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Original Article

# 5′ UTR and NS5B-based genotyping of hepatitis C virus in patients from Damietta governorate, Egypt

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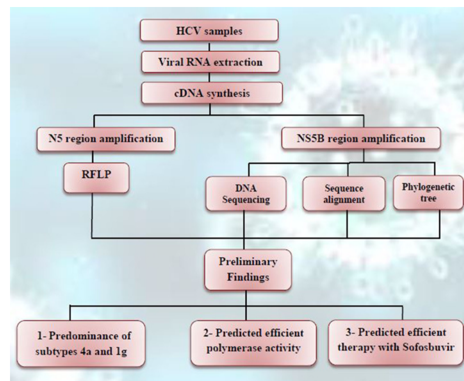
## GRAPHICAL ABSTRACT

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## ABSTRACT

Chronic hepatitis C virus (HCV) infection is a main health problem in Egypt causing high rates of mortalities. Egypt has the highest HCV prevalence in the world, with specific HCV subtypes epidemic and circulating extensively in the country. Different antiviral therapy protocols have been implemented for treating Egyptian HCV patients. Due to the limited data about HCV in Egypt, this study aimed to genotype HCV strains circulating in the Nile Delta Damietta governorate and to investigate the variation in the non-structural 5B (NS5B) region targeted by the newly approved antiviral drugs. Thirty HCV samples from treatment-naïve patients were genotyped by restriction fragment length polymorphism. Some samples were genotyped by direct sequencing of their 5′ untranslated region (UTR) and NS5B regions. Phylogenetic analysis was also performed on the sequences of their NS5B regions. Fourteen new sequences have been deposited in the GenBank database. Results showed that subtype 4a was prevalent in addition to subtype 1g. None of the previously reported NS5B substitutions were detected in the sequenced isolates from treatment-naïve patients, which may be a good predictor for efficient treatment of HCV Egyptian patients with Sofosbuvir. Further studies on Sofosbuvir treated-HCV Egyptian patients are required to investigate whether any NS5B substitutions can confer resistance to treatment.
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## Introduction

Hepatitis C virus (HCV) is the main cause of chronic liver disease and it is an epidemic pathogen worldwide. It is estimated that

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around 71 million people are chronically infected with HCV and approximately 400 thousand people die each year mostly because of liver cirrhosis and hepatocellular carcinoma [1]. HCV is a blood-borne, positive sense, single-stranded RNA virus. Its RNA strand is approximately 9.6 kb with a long open reading frame encoding 3 structural (C, E1, E2) and 7 non-structural (P7, NS2, NS3, 4A, NS4B, NS5A, NS5B) proteins [2]. HCV strains are classified into 7 genotypes (1–7) and several subtypes based on the phylogenetic and sequence analyses of the complete viral genomes [3]. At the nucleotide level, HCV genotypes differ from each other by 31–33% and the subtypes within the same genotype differ from each other by 20–25% [4]. HCV genotypes are geographically distributed worldwide, with HCV genotypes 1, 2 and 3 widely distributed and subtypes 1a, 1b and 2a specifically called epidemic subtypes [5,6]. Genotype 3 circulates mainly in south Asia, genotype 4 in central Africa and Middle East, genotype 5 in Southern Africa, genotype 6 in South East Asia and genotype 7 in Congo [7–9].

Egypt records the highest HCV prevalence worldwide [10]. According to the Demographic Health Survey (DHS) of 2015, seroprevalence of HCV was 10% compared to 14.7% in 2008 [11]. It has been reported that around 93% of infections are due to genotype 4 [12]. HCV subtype 4a became epidemic and extensively distributed in Egypt due to the unsafe injections during the anti-schistosomal public health campaigns in the past [13]. Subtype 4a has been reported as the predominant subtype in Egypt in some studies [14–16], while infection with genotype 1 was thought to never exceed 10% [15]. In Egypt, an increased risk of hepatocellular carcinoma was significantly associated with the infection with HCV subtype 4o [17]. HCV genotype 4 responsiveness to treatment with PEG-INF/RBV is better than genotype 1 but worse than genotype 2 and 3 [18]. However, genotype 4 became the most difficult to treat genotype after the effective response of genotype 1 to protease inhibitors [19].

PEG-INF/RBV treatments have been implemented in Egypt for long time and they achieved sustained virological response (SVR) of approximately 40–69% [20]. One of the recently approved new treatments for HCV in Egypt is Sofosbuvir [21]. Sofosbuvir plus Ribavirin (interferon-free regime) for 12 or 24 week became the effective treatment protocol for both naïve and previously treated Egyptian patients [22]. Combination of Elbasvir (NS5A inhibitor) and Grazoprevir (NS3/4A protease inhibitor) for treating genotypes 1 and 4 produced high SVR rates [23]. HCV genotyping is clinically important in predicting the efficiency of antiviral therapy, in determining the duration of treatment [24] and, also, in detecting HCV molecular epidemiology [25]. This study aims to genotype HCV strains circulating in Damietta Governorate and to investigate the variation in HCV NS5B region that is targeted by the newly approved antiviral drugs.

## Patients and methods

### *Blood sampling*

Blood samples were collected in EDTA-containing vacuotainer tubes from 30 HCV-infected but treatment-naïve patients from Damietta governorate. *The study has been approved by our institution board (5/2/2/1) and all patients have provided an informed consent.*

### *Viral RNA extraction*

Viral RNA was extracted from all blood samples by GeneJET Viral DNA/RNA Purification Kit (#K0821, Thermo Scientific, Waltham MA, USA) according to the manufacturer's instructions. The purified RNA was immediately used or stored at −20 °C.

### *Polymerase chain reaction (PCR)*

All viral RNA samples were converted into cDNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (#K1631, Thermo Scientific, Waltham MA, USA). PCRs were performed to amplify 302 nt-fragment of the 5′ UTR (47–348) using the forward primer 5′-GTGAGGAACTACTGTCTTCACGCAG-3′ and the reverse primer 5′-TGCTCATGGTGCACGGTCTACGAGA-3′ [15], and to amplify 381 nt-fragment of the NS5B region (8256–8636) using the forward primer 5′-TATGAYACCCGCTGYTTTGAC-3′ and the reverse primer 5′-CCTGGTCATAGCCTCCGTGAA-3′ [17]. Each PCR included 25 μL of Maxima Hot Start PCR master mix kit (#K1051, Thermo Scientific, Waltham MA, USA), 2 μL of the forward primer, 2 μL of the reverse primer and 4 μL of the cDNA sample. Each reaction was brought to a final volume of 50 μL with nuclease-free water. Reactions were loaded to a thermal cycler (Multigene, Labnet, Edison NJ, USA) and subjected to 40 cycles of 30 s at 95 °C, 30 s at the optimized annealing temperature and 40 s at 72 °C. An initial activation step (4 min at 95 °C) and a final extension step (10 min at 72 °C) were also included in the program.

### *Restriction digestion*

PCR products of the 5′ UTR region were digested using two combinations of restriction endonucleases; *Mva*I/*Hinf*I and *Rsa*I/*Hae*III. Restriction reactions were run in 2.5% agarose gels for 40 min in TBE (#B52, Thermo Scientific, Waltham MA, USA) buffer, stained for 25 min with Ethidium Bromide and the gels were documented in Photo Doc-IT Imaging system (UVP, Upland CA, USA).

### *DNA sequencing*

PCR products of the 5′ UTR and NS5B regions were purified using GeneJET Gel extraction kit (#K0691, Thermo Scientific, Waltham, MA USA) and sequenced using the standard Sanger method on ABI 3730XL DNA Sequencer at Macrogen sequencing services (Macrogen, Seol, South Korea). Chromatograms were checked and corrected using a sequence viewer. The genotype of each sample was determined by comparing its sequence with those of HCV prototypes deposited in the GenBank database, followed by further genetic analysis.

### *Phylogenetic analysis*

The phylogenetic tree has been constructed using Neighbor-Joining method and the evolutionary distances were computed using the Kimura 2-parameter method with discrete gamma distribution using MEGA7 software. Bootstrap values were determined using 1000 replicates. 281 HCV sequences of other HCV isolates retrieved from HCV database and the GenBank database were used for constructing the phylogenetic tree.

## Results

### *Genotyping by restriction fragment length polymorphism (RFLP)*

Viral RNA was extracted from 30 HCV-infected patients, cDNA was synthesized and a 302 nt-fragment of HCV 5′ UTR was amplified by PCR. PCR products were subjected to double restriction digestion reactions using 2 pairs of enzymes; *Mva*I/*Hinf*I and *Rsa*I/*Hae*III. Digestion of the 30 patient samples with *Mva*I/*Hinf*I resulted in a clear ∼ 177 bp band and 2 close bands (∼56 and ∼69 bp) in 28 samples (Fig. 1a samples 2–8), while in the other 2 samples one separate 129 bp band, 2 close bands (∼63 bp and ∼69 bp) and ∼41 bp band have been obtained (Fig. 1a, sample 1).

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