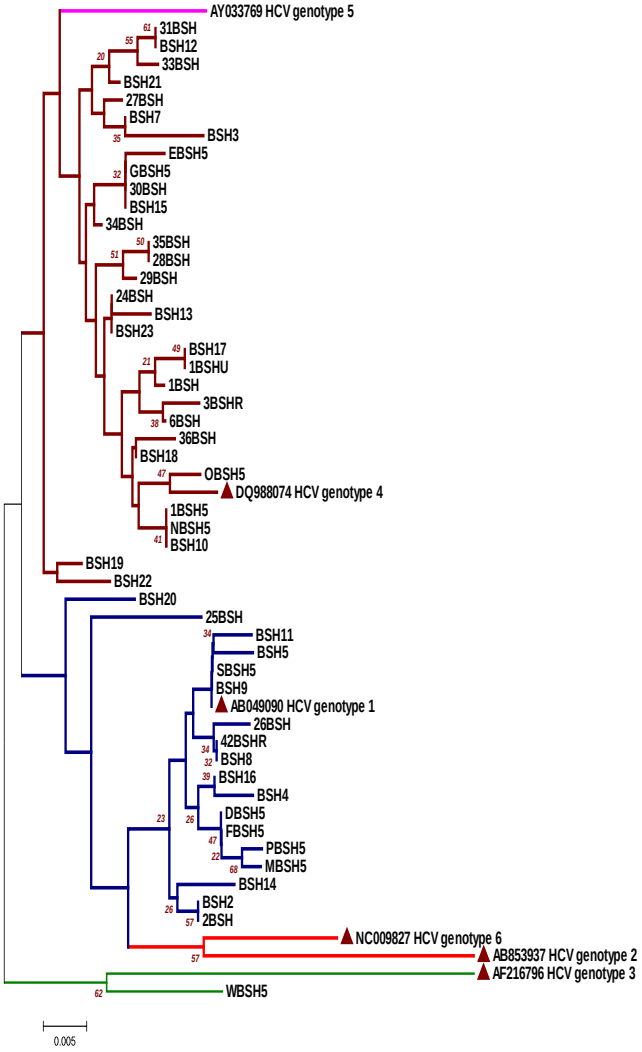

Figure 2: Phylogenetic tree constructed by the neighbor-joining method (NJ), based on the 230 bp of HCV-5'UTR reference sequences retrieved from GenBank database and 50 present HCV isolate sequences. Six reference HCV genotypes isolates indicated by different colored branches, red closed circles, and their accession numbers. Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 resampling.

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HCV Biochemical Markers

HCV genotype 1has been verified normal ALT, AST and AFP levels in 13 (72.22%), 8 (44.44%) and 16 (88.88%) respectively in patients samples, while HCV/1 samples illustrated a high significant increase (*P* value= 0.000) in 7 (38.8%), 10 (55.55%) and 2 (11.11%)of HCV/1 patients samples(*P* value= 0.00) respectively(Figure 3). Although AFP reported a high significant correlation (*P* value= 0.000) between both ALT and AST in the present HCV/1 samples, no significant association was verified between ALT and AST (*P* value= 0.956).

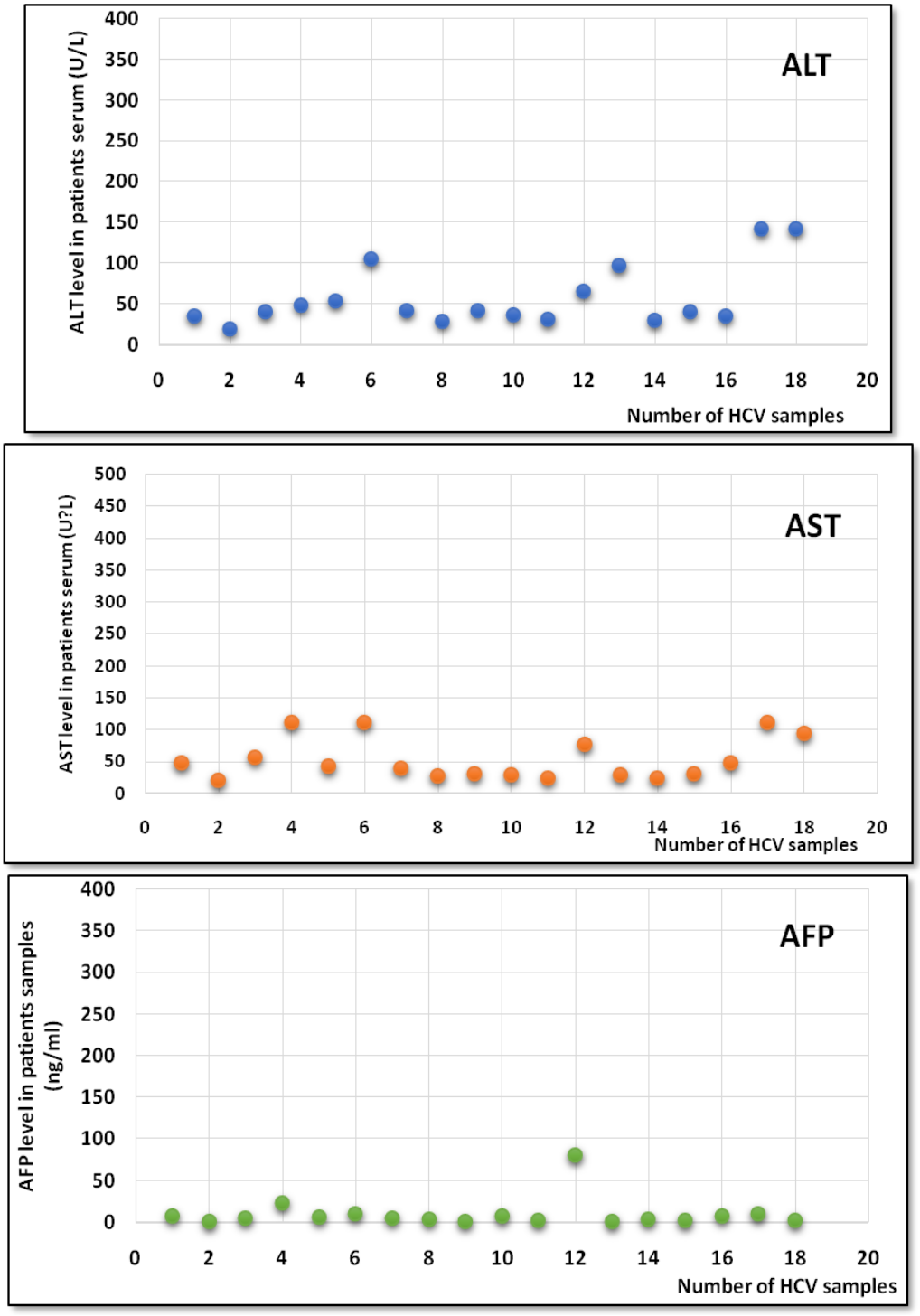

Figure 3: Levels of ALT, AST, AFP in 18 HCV plasma samples identified as genotype 1.Baseline ALT. AST and AFP values were within the normal range according to the new cut–off values (5-55 U/L), (5-34 U/L) (0-15 ng/ul) respectively.

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As for the present 31 samples identified as HCV/4, normal ALT, AST and AFP levels were observed in 22 (70%), 14 (45.16%) and 25 (80.64%) of HCV patient's samples. Meanwhile, ALT, AST and AFP demonstrated a significant increase in9 (29.03%), 17 (54.83%), and6 (19.35%) of the patient's samples (*P* value= 0.000) (Figure 4). Notably, AFP reported a marked significant correlation between both ALT and AST (*P* value= 0.027 and 0.0231) respectively, in the current HCV/4 samples, no significant association was verified between ALT and AST (*P* value= 0.940).

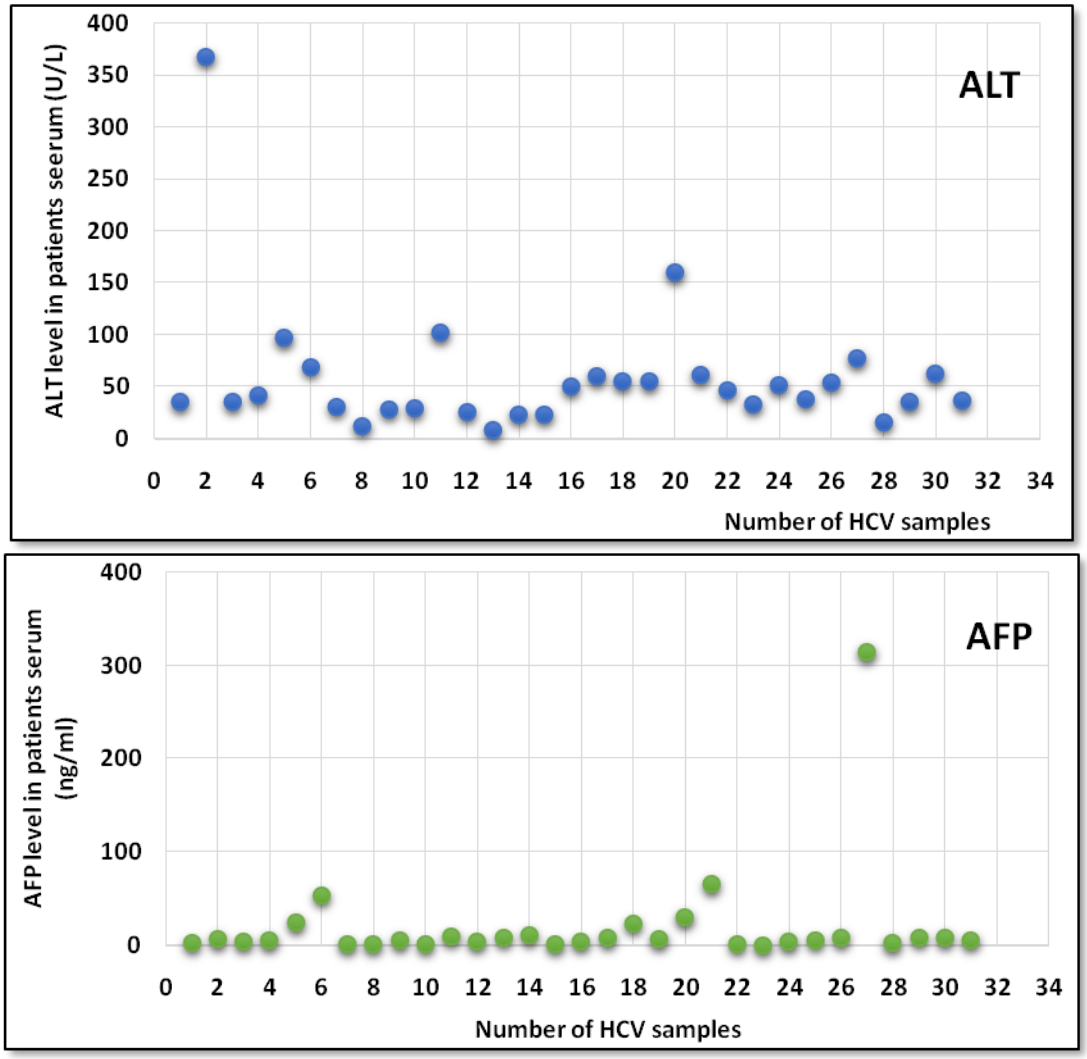

Figure 4: Levels of ALT, AST, AFP in 31 HCV plasma samples identified as genotype 4. Baseline ALT. AST and AFP values were within the normal range according to the new cut–off values (5-55 U/L), (5-34 U/L) (0-15 ng/ul) respectively.

Although the present sample identified as HCV genotype 3 verified normal ALT and AFP level in the patient sample, a slight increase in the AST level was recorded (Figure 5).

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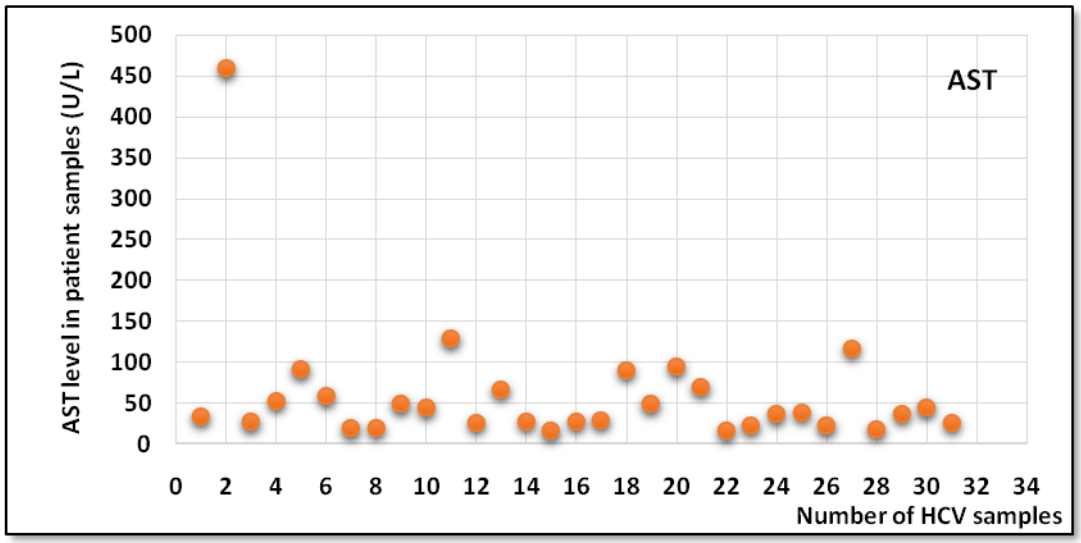

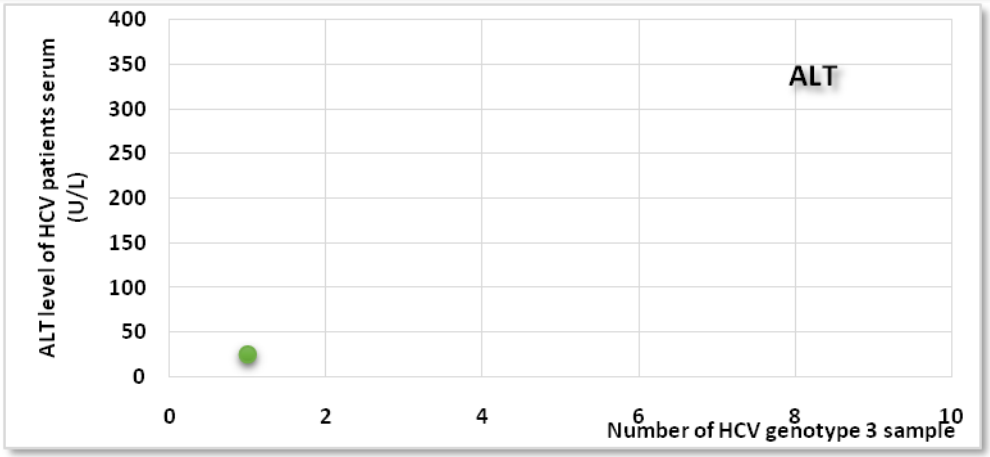

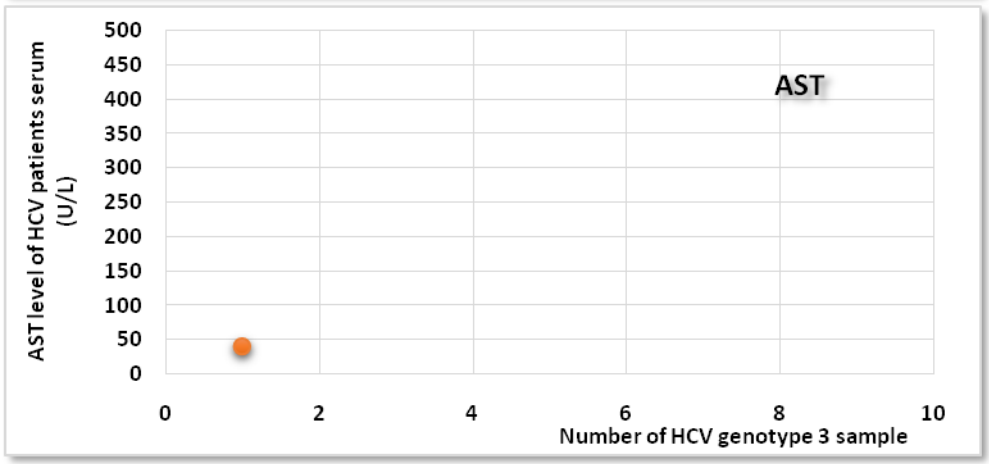

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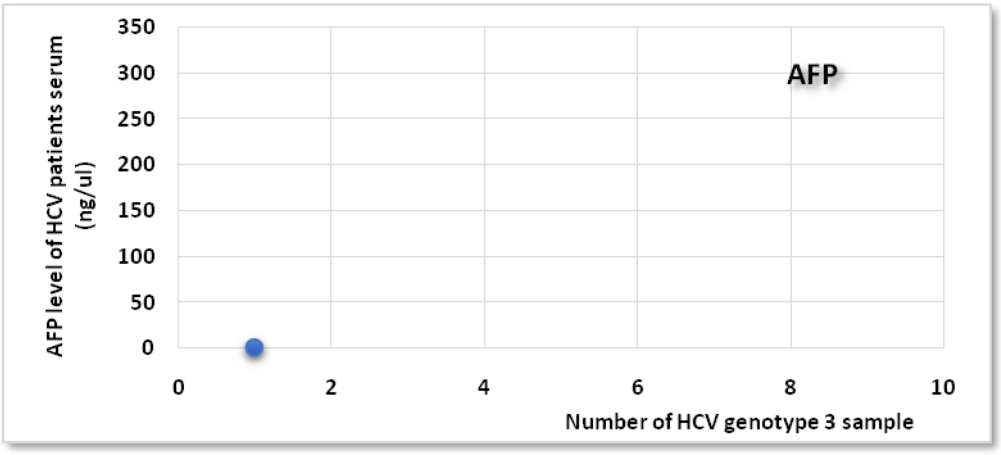

Figure 5: Levels of ALT, AST, AFP in 1 HCV plasma sample identified as genotype 3. Baseline ALT. AST and AFP values were within the normal range according to the new cut–off values (5-55 U/L), (5-34 U/L) (0-15 ng/ul) respectively.

No significant association was observed in between both genotype HCV/1 and HCV/4 in ALT, AST and AFP levels (*P* value = 0.956, 0.575, and 0.472) respectively.

DISCUSSION

HCV infections account for a considerable ratio of liver diseases worldwide and major problem in public health. Even though evolution in the diagnosis and treatment of viral hepatitis, their incidence is still extraordinary in some regions of the world [29].Saudi Arabia has been assigned as a country with an intermediate HCV prevalence [8].The current study attempted to demonstrate the association of the HCV genotypes and some HCV biomarkers. The choice of the genome region to be examined in the recognition of HCV genotypes is critical [30]. Many precursor studies demonstrated the uses of the 5'UTR region for HCV genotyping whereas, it is the region of choice for qualitative and quantitative HCV-RNA based on either hybridization or sequence analysis[31].The currentHCV-5'UTR phylogenetic tree announced the HCV/4 as the predominant HCV genotype which may reach 60.79% among the current HCV samples collected from Saudi chronic patients, followed by HCV/1 and HCV/3 genotypes (37.29% and 1.96% respectively), without any traces for either HCV/ 2, 5 or 6 genotypes.

Investigations that measure the level of serum ALT and, AST and AFP usually reflect hepatic cells integrity instead of liver function[16].The present results demonstrated normal ALT levels in 72%%, 100% and 70% in the serum of patients infected with HCV/1, HCV/3, and HCV/4respectively.ALT increased significantly in 9.7227.77% and 29.9% of chronic patients infected with HCV/1 and HCV/4 respectively. Although the ratio of the patients reported in HCV/4 infection was higher than those infected with HCV/1, no association was reported in the ALT level between HCV4 and HCV/1 genotypes Meanwhile, Approximately 44.44% of the patients infected with HCV/1and 45% of HCV/4patients had normal AST levels, while AST increased significantly in approximately in 55.55% of the HCV/1 chronic patients as well as 54.83% of HCV/4 patients. The non-significant increase was observed in both ALT and AST levels in patients infected with HCV/4 in comparing to those infected with HCV/1.The present result confirmed no significant association between the biochemical markers studied ALT and AST and HCV genotypes1 and 4 [32]. Additionally, the present study verified non-significant increase to normal ALT and AST levels in patient had infected with HCV/3, whereas the serum ALT and AST levels were slightly higher in the genotype 1 and 4 compared to other genotypes [33, 34], this may promote the fibrosis progression that was found to be influenced by increased duration of HCV infection, this suggestion agrees with the results demonstrated by [32, 35].

Although, levels of serum AFP considered as a routine screening tool for HCC in patients with chronic liver diseases [36]. Raised levels of AFP are not identifiable for HCC and can be established in chronic HCV without HCC, as well as liver cell dysplasia [37, 38].The AFP levels in the current HCV/1 and HCV/4 plasma samples established a normal level in approximately 86.11% and 80.64% of the patients respectively, whereas either 11.11% of the HCV/1 and 19.35% of the HCV/4 infected patients demonstrated a significant increase in

AFP levels. Normal AFP level was observed in patient had infected with HCV/3. The present study verified a significant correlation between the elevation of AFP and increasing ALT and AST levels in the all patient subjected to this study with no regarding the HCV genotypes. A significant correlation was detected between serum ALT levels and serum AFP levels in all patients as patients with higher ALT levels had higher AFP serum levels [39]. Simultaneously, patients with high AST levels had high levels of AFP [40], where, elevated serum AFP is independently associated with elevated levels of AST. AFP is usually elevated in patients with chronic liver disease due to the presence of chronic active hepatitis or cirrhosis, this did not necessarily signify the development of HCC[41].

CONCLUSION

The study presented here is the first comprehensive research addressing genotypes analysis of HCV virus in Saudi Arabia, Jeddah using the 5UTR nucleotide sequencing analysis method. Our study announced the HCV genotype 4 is the predominant followed by HCV/1 then HCV/ 3. Our study was unable to find any significant difference between biochemical markers (ALT, AST, and AFP) of the patients infected with genotype 1 or those infected with genotype 4. Otherwise, a significant correlation was detected between plasma ALT and AST and plasma AFP levels in all patients with higher ALT and AST levels had higher AFP levels without respect to HCV genotypes.

Conflict of interest: All authors have announced that no competing interests occur.

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