

## Genetic subtypes and natural resistance mutations in HCV genotype 4 infected Saudi Arabian patients

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## Abstract

This study aimed to characterize the genetic subtypes of HCV-GT4 and identify the presence of natural occurring resistance-associated substitutions (RASs) in Saudi Arabia patients.

A total of 17 GT4 patients was analyzed. Sequence analysis of NS3, NS5A and NS5B regions was performed by direct sequencing. In addition, phylogenetic analysis was used to determine genetic subtypes, RAS and polymorphisms.

Nine patients were infected by a GT4a, one with GT4o, 3 with GT4d. The remaining four patients were infected with a recombinant virus (GT4a+GT4o in three patients, GT4c+GT4d in a patient).

Natural RASs were found in six patients (35%), including three infected by GT4a, two by GT4a+GT4o and one patient infected by GT4c+GT4d. In particular, NS3-RAS V170I was demonstrated in three patients, while NS5A-RASs (L28M, L30R, L28M+M31L) were detected in the remaining three patients.

All patients were treated with sofosbuvir plus daclatasvir; three patients were lost to follow-up whereas 14 patients completed the treatment. A sustained virological response (SVR) was obtained in all but one patient carrying NS3-RAS V170I who later relapsed.

GT4a is the most common subtype in this small cohort of Saudi Arabia patients infected with hepatitis C infection. Natural RASs were observed in about a third of patients, but only one of them showed a treatment failure.

**Keywords:** HCV; Genotypes; Subtypes; DAA

## 1. Introduction

Hepatitis C Virus (HCV) is a leading cause of cirrhosis, hepatocellular carcinoma, liver transplantation, and liver-related death worldwide [1]. The epidemic caused by HCV affects all regions, with significant differences between and within countries. Globally, an estimated 71 million people are chronically infected by HCV. The WHO Eastern Mediterranean Region and the European Region have the highest reported prevalence of HCV [1,2,3].

HCV exhibits high genetic diversity and is currently classified into eight genotypes (GT1 to GT8), with varied geographic prevalence [4].

The GT1 is prevalent in North and South America and Western and Northern Europe [5]; the GT2 circulates in Japan and part of Europe and America; the GT3 is widely spread in South Asia, Australia and Europe [5]. However, the distribution of HCV genotypes in the Middle East is highly variable, with a higher prevalence of GT1 in Iran, Oman and UAE. In contrast, GT2 is prevalent in Bahrain and Libya, GT3 in Afghanistan and GT4 in Egypt, Iraq, Jordan, Palestine, Qatar, Saudi Arabia, and Syria.

In Saudi Arabia, the GT4 is the prevalent genotype [6]; however, GT4 subtypes are still poorly investigated. In addition, Even fewer data are available on the presence of naturally occurring RAS and their possible impact on the response to treatment with direct-acting antivirals (DAAs). Recently, the natural presence of RASs in the viral genome (NS3, NS5A and NS5B) was associated with a decreased virological response rates to DAA [7-9].

This study aimed to characterize the genetic subtypes of HCV-GT4 in Saudi Arabian patients and analyse the prevalence and characteristics of resistance-associated substitutions (RASs) in DAA-naïve patients. The impact of natural RASs on the response to DAA treatment was also evaluated.

## 2. Material and Methods

### 2.1 Study Population

A total of 17 HCV-infected attending the Hepatology Clinic patients at Gastroenterology section at the King Fahad Hospital of the University, Al Khobar, Saudi Arabia, were included in the study. Of the 17 patients, twelve patients were Saudi, three were Egyptian, one was Sudanese, and one was Palestinian. All patients infected with HCV with detectable HCV RNA were included and we excluded coinfection with HBV and HIV.

Eleven patients were males and six were females; the mean age was 48 years (range 24 to 73 years). The risk factors included blood transfusion in 7/17 patients (41%), surgery in 1/17 patients (6%), intrafamilial transmission in 1/17 patients (6%), while in the remaining eight patients, the risk factor was unknown (47%). All patients were infected with GT4 and were DAA-naïve. At baseline, HCV-RNA levels ranged from 1,813 to 4,977,155 IU/ml.

### 2.2 Serum samples

Serum samples were stored at -80°C until testing.

All specimens were tested for HCV-RNA levels by a commercially available method (ABBOTT GmbH & Co KG Max Plank-Ring 2 65205 Wiesbaden Germany); detection limit was 12 IU/ml.

### 2.3 Amplification and NS3, NS5A and NS5B sequencing

HCV RNA was extracted as reported elsewhere using the Qiamp viral RNA minikit following the manufacturer's instructions (Qiagen GmbH, QiagenStrasse 1, 40724 Hilden, Germany) [10]. Synthesis and amplification of cDNA were then carried out in a single step using the commercial Superscript III One-step RT-PCR system with Platinum Taq (Invitrogen by Life Technologies, 5791 Van Allen Way Carlsbad, CA, 92008 USA). Primers for RT-PCR for each HCV genotype/subtype were designed from known sequences based on the NS3, NS5A and NS5B; if necessary, nested PCR was also performed with specific HCV genotype/subtype primers. Finally, NS3, NS5A and NS5B amplified products were purified and sequenced by an automated DNA sequencing analyzer (ABI-3130) in sense and antisense orientation using the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA). Wild-type amino acids were defined according to reference sequences from Geno2Pheno HCV tool [11].

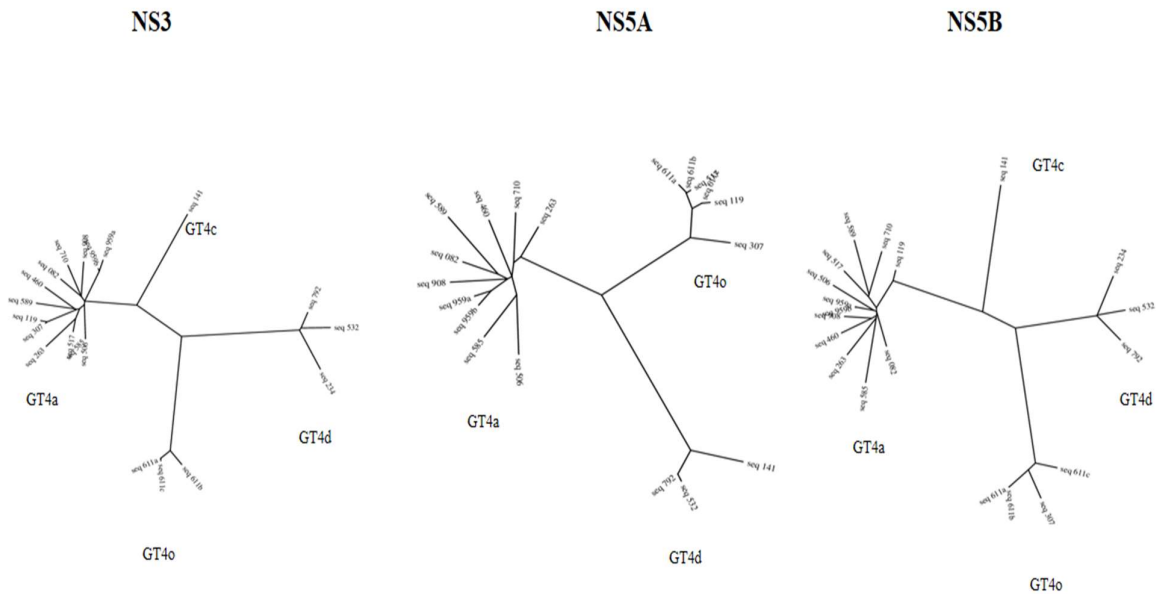
The primary local alignment search tool (BLAST) was used to identify HCV genotype.

First, the nucleotide sequences from NS3, NS5A and NS5B regions were analyzed by HCV BLAST in the Los Alamos HCV sequence database (<http://hcv.lanl.gov/content/index>). Then, the NS3, NS5A and NS5B sequences were aligned using the Clustal W algorithm integrated into the BioEdit software. The sequences of HCV strains were then aligned with a reference panel of sequences representative of each subtype (Genebank Accession numbers: Y11604, DQ418786; FJ462436; FJ462440), the same proposed by Geno2pheno HCV tools [11]. The sensitivity for detection of RASs using Sanger sequencing is approximately 10-20% [12].

GT4 subtypes were assessed by the construction of phylogenetic trees for the NS3, NS5A and NS5B regions, using the Phylogeny.fr platform [13]. The study was performed according to the Declaration of Helsinki, and ethical approval was obtained from Imam Abdulrahman Bin Faisal University (IRB Number -2020-01-313). Professor Badr Abdulrahman Aljandan was the chairman of the Institutional Review Board.

### 3. Results

Phylogenetic analysis showed the presence of different GT4-subtypes. In particular, the GT4a subtype was observed in five Saudi patients, in three Egypt and one Sudan patient. Three patients (two were Saudi and one was Palestinian) were infected with subtype GT4d and one Saudi patient was infected with subtype GT4o (Figure 1).



**Figure 1.** Genetic subtypes in 17 GT4-infected patients

Recombinant viruses were observed in four Saudi patients infected with GT4a + GT4o (in three patients) and GT4c+GT4d (in a patient).

In the NS3 region, the V170I mutation, associated with resistance to NS3 protease inhibitors, was detected in three patients, two infected with GT4a + GT4o and one with GT4a (Table 1).

GT4 NatuSubtypes	Patient's identified	NS3	NS5A	Main Geographical Area
A	908	V170I		EGYPT
A	710		L28M	SAUDI
A	460		L30R	SAUDI
A+O	119	V170I		SAUDI
A+O	307	V170I		SAUDI
C+D	141		L28M+M31L	SAUDI

**Table 1.** Naturally occurring resistance mutations detected in 6 GT4 HCV patients

Several polymorphisms not associated with resistance to DAA treatment were found in all GT4 subtypes, as reported in table 2. In particular, nine GT4a and 3 GTa+ GT4o recombinant patients showed A61S, S101A, A102S, I114V/L, I134T, R150A/V (reference sequence Y11604); three

GT4d patients had T95S/A (reference sequence DQ418786); a GT4o patient presented with L14F, V28A, T95A, S98T and R149H (reference FJ462440) (table 2).

SUBTYPES	HCV PATIENTS	NS3 POLYMORPHISMS	NS5A POLYMORPHISMS	NS5B POLYMORPHISMS
GT4a	9	A61S; S101A; A102S; I114V/L; I134T; R150A/V	V53M; K56T; I99V/T	N213T/S
GT4d	3	T95S/A	D105N; D126E, A164P/T; L168M	R127L; T130N/I/V
GT4o	1	L14F; V28A; T95A; S98T; R149H	M56I; E62N; K107E; I121V; S127F; L158I; C174S	R100K; A130T
GT4a+GT4o	3	A61S; S101A; A102S; I114V; I134T; R150A	M56I; E62N; I121V; S127F	R100K; A130T; N213T/S
GT4c+GT4d	1		D126E; A164Q; L168M	

**Table 2.** NS3, NS5A and NS5B polymorphisms in HCV GT4 not associated with DAA

The sequence analysis of the recombinant virus GT4c+GT4d showed several polymorphisms compared to wild-type FJ462436. However, their significance is uncertain as it is a single isolate.

In the NS5a region, two relevant mutations associated with resistance to NS5A inhibitors were found in two GT4a infected patients, the L28M in one case and L30R in the other one. In addition, a patient who harboured a recombinant virus (GT4c+GT4d) displayed an association of both L28M and M31L mutations which correlated with DAA resistance (table 2).

In all GT4 isolates polymorphisms not associated with drug resistance were observed in NS5A regions. In particular, V53M, K56T and I99V/T were observed in all GT4a isolates; D105N, D126E, A164P/T/Q and L168M were present in GT4d; M56I, E62N, K107E, I121V and S127F were found in GT4o and GT4a+GT4o patients. In addition, the recombinant virus showed two further mutations: L158I and C174S compared to the reference sequence (table 2).

Although no mutations associated with resistance were observed among NS5B sequences; however, also in this region, some polymorphisms were found according to the reference sequence. GT4a showed amino acids changes at position 100, 130 and 213; GT4d showed two changes at position 127 and 130, whereas in GT4o, changes were observed at position 100 and 130 compared to sequence reference.

All patients were treated with a sofosbuvir (400mg) plus daclatasvir (60mg) regimen for 12 weeks. Three patients were lost to follow-up whereas fourteen patients completed the treatment. A sustained virological response (SVR) was obtained in all but one who experienced a relapse. At baseline, a V170I mutation in the NS3 region was detected in the patient who relapsed, who nevertheless successfully retreated with glecaprevir plus pibrentasvir.

## Discussion

The study of genotype, subtype characteristics, and circulation is critical to defining HCV epidemiology and driving more appropriate therapy choices.

In the present study, GT4 subtypes were assessed in 17 HCV GT4-infected patients from Saudi Arabian. The prevalent subtype was GT4a, and the other identified subtypes were GT4o and GT4 d. Interestingly, three patients were found to be infected with a recombinant virus having both GT4a and GT4c.

This study further analyzes mutations in the NS3, NS5A, and NS5B regions associated with drug resistance.

The NS3 protease gene was successfully sequenced in all patients. Several reports demonstrated the presence of mutations associated with resistance to NS3 protease inhibitors; however, these studies were limited to GT1 and GT3 ([7,8,9]. In the patients studied, we found the V170I mutation associated with resistance to NS3 protease inhibitors in three patients, of whom two were infected with GT4a+GT4o and one with GT4a. These results support two previous studies [14, 15], which reported a V170I in GT4a isolates.

All patients with V170I RAS at baseline achieved an SVR after treatment with SOF/DAC except one patient who relapsed, even though he was treated with a regimen not containing a protease inhibitor. This mutation has not yet been associated with resistance in the GT4-infected patients, but this could be due to their low recruitment in clinical trials [16].

Paolucci and collaborators, reported a mutation at position 168 was found in the NS3 region of GT4a-infected patients with DAA-failure [17]. Still, this mutation was not detected in any of our isolates.

Further, the sequence analysis of NS3 region of our HCV isolates showed the presence of several polymorphisms not associated with DAA resistance. Whether the NS3 polymorphisms observed in Saudi patients could be involved in treatment failures is not yet known.

In the NS5A region, two clinically relevant RAS (L28M, L30R) were found in two patients harbouring GT4a and GT4d. In contrast, in a patient with a recombinant virus (GT4c+GT4d), the presence of an association of L28M+M31L was detected. In other non-GT4 genotypes, these mutations have been associated with resistance to DAA [18].

Paolucci and collaborators did not find neither L28M or L30R associated with resistance to NS5A inhibitors in their HCV isolates; however this study was only focused on GT1a and GT1b [19]. No mutations were observed in the NS5B region of our isolates. However, several polymorphisms were found in this region compared to the reference sequence with no apparent clinical impact and were not associated with DAA resistance.

In conclusion, in this small GT4 cohort, most patients were infected with GT4a. Although we found natural RASs were found they were not associated with DAA failure was not observed. Further studies on larger case series are needed to confirm these results.

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**Conflict of Interest:** The authors declare no conflict of interest.

**Statement of Ethics:** The project was reviewed and approved at Imam Abdulrahman Bin Faisal University IRB (IRB Number: IRB-2020-01-313) through an Expedited Review on Monday, October 26, 2020 by Professor Badr Abdulrahman Aljandan

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