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Characterization of a Partial Sequence Encoding Envelop Protein of HCV-Genotype 4a Egyptian Isolate

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ABSTRACT: *In this study, RNA isolated from sera of Egyptian HCV-patients was used to amplify a fragment of a M. wt. of ~800pb corresponding to a partial sequence of the HCV-E2 encoding gene. The amplified fragment was cloned, sequenced and the nucleotide blast analysis of our sequence revealed partial homology with previously published E2-genes of viral isolates from different locations; the highest match (88%) was annotated with a Japanese isolate suggesting that our herein characterized HCV-E2 partial sequence is a novel one. The impact of HCV-E2 sequence variability will be discussed.*

I. Introduction

About 150 million people are chronically infected with hepatitis C virus worldwide, and more than 350 000 people die every year from hepatitis C-related liver diseases **[1].** Egypt has the highest worldwide prevalence of genotype 4 as the most common in the Middle East and Africa **[2]**. The HCV genome is a positive-strand, ~ 9.6-kb RNA molecule consisting of a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). The 5′UTR contains a highly structured internal ribosome entry site while the 3′ UTR is essential for replication **[3, 4]**. The HCV-ORF encodes a single polyprotein of ~ 3000 amino acids in length and is post translationally processed to produce at least 10 different proteins: the core, envelope proteins (E1-E2), p7 and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B **[5, 6, 7].** The envelope glycoprotein genes display some of the highest levels of HCV genetic heterogeneity, with E2 exhibiting greater variability than E1. The hypervariable region 1 (HVR1) is located at the N-terminus of E2 and this region is the major determinant for strain-specific neutralizing-antibody responses **[8]**. The rate and nature of nucleotide substitutions within HVR1 during the early stages of infection appear to be correlated with outcome: patients harboring a stable HVR1 quasispecies frequently resolve infection, whilst those with evidence of a rapidly evolving population develop chronic infection. In spite of the high variability of this region there is a strong negative selection against some amino acid substitutions since, at most codons, there is selection for conservative amino acid replacement, pointing to a biological role in the virus life-cycle **[9, 10]**. E2; one of the possible targets for the development of an effective vaccine, encodes as many as 11 N-linked glycosylation sites, many or all of which may be utilized during the post-translational processing of nascent E1-E2 complexes **[11]**. Multiple N-linked glycans, in addition to assisting in the folding of antigenically complex proteins, may have other functions, such as masking proteins from reactivity with virus-specific antibodies, facilitating escape from neutralization by antibodies or the complement, and interfering with antigen processing. E2 is thought to mediate attachment to target cells and binds to human CD81, a member of the tetraspanin family of proteins. Interaction of E2 with CD81 on B or T cells has been reported to result in B-cell aggregation and a lowering of the threshold for T- and B-cell activation **[12**, **13, 14]**. The N-terminal 27 residues of E2 (HVR1); aa 384–410, show a very high degree of variation, both within isolates and genotypes, and this portion of the sequence is considered as a leading contributor to disease progression due the emergence of new viral mutants or "quasispecies" induced by the host immune system **[15**, **16, 17]**. This study was designed to amplify HCV-E2 protein encoding sequence from HCV-infected Egyptian patients and compare it to other HCV-envelope sequences from different geographical settings.

II. Materials and Methods

2.1. Human Sera

Blood samples were collected from infected Egyptian patients with HCV who were examined at the Medical Unit of the National Research Center. Additional blood samples were collected from humans with no history of liver diseases or infections and used as negative control. Both were centrifuged at 14,000*xg*, sera were separated, divided into aliquots and stored at -80 ^{o}C until used for viral RNA extraction, measuring liver enzymes and detecting of anti-HCV antibodies (Axium; Florida, USA).

2.2. Approval of the Ethical Committee

Collection of human blood samples was approved by the Medical Ethical Committee of the National Research Center in Egypt according to the ethical guidelines approved by the Ethical Committee of the Federal Legislation and ethical guidelines of the National Institutes of Health in the USA.

2.3. Amplification of HCV-E2 Gene

Viral RNA was extracted from sera of HCV infected patients according to the manufacturer's instructions, (Qiagen, Hilden, Germany). The HCV-E2 encoding sequence was amplified using the listed primers in **(Table 1)** in either a single round or nested reverse transcriptase polymerase chain reaction (RT-PCR) and the RevertAid premium RT (Thermofisher Scientific; USA). The RT program included 60 min at 50 ^{o}C followed by enzyme deactivation at 85^{o}C for 5 min. The first PCR program included 35 cycles each of 30 seconds (s) at 94^{o}C, 45 seconds at 54^{o}C and 2 min at 72^{o}C using a Dream*Taq* DNA polymerase (Thermofisher Scientific, USA), followed by a final extension at 72 ^{o}C for 10 min. The PCR product was used in the nested PCR and the program included 35 cycles of 30 s at 94^{o}C, 30 s at 58^{o}C, 1 min at 72^{o}C and a final extension of 72 ^{o}C for 10 min. PCR products were resolved by electrophoresis on 1% agarose gels, gel slices carrying the amplified fragments were cut and subjected for DNA extraction (Gel cleaning kit; Thermofisher Scientific, USA).

2.4. Cloning, Plasmid Purification and Sequencing

The purified envelope fragment was first cloned into the pSC-TA plasmid (Stratagen) according to the user manual and the generated construct (pSC-E2) was used to transform the DH5α-*E. coli* competent cells. Successful cloning was confirmed by colony PCR on grown bacteria on LB/ampicillin agar plates. PCR-positive colonies were subjected to small-scale plasmid preparation using the GeneJet plasmid DNA miniprep kit (Thermofisher Scientific, USA), the purified plasmid was subjected to automated sequencing from both directions using the HCV-E2 sequence specific forward (F) and reverse (R) primers. The obtained sequences were aligned to the previously published sequences in the GenBank using the basic nucleotide blast application. Construction of a neighbor-joining phylogenetic tree was done using the Bioedit software (http://www.mbio.ncsu.edu/BioEdit/ bioedit.html).

III. Results & Discussion

3.1. Successful Amplification and Cloning of the Encoding Fragment for the HCV-E2 Protein

A fragment of a M. wt. of ~800pb corresponding to a partial sequence of the HCV-E2 encoding gene was visualized upon subjecting extracted RNA from HCV-infected human sera to RT-PCR **(Fig. 1.A).** Successful amplification of HCV-E2 fragment was first confirmed by internal PCR that resulted in a shorter fragment of 660pb when as expected molecular weight predicted from the published sequence of HCV.Ed43 (Accession NO.: **Y11604**) **(Fig. 1.B).** Moreover, successful cloning to pSC-TA vector was verified when the same parent fragment (800 bp) was detected by direct PCR on transformed bacteria with the construct carrying such a fragment **(Fig 2)**.

3.2. Homology of the Obtained Sequence with Previously Published HCV-E2 Gene Sequences from Various Geographical Settings

Nucleotide blast analysis (ncbi.nlm.nih.gov) of our HCV-E2 partial sequence revealed partial homology with previously published E2-genes of viral isolates from different locations such as Egypt, Japan, USA, UK and others .The highest extent of homology (88%) was annotated with the sequence of hepatitis C virus subtype 4a genomic RNA, complete genome, isolate: HCV genotype 4a-KM (AB795432.1,Tsukiyama-Kohara, K. and Michinori Kohara, 2013 (unpublished)), which further confirms the successful amplification of the HCV-E2 fragment and strongly suggest a correlation between such sequence variation and the resistance of Egyptian HCV 4a –infected patients to any HCV therapy including the novel ones containing the protease inhibitors **[18].** The descending order of the homology of our HCV-E2 partial nucleotide sequence with previously published ones is presented in **Table (2).** Nucleotide sequence-based phylogenetic tree is demonstrated in **(Fig. 3).**

IV. Conclusion

Both partial homology and unique features of our newly characterized HCV-E2 sequence compared to previously published HCV-envelope sequences might be among the reasons for the poor response of HCV-4a to effective anti-HCV therapy against other genotypes of the virus.

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Figures And Tables

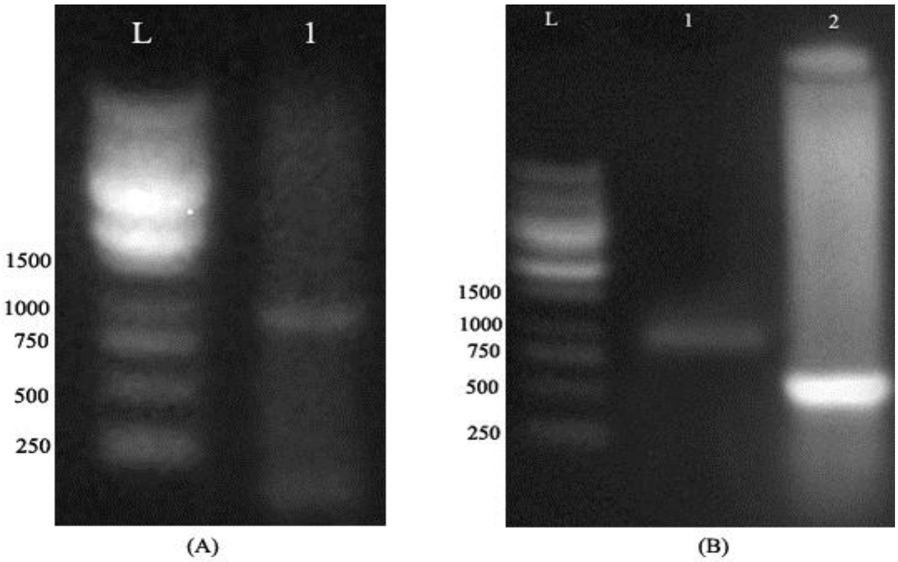

Fig. (1): Amplification of HCV-E2 fragments. Fig (1A) shows the PCR product following RT-PCR. A homogenous band of ~800 pb was detected at the expected M.wt. Fig (1B) shows the nested PCR amplification of smaller band of ~660 pb (lane 2) when the purified HCV-E2 fragment (lane 1) was used as template. Electrophoresis was carried out on 1% agarose gel and a 1kb DNA ladder was run on the same gel (lane L).

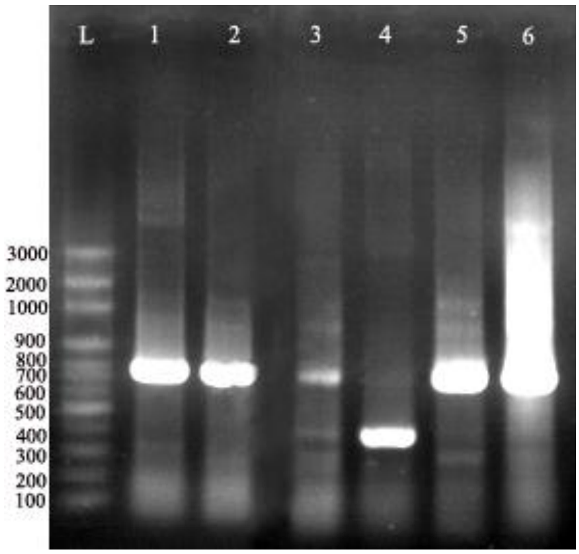

Fig. (2): Amplification products obtained from direct PCR on individual *E. coli* BL21 (DE3) colonies transformed with the pSC-A constructs into which the HCV-E2-encoding sequence was cloned using insert-specific primers. Specific bands of a M.wt. 800 bp were visualized in 4 out of 6 tested colonies reflecting high cloning efficiency. Electrophoresis was carried out on 1% agarose gel and a 1kb DNA ladder was run on the same gel (lane L).

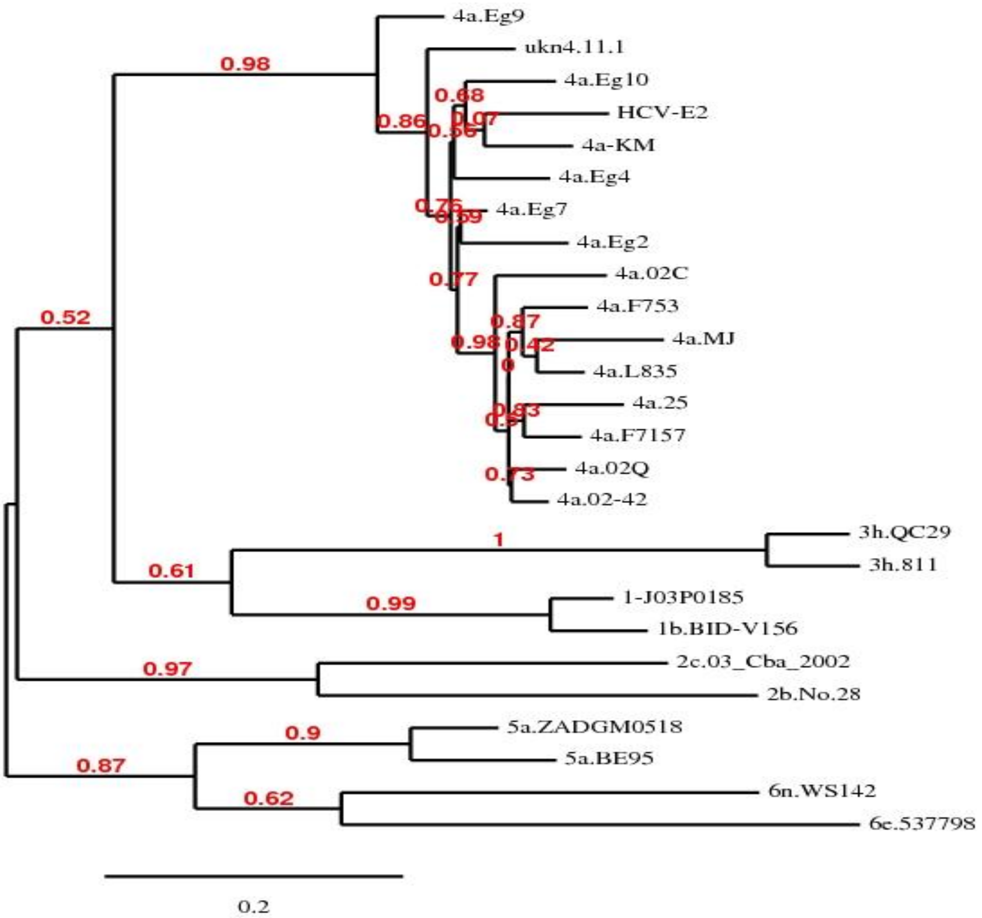

Fig. (3): Phylogenetic comparison of HCV-E2 sequence from Egyptian isolate with published ones with highest degree of homology.

Description	Country	Total Score	Max Ident	Accession	Year Of Submission	Publication
Hepatitis C virus subtype 4a genomic RNA, complete genome, isolate: HCVgenotype4a-KM	Japan	514	88%	AB795432	2013	-
Hepatitis C virus isolate Eg7 polyprotein gene, partial cds	Egypt	483	86%	DQ988076	2007	-
Hepatitis C virus isolate Eg4 polyprotein gene, partial cds	Egypt	466	86%	DQ988075	2007	-
Hepatitis C virus subtype 4a isolate 02-42 polyprotein gene, complete cds	USA	460	87%	DQ418783	2007	**[19]**
Hepatitis C virus isolate Eg10 polyprotein gene, partial cds	Egypt	455	85%	DQ988078	2007	-
Hepatitis C virus subtype 4a isolate L835 polyprotein gene, complete cds	USA	448	86%	DQ418789	2007	**[19]**
Synthetic construct clone ukn4.11.1 envelope protein gene, complete cds	UK	448	85%	AY734986	2007	**[20]**
Hepatitis C virus isolate Eg2 polyprotein gene, partial cds	Egypt	446	85%	DQ988073	2007	-
Hepatitis C virus isolate 02Q polyprotein gene, partial cds	USA	442	86%	DQ418785	2007	**[19]**
Hepatitis C virus subtype 4a isolate 25 polyprotein gene, complete cds	SP	436	85%	DQ516084	2007	**[21]**

Hepatitis C virus subtype 4a isolate 02C polyprotein gene, complete cds	USA	433	85%	DQ418784	2007	**[19]**
Hepatitis C virus subtype 4a isolate F7157 polyprotein gene, complete cds	USA	425	86%	DQ418788	2007	**[19]**
Hepatitis C virus isolate Eg9 polyprotein gene, partial cds	Egypt	412	84%	DQ988077	2007	-
Hepatitis C virus subtype 4a isolate F753 polyprotein gene, complete cds	USA	411	84%	DQ418787	2007	**[19]**
Hepatitis C virus isolate 4a-MJ polyprotein gene, partial cds	USA	409	84%	GQ379230	2009	**[22]**
Hepatitis C virus (1) isolate J03P0185 envelope glycoprotein (env) gene, partial cds	USA	304	73%	JQ064300	2011	**[23]**
Hepatitis C virus subtype 1b isolate HCV-1b/US/BID-V156/2004,complete genome complete genome	USA	291	73%	EU155226	2009	-
Hepatitis C virus (2c) isolate 03_Cba_2002 polyprotein gene, partial cds	Argentina	285	72%	JF511047	2011	-
Hepatitis C virus (3h) isolate QC29, complete genome	Canada	277	70%	JF735121	2013	**[24]**
Hepatitis C virus (3h) isolate 811, complete genome	Somalia	277	70%	JF735126	2013	-
Hepatitis C virus (5a) isolate ZADGM0518 polyprotein gene, partial cds	South Africa	272	70%	KC767832	2013	-
Hepatitis C virus (6n) isolate WS142 polyprotein gene, partial cds	China	271	69%	EU119974	2008	**[25]**
Hepatitis C virus subtype 2b gene for polyprotein, complete cds, isolated from patient No.28 isolated from patient No.28	Japan	269	70%	AB661432	2011	-
Hepatitis C virus (6e) isolate 537798 polyprotein precursor, gene, complete cds	USA	263	69%	EU408326	2009	-
Hepatitis C virus type 5a (BE95) envelope protein (E1) gene, partial cds; envelope protein (E2) gene, complete cds	Belgium	258	69%	L29578	1995	**[26]**

Table (1) list of primers used in gene amplification and screening

Table (2): Homology results of nucleotide sequence of our HCV-E2 gene to published HCV-envelope

	Primer ID	**Primer sequence**	**Nucleotide position**
1	Outer sense	5'GGCTCTTGTCCCCCCGTGGC3'	566-588
2	EF1	5′CACTGGACCACCCAGGATTGCA3′	1171-1196
3	E2F2	5′CACTGGGGTGTCCTCGTGGG 3′	1228- 1253
4	E2R1	5′TGAACAGTACCGGGTTCACCAA 3′	1947-1967
5	E2R2	5′TGGATGAACAGTACCGGGTTCA3′	1942-1963

sequences in GenBank database