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Phylogenetic Analysis and Genotyping Distribution of Hepatitis C Virus Isolates Based on the 5`Untranslated Region among some Egyptian Populations

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ABSTRACT

Hepatitis C virus (HCV) is a global problem and the genetic diversity in non-Western countries particularly in Egypt, where the highest prevalence of HCV is very important because of showing distribution of different HCV genotypes . This study aimed to report phylogenetic analysis of hepatitis C virus isolates based on the 5` untranslated region (5`UTR) in Egyptian populations using 51 samples with high and moderate viral load , selected from different regions in Egypt. Samples were identified based on RT-PCR amplification of the 5` UTR. The data were confirmed by sequencing and phylogenetic analysis using Multiple Sequence Alignment to describe the similarities of different isolates of HCV. The results appeared a high level of genetic diversity of genotype 4 with 90.2% that was commonly found with its subtypes ranged 4a (74.5%) ,4l (5.9%) ,4v (5.9%) and 4n (3.9%) while genotype 1 recorded 9.8% with its subtypes ranged 1a (3.9%) ,1b (2.0%) and 1g (3.9%). Using Multiple Sequence Alignment of HCV isolates indicated high percent of similarities between different genotypes of isolates. The results obtained in this study provide a strong evidence for high percent of similarities between different genotypes of isolates obtained from different Egyptian regions.

INTRODUCTION

Hepatitis C virus (HCV) is considered a widely common infectious disease all over the world, as it affects about 170 -210 million people (Stevenson and Utay, 2016).The lowest ranges of prevalence (0.01–0.1%) are found in Netherlands and the highest (14.7%) in Egypt (Gower *et al.*, 2014).

HCV is developed to chronic after infection period with more than six months, there is a very low rate of spontaneous clearance (Zeuzem *et al.*, 2009). The manifestations of chronic HCV range from an asymptomatic state to cirrhosis, and hepatocellular carcinoma (Westbrook and Dusheiko, 2014). HCV infection is usually slowly progressive (Chen and Morgan, 2006 and Hirofumi *et al.*, 2012). Thus, it may not result in clinically apparent liver disease in many patients if the infection is acquired later in life. Approximately 20 - 30% of chronically infected individuals develop cirrhosis over a 20 - 30 years period of time (Yang *et al.*, 2017).

HCV is an enveloped icosahedral viron of 55-65 nm size and it is closely related genetically to both the pestiviruses and flaviviruses (Pfaender *et al.*, 2014). HCV is the only known member of genus *Hepacivirus* in family *Flaviviridae,* that affects man as its primary host (Simmonds *et al.*, 2005 and Blackard *et al.*, 2012). It is has a positive sense, single-stranded RNA (+ ssRNA) genome and HCV genome is 9.6 kb and encodes a unique large polyprotein, that is processed by cellular and viral encoded proteases to give at least 10 mature structural and nonstructural proteins (NSP) (Catanese and Dorner, 2015) .

HCV can be classified by RNA sequence analysis into up to seven genotypes and more than 100 subtypes (Lamoury *et al.*, 2015). Genotypes differ from each other by 25–35% at the nucleotide level , but subtypes differ from each other by 15–25% (Smith *et al.*, 2014).

The 5` untranslated region (UTR) has 341 nucleotides long and contains four domains that are highly structured numbered I to IV( Kamal and Nasser 2008). The 5`UTR is highly conserved and is used for diagnostic testing, whereas sequences encoding the viral envelope may have 60% identity between isolates collected worldwide (Ray *et al.*, 2000 and Kumthip and Maneekarn, 2015) on the basis of the phylogenetic relations of sequences from the core/E1 and NS5B genomic regions, there are 7 distinct HCV genotypes have been described, and each contains multiple subtypes (e.g., HCV 1a, 1b) (Lamoury *et al.*, 2015). The performance of diagnostic assays all may differentiated according to genotype (Costafreda *et al.*, 2006).

5'UTR heterogeneity correlates with interferon and ribavirin treatment outcome as its stability correlated with non-response, whereas changes in this region were associated with Sustained virologic response (SVR) (Bukowska-Ośko *et al.,* 2015) .

There is a distinct geographical distribution of HCV genotypes; Genotype 1 is the most common one in the USA and Europe; and genotypes 2 and 3 have the lowest prevalence in these regions and genotypes 4, 5, and 6 are rare (Ruane *et al*., 2015). Genotype 3 is the most common in India, Far East and Australia and genotype 4 is the most common in Africa and the Middle East.(Mousavi *et al.*, 2013) Genotype 5 is the most common in southern Africa, and genotype 6 is the most common in Hong Kong, Vietnam and Australia (Messina *et al.*, 2015). The novel genotype 7 was identified in patients from Canada and Belgium, possibly infected in Central Africa (Chen *et al.*, 2016) . This study was conducted to investigate phylogenetic analysis of HCV isolates based on 5`UTR region in some Egyptian populations.

MATERIALS AND METHODS

Patients and samples

Fifty one samples were collected from different Egyptian patients inhabit various regions i.e. (Cairo, Mansoura and Asiut).

In vacutainer blood collection tubes each 3 ml blood samples were withdrawn from each patient by vein puncture then allowed to clot for 15 minutes and centrifuged at 7000 rpm for 10 minutes for serum separation, then the sera were stored in 2 ml micro tubes at -80°C (Jean *et al* , 2003).

HCV antibodies and RNA detection

HCV serostatus of each participant was determined by testing for anti-HCV antibodies (anti HCV Ab) using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) test using Murex anti-HCV version 4.0 Kit (Murex Biotech S.A. Kyalami, South Africa) (Conroy-Cantilena, 1997) and then determine: Quantitative estimation of viral load, HCV RNA in sera were detected by real time PCR (RT-PCR) based assay for quantification of HCV RNA using ABI 7500 real time PCR (Applied Biosystems, Foster City,CA, USA) (El-Bendary *et al* , 2016).

RNA preparation and PCR amplification

Viral RNA was extracted and purified from serum using Qiagen RNA extraction kit (Qiamp® RNA Minikit, Qiagen, Germany) , according to manufacturer's instructions (El-Bendary *et al* , 2014). The extracted viral RNA was then converted to cDNA and amplified using Quant One Step RT-PCR Kit (TIANGEN Biotech (China) according to manufacturer's instructions using specific primer (A) for 5`UTR with forward 5'-GAAAGCGTCTAGCCATGGCGTTAGT-3' and reverse 5'-CTCGCAAGCACCCTATCAGG-3' (Abdel-Moneim *et al* , 2012). Then set up thermal cycler conditions as following: reverse transcription at 50 ℃ for 30 min ,then initial denaturation at 94 ℃ for 2 min followed by 45 cycles as denaturation at 94 ℃ for 45 sec and annealing at 55 ℃ for 30 sec, then extension at 65 ℃ for 45 sec for each cycle with a final extension at 65 ℃ for 10 min then analyze the PCR products using 2% agarose gel electrophoresis(Jean *et al* , 2003).

cDNA purification from gel

cDNA was purified from gel using MEGA quick-spin™Total fragment DNA purification kit, Intron (iNtRON Biotechnology, Korea) according to manufacturer's instructions (El-Bendary *et al* , 2016).

5` UTR sequencing of cDNA

Each isolate of HCV was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) .The data collection was done on an automated ABI PRISM 310 genetic analyser (Applied Biosystems), then nucleotide sequences of each isolate were used for phylogenetic analysis (Ohtsuru *et al.*, 2013) .

Sequencing data analysis

Computational blast data analysis for nucleotide sequences of each isolate were assigned online by alignment using (http://www.blast.ncbi.nlm.nih.gov) and detecting different genotypes (Omran *et al.,* 2009).

Phylogenetic analysis

Sequences applied for specific primer of 5` UTR and obtained sequencing strains from different regions in Egypt that aligned using MAFFT (Multiple Alignment using Fast Fourier Transform). Multiple Sequencing Alignment through Jalview program version 2.10.2b1 (Vieira *et al.,* 2011).

RESULTS AND DISCUSSION

Detection of HCV using ELISA kit Murex anti-HCV version 4.0, with Cut-off Value 0.690 and positive results are more than 0.8 and obtained 51 patients of positive HCV (Table 1).

Table 1. ELISA results for positive 51 samples of HCV.

No.	Virus sample	ELISA Value (OD)	Result	No.	Virus sample	ELISA Value	Result
1	Ma1	2.7	+ve	27	As14	3.1	+ve
2	Ma2	0.89	+ve	28	As15	3.6	+ve
3	Ma3	1.2	+ve	29	As16	2.8	+ve
4	Ma4	0.96	+ve	30	As40	1.6	+ve
5	Ma5	2.9	+ve	31	As44	2.5	+ve
6	Ma6	3.1	+ve	32	As45	0.90	+ve
7	Ma7	2.4	+ve	33	As46	1.7	+ve
8	Ma8	2.2	+ve	34	As47	2.4	+ve
9	Ma9	2.7	+ve	35	As54	3.2	+ve
10	Ma10	0.99	+ve	36	Ca17	2.8	+ve
11	Ma25	0.87	+ve	37	Ca18	3.3	+ve
12	Ma26	2.6	+ve	38	Ca19	1.8	+ve
13	Ma28	1.7	+ve	39	Ca20	2.9	+ve
14	Ma31	0.94	+ve	40	Ca21	1.3	+ve
15	Ma32	3.9	+ve	41	Ca22	4.1	+ve
16	Ma33	1.4	+ve	42	Ca23	0.97	+ve
17	Ma35	2.8	+ve	43	Ca24	1.6	+ve
18	Ma36	3.3	+ve	44	Ca34	2.3	+ve
19	Ma37	3.6	+ve	45	Ca41	1.9	+ve
20	Ma38	1.7	+ve	46	Ca42	2.6	+ve
21	Ma39	2.9	+ve	47	C43	4.4	+ve
22	Ma48	3.8	+ve	48	Ca50	0.88	+ve
23	Ma49	4.2	+ve	49	Ca51	1.1	+ve
24	As11	0.91	+ve	50	Ca52	2.6	+ve
25	As12	1.8	+ve	51	Ca53	2.2	+ve
26	As13	2.6	+ve				

Note: OD. = Optical Density.

Determination quantitative estimation of viral load: HCV RNA in serum was detected by RT-PCR-based assay for quantification of HCV RNA (ABI 7500 real time PCR) and the samples detected between 100,000 IU/ml – 1000,000 IU/ml with moderate and high titers to obtain 51 samples then the extracted RNA amplified through PCR amplification and obtaining cDNA that identified on agarose gel (2%) giving sharp bands of specific primer (A) of 5` UTR and checked in each lane with product at 241 bp (Figure 1) .

Hepatitis C is a world wide health problem (Mohamed *et al.,* 2015) and on the basis of the phylogenetic relations of sequences from 5` UTR, there are 7 distinct HCV genotypes have been described, and each contains multiple subtypes (e.g., HCV 1a, 1b) and treatment recommendation was based on the HCV genotype (Lamoury *et al.*, 2015).

Different genotypes were found in this study with 56.9% males ,43.1% females of a total 51 samples , as genotype 4 with its subtype 4a which is the most common subtype in Egypt and other subtypes that are less common such as 4n,4l,,4v and genotype 1 the less prevalent in Egypt and its subtypes as 1a,1b and 1g (Table 2)

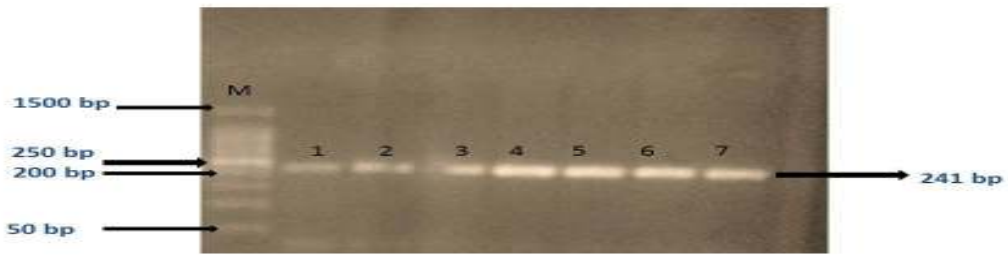

Figure 1. Agarose gel (2%) stained with ethidium bromide showing RT-PCR (PCR amplification of HCV) for 5` UTR from positive samples using specific primer A (M : DNA molecular weight marker (ladder with 50 bp) ; Lanes 1-7 PCR product bands at 241bp).

Table 2. Genotyping distribution of HCV based on the 5`UTR in some Egyptian patients from different regions in Egypt.

Virus sample	Age	Sex	Genotype	Location/Governorate
Ma1	48	M	4a	Mansoura/ El-Dakahlya
Ma2	41	F	4a	Mansoura/ El-Dakahlya
Ma3	47	M	4l	Mansoura/ El-Dakahlya
Ma4	18	F	4l	Mansoura/ El-Dakahlya
Ma5	51	M	4a	Mansoura/ El-Dakahlya
Ma6	44	F	4a	Mansoura/ El-Dakahlya
Ma7	54	M	4n	Mansoura/ El-Dakahlya
Ma8	32	F	4n	Mansoura/ El-Dakahlya
Ma9	51	F	4a	Mansoura/ El-Dakahlya
Ma10	28	M	4a	Mansoura/ El-Dakahlya
Ma25	38	F	1b	Mansoura/ El-Dakahlya
Ma26	43	M	1g	Mansoura/ El-Dakahlya
Ma28	51	M	4a	Mansoura/ El-Dakahlya
Ma31	36	F	4a	Mansoura/ El-Dakahlya
Ma32	39	F	4a	Mansoura/ El-Dakahlya
Ma33	44	M	4a	Mansoura/ El-Dakahlya
Ma35	18	M	4a	Mansoura/ El-Dakahlya
Ma36	22	M	4l	Mansoura/ El-Dakahlya
Ma37	23	M	4a	Mansoura/ El-Dakahlya
Ma38	21	F	4a	Mansoura/ El-Dakahlya
Ma39	28	F	4a	Mansoura/ El-Dakahlya
Ma48	38	F	4a	Mansoura/ El-Dakahlya
Ma49	49	M	4a	Mansoura/ El-Dakahlya
As11	39	F	4a	Asiut/Asiut
As12	43	M	4a	Asiut/Asiut
As13	47	M	4a	Asiut/Asiut
As14	41	F	4a	Asiut/Asiut
As15	50	M	4a	Asiut/Asiut
As16	44	F	4a	Asiut/Asiut
As40	18	M	4a	Asiut/Asiut
As44	22	M	4a	Asiut/Asiut
As45	29	F	4a	Asiut/Asiut
As46	49	M	4a	Asiut/Asiut
As47	19	M	4a	Asiut/Asiut
As54	46	F	4a	Asiut/Asiut
Ca17	45	M	4v	Cairo/Cairo
Ca18	38	F	4v	Cairo/Cairo
Ca19	49	F	4a	Cairo/Cairo
Ca20	46	M	4a	Cairo/Cairo
Ca21	49	M	4a	Cairo/Cairo
Ca22	41	F	4a	Cairo/Cairo
Ca23	54	M	1g	Cairo/Cairo
Ca24	48	M	1a	Cairo/Cairo
Ca34	44	M	1a	Cairo/Cairo
Ca41	23	M	4a	Cairo/Cairo
Ca42	29	F	4a	Cairo/Cairo
C43	49	M	4a	Cairo/Cairo
Ca50	51	M	4v	Cairo/Cairo
Ca51	21	F	4a	Cairo/Cairo
Ca52	22	M	4a	Cairo/Cairo
Ca53	19	F	4a	Cairo/Cairo

Note: Ma= Mansoura, As= Asiut, Ca= Cairo, F= Female, M= Male

The sequence analysis of 5` UTR indicated that the samples belonged to HCV and different genotypes were detected as genotype 4 with its subtype 4a which is the most common subtype in Egypt as reported by DeWolfe Millera and Abu-Raddad (2010). Other subtypes that are less common such as 4n,4l,4v and genotype 1 the less prevalent in Egypt and its subtypes as 1a,1b and 1g. Genotype 1 with 9.8% and percent of females (4.5%) in genotype 1 is lower than that found in males (13.8%). These data differs with the study of Niu *et al.* (2016), who found that females were higher in genotype 1 than males, and genotype 4 with 90.2% and percent of females (95.5%) in genotype 4 is higher than that found in males (86.2%). These observations were previously reported in many studies (Chamberlain *et al.,*1997 and Khattab *et al*.,2011). Also detection subtypes for genotype 4, 4a with 74.5%, 4l, 4v, with 11.8% (5.9% for each) and 4n with 3.9% (Abdel-Ghaffar *et al.* , 2015) and subtypes for genotype 1 are 1g & 1a (with 3.9% for each) & 1b with 2.0 % (Andriulli *et al.* ,2015) (Table 3).

Table 3. Genotypes and subtypes percentages found in Egyptian populations.

Genotype/subtype	%	% of Male	% of Female
Genotype 4	90.2	86.2	95.5
Subtype 4a	74.5	68.9	81.9
Subtype 4l	5.9	6.9	4.5
Subtype 4v	5.9	6.9	4.5
Subtype 4n	3.9	3.5	4.6
Genotype 1	9.8	13.8	4.5
Subtype1g	3.9	6.9	0
Subtype 1a	3.9	6.9	0
Subtype 1b	2.0	0	4.5

Different isolates of Egyptian populations are aligned using MAFFT. It is a high speed multiple sequence alignment program and alignment format is FASTA. On the other hand, it illustrated the % of identity is not small between different regions in Egypt that have similarities of their sequences in different positions of each HCV isolate (Lole *et al.,*2003) (Figure 2).

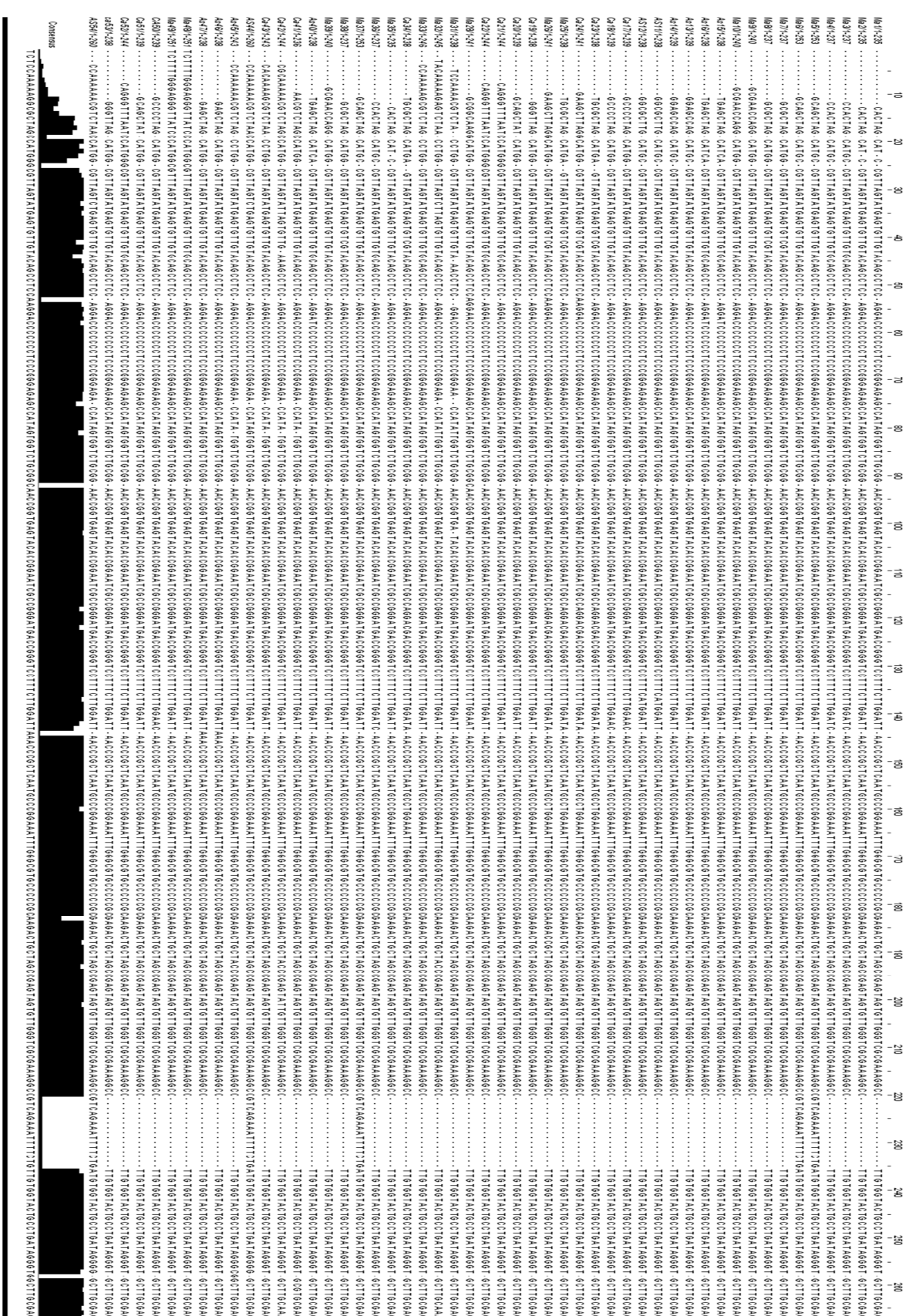

Figure 2. Multiple alignments of the neucleotide sequence of 5`UTR for the 51 isolates from 3 different regions (Mansoura ,Cairo & Asiut) in Egypt using MAFFT .

The phylogeny between different regions in Egypt illustrated in Figure 3. Genotype 4 isolates display great genetic diversity in 51 HCV isolated from Egypt with the most common genotype 4a. This phylogenetic tree was drawn according to percentage of identity between nodes and related branches, each branch of the phylogenetic tree is assembled with its adjacent nodes, nodes represent the similar sequences as found Ma48, Ma49 that are very similar sequences have length 2.27 and Ma32 & Ca42 that considered two adjacent nodes. On the other hand, there is high similarities between them and have branch length 2.22 then this has 2 clusters Ca23,Ma25,Ca34 similar sequences & Ca24,Ma26 with the same length 1.30, although it belongs to different regions reaching to Ma1 has two pairs with Ma2,Ma35 and has other branch with Ma3,Ma4,Ma36 with the same length 0.37.

In conclusion, each branch have two adjacent nodes (similar sequences) either these sequences found in the same region of Egypt or different regions, there is a relation between all of them according to % of identity between sequences and length of branches. Different genotypes of HCV have % of identity is not small between different regions in Egypt and have similarities of sequences in different positions.

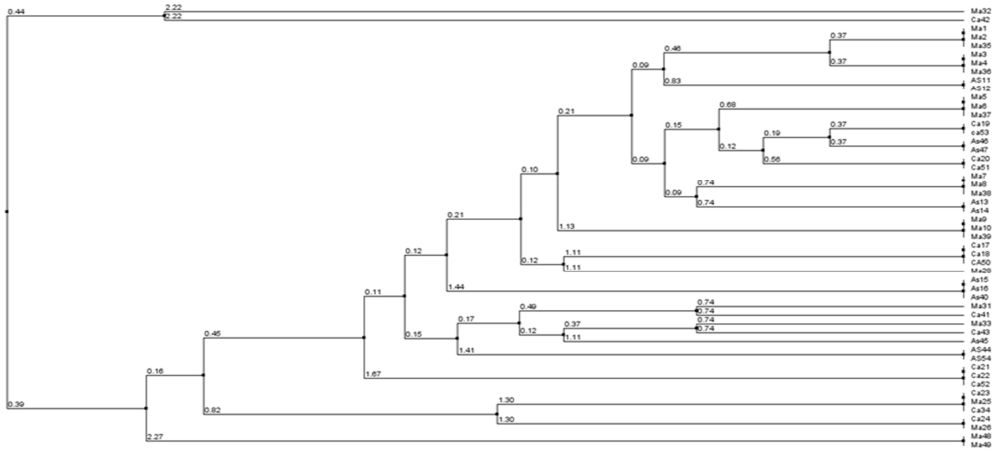

Figure 3. Phylogenetic tree based on the nucleotide sequences of HCV 5` UTR Showing the genetic relationship between all the 51 positive isolates.

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تحليل التطور الوراثى والتوزيع الجينى لعزلات فيروس الالتهاب الكبدى الوبائى "سى" معتمدا على المنطقة الغير مترجمة من النهاية `5 على مستوى بعض العشائر المصرية

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يعتبر فيروس الالتهاب الكبدى الوبائى "سى" مشكلة عالمية ,ويشكل التنوع الوراثى ذو أهمية قصوى فى البلدان غير الغربية ولاسيما فى مصر حيث يوجد بها أعلى معدلات انتشار فيروس التهاب الكبدى "سى" وذلك بسبب توزيع مختلف الانماط الجينية للفيروس. تهدف هذه الدراسة الى تحليل النشوء والتطور لعزلات الفيروس استنادا الى منطقة 5`UTR من مجموعة من المصريين وذلك باستخدام 51 عينة ذات قوة فيروسية متوسطة وعالية تم اختيارها من مناطق مختلفة فى مصر, وذلك من خلال تفاعل البلمرة المتسلسل لمنطقة 5` UTR . بعد ذلك تم التأكد من النتائج عن طريق تقنية التسلسل المتتابع وتحليل النشوء والتطور باستخدام المحاذاة التتابعية المتعددة لتصف ما اذا كان هناك تشابه بين مختلف العزلات للفيروس. أوضحت النتائج بأن هناك تنوع وراثى للنمط الجينى 4 بنسبة 90.2% والذى يعتبر أكثر شيوعا وتم تعيين الأنماط التحت جينية للنمط الجينى 4 وهى النمط التحت جينى 4a بنسبة 74.5% ,4l,4v بنسبة 5.9% لكل منها و 4n بنسبة 3.9% , بينما النمط الجينى 1 بنسبة 9.8% أما الأنماط التحت جينية للنمط الجينى 1 هى 1a , 1g بنسبة 3.9% لكل منها و 1b بنسبة 2.0 % , وكنتيجة لاستخدام المحاذاة التتابعية المتعددة لعزلات الفيروس اتضح أن هناك نسبة تشابه عالية بين مختلف الأنماط الجينية لعزلات الفيروس. هذه النتائج تعكس دليل قوى على وجود نسبة تشابه عالية بين مختلف الأنماط الجينية لعزلات فيروس "سى" الموجودة فى مناطق مختلفة فى مصر