

1-1-2015

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KAYMAN, TUBA; POLAT, CEYLAN; ERGÖR, GÜL; and ABACIOĞLU, YUSUF HAKAN (2015) "Characterization of HCV genotype 4d infections in Kayseri, Turkey*," *Turkish Journal of Medical Sciences*: Vol. 45: No. 3, Article 11. <https://doi.org/10.3906/sag-1405-43>

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Characterization of HCV genotype 4d infections in Kayseri, Turkey*

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Received: 13.05.2014

Accepted/Published Online: 30.06.2014

Printed: 30.06.2015

Background/aim: The frequency of genotype 4 hepatitis C virus (HCV) infections is significantly higher in Kayseri compared to other provinces in Turkey. We aimed to characterize genotype 4 infections in Kayseri by analyzing the demographic and laboratory data of 218 HCV RNA-positive, treatment-naïve patients admitted to the Kayseri Training and Research Hospital in 2010 and 2011.

Materials and methods: The distributions of sex, age, and viral loads of these patients with respect to HCV genotypes were analyzed. We also characterized the type 4 sequences at the subtype level. Randomly selected sera from 32 of the 72 genotype 4 patients from this cohort were subjected to PCR amplification in the NS5B region and further characterized by sequencing and phylogenetic and molecular clock analysis.

Results: Distribution rates of HCV genotypes 1, 4, and 2 in the 218 patients were 62.4%, 33.0%, and 4.6%, respectively. Most of the patients infected with types 1 and 4 were over the age of 40 and female. The NS5B sequences of 32 Kayseri genotype 4 isolates were closely related with type 4d sequences but formed a separate cluster.

Conclusion: The introduction of type 4d HCV into the Kayseri region probably took place 30–75 years ago, as predicted by molecular clock analysis.

Key words: Hepatitis C virus, genotype 4d, phylogenetic analysis, Kayseri, Turkey

1. Introduction

It is estimated that 130–210 million people worldwide are chronically infected with the hepatitis C virus (HCV). Complications of chronic liver disease, like cirrhosis with or without hepatocellular carcinoma, will develop over many years in around 20% of these patients, making HCV infections a major public health issue (1).

HCV has six major genotypes and more than 80 subtypes (2). Recently a seventh genotype was proposed (3). Genotypes may differ in their epidemiology, pathogenesis, and response to treatment (4). Type 1, 2, and 3 HCV infections are common throughout the world. Type 4 is prevalent in the Middle East and Africa and responsible for more than 80% of all HCV infections in some countries (5). Genotypes 5 and 6 are mainly found in South Africa and in Southeast Asia, respectively (6). Sustained virological response to standard pegylated interferon plus ribavirin treatment is usually lower for types

1 and 4 than types 2, 3, 5, and 6 (7), even with treatment regimens tailored to the on-treatment virological response (8). Therefore, genotyping at the pretreatment stage has become a standard of care for patient management. For prediction of treatment response and dose scheduling, 5'-UTR sequences provide fairly accurate genotype information. As 5'-UTR-based assays are not reliable for subtyping and for epidemiological investigations, analysis of sequences from subgenomic regions such as NS5B and core/E1 are required (9).

In Turkey, it is estimated that around 0.75 to 1.5 million people are infected with HCV (10). Genotype 1, and especially the type 1b virus, causes approximately 90% of these infections, while types 2, 3, and 4 exist, albeit in low prevalences (11). However, two recent reports from Kayseri, a relatively large province in Central Anatolia with a population of 1,274,968 (as of 2012), indicated unusually high prevalence of type 4 infections in the province,

* This study was presented in part at the 35th Turkish Microbiology Congress, 3–7 November 2012, Kuşadası, Aydın, Turkey.

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reaching 35% among patients admitted to hospitals for treatment of chronic hepatitis C (12,13).

We therefore sought to characterize the type 4 HCV infections in Kayseri Province by analyzing the data of treatment-naive chronic hepatitis C patients for the distribution of genotypes with respect to sex, age, and viral load. We further characterized the type 4 viruses by phylogenetic analysis of NS5B sequences in a randomly selected group of samples.

2. Materials and methods

2.1. Dataset and serum samples

The dataset consisted of laboratory records of 218 treatment-naive chronic hepatitis C patients admitted to the Kayseri Training and Research Hospital in 2010 and 2011. All patients were anti-HCV- and HCV RNA-positive. The dataset was utilized to analyze the distribution of genotypes with respect to sex, age, and viral load. To investigate the phylogeny of type 4 viruses, 32 samples were randomly selected among the 72 type 4 samples represented in the dataset and subjected to further molecular analysis.

This study was approved by the Dokuz Eylül University Noninvasive Clinical Research Ethics Committee.

2.2. Routine clinical virology analyses

Viral load levels of the samples were measured by COBAS AmpliPrep/COBAS Taqman HCV (CAP/CTM v1.0) (Roche Molecular Systems, USA). Genotyping was performed with the Abbott m2000_{SP}/Abbott m2000_{RT} (Abbott Diagnostic, USA).

2.3. Molecular characterization of type 4 samples

2.3.1. RNA extraction

Extraction of viral RNA from serum samples was carried out with the EZ-1 Virus Mini Kit v.2.0 (QIAGEN, Germany) on the EZ-1 platform as recommended by the manufacturer.

2.3.2. cDNA synthesis

Five microliters of RNA extract was reverse-transcribed using 100 pmol of random hexamers and a First Strand cDNA Synthesis Kit (Fermentas, Latvia) at 37 °C for 60 min in a total volume of 20 µL according to the manufacturer's recommendations.

2.3.3. Heminested PCR

A heminested PCR approach was adopted to amplify the 380-bp stretch in the NS5B gene between positions 8256 and 8636 (nucleotide positions with respect to reference strain H77, GenBank accession number AF 009606) using primers PR3, PR4, and PR5 (14). Using 41 reference sequences from the Los Alamos HCV database (www.hcv.lanl.gov) (15), we have shown that a degenerate base at position 21 (Y: C or T) would enhance the sensitivity of primer PR3 for different genotypes, and we therefore designated this PR3 variant as PR3.1, as shown in Table 1 (data not shown).

PCR reaction was carried out in volumes of 50 µL using 1.5 mM MgCl₂, 10 mM dNTP (Fermentas), 10 pmol of sense and antisense primers, and 2.5 U of Taq DNA polymerase (Fermentas). Cycling parameters for the first PCR were predenaturation at 95 °C for 4 min followed by denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min (30 cycles), with a final extension step of 72 °C for 10 min. For the second PCR, following predenaturation at 95 °C for 5 min, there were 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products were run and visualized in 1% agarose gels.

2.4. Sequencing and phylogenetic analysis

The 380-bp PCR products were directly sequenced using the primers shown in Table 1 by a commercial company (RefGen Ltd., Turkey).

For phylogenetic analysis, the sequences of Kayseri isolates and 52 reference sequences from different genotypes downloaded from the Los Alamos HCV database were used (15). GenBank accession numbers of these sequences are EU155379, AJ000009, EU482875, EU155339, EU155306, EU482834, AY051292, EU482858, EU155243, EU155279, EU155274, D14853, AY651061, AF238483, AB031663, D10988, AF238481, D50409, AY746460, AF238486, NC009823, AF169004, AB030907, X76918, D28917, AF046866, D63821, AY515261, D49374, NC009824, DQ418788, DQ418787, Y11604, DQ418783, DQ418786, Y13184, AF064490, EF424627, EF424626,

Table 1. Primers used in amplification and sequencing.

Primers*		Sequence**	Positions***
PR3.1	sense	5' - TATGAYACCCGCTGYTTTGAYTC - 3'	8256-8278
PR4	antisense	5' - GCNGARTAYCTVGTCATAGCCTC - 3'	8622-8644
PR5	antisense	5' - GCTAGTCATAGCCTCCGT - 3'	8619-8636

*: C or T (Y) in position 21 in Primer 3.1 is shown in bold.

** : N: A, C, G or T; R: A or G; V: A, C or G; Y: C or T.

***: Positions are given with respect to reference sequence H77, GenBank accession number AF 009606.

DQ835770, DQ835764, EF632069, D84265, DQ278893, DQ835761, D84264, DQ835766, DQ278894, NC009827, D63822, D84263, and EF108306.

All sequences were aligned using the Clustal X modality provided in MEGA 5.02 software (16). Phylogenetic trees were constructed using the Jukes–Cantor model of substitution and maximum likelihood or neighbor-joining methods. Bootstrap analysis (1000 replicates) was used to check the reliability of the trees.

2.4.1. Nucleotide sequence accession numbers

The sequences of Kayseri isolates obtained in this study were submitted to GenBank and were assigned the accession numbers KF463156, KF463157, KF463158, KF463159, KF463160, KF463161, KF463162, KF463163, KF463164, KF463165, KF463166, KF463167, KF463168, KF463169, KF463170, KF463171, KF463172, KF463173, KF463174, KF463175, KF463176, KF463177, KF463178, KF463179, KF463180, KF463181, KF463182, KF463183, KF463184, KF463185, KF463186, and KF463187.

2.5. Molecular clock analysis

Molecular clock analysis was performed using MEGA software 5.02 (16). The minimum (1×10^{-3} substitutions site⁻¹ year⁻¹) and the maximum (0.41×10^{-3} substitutions site⁻¹ year⁻¹) evolutionary rates reported in the literature for the NS5B region (17,18) and the maximum likelihood method were used to estimate the date of entry of HCV type 4d into Kayseri Province.

2.6. Statistical analysis

The relationships among genotypes, sex, age, and RNA levels were examined using SPSS 15.0. Categorical data and continuous variables were analyzed by chi-square test, t-test, and one-way ANOVA with post hoc Bonferroni test, respectively. Means and standard deviations were given for continuous variables. The RNA levels not suitable

for normal distribution were analyzed using logarithmic transformations with t-test.

3. Results

3.1. Genotypes and demographics

Of the 218 treatment-naive patients, 136 were infected with type 1 (62.4%), 5 of whom were infected with type 1a and the remaining 131 with type 1b; 72 were infected with type 4 (33.0%); and 10 were infected with type 2 (4.6%) HCV.

The sex distribution of genotype 2 patients was different from that of patients infected with genotypes 1 and 4. While approximately two-thirds of patients infected with HCV genotypes 1 and 4 were female, most patients infected with genotype 2 were male ($P = 0.009$) (Table 2).

The mean ages of patients infected with types 1, 2, and 4 HCV were 57 ± 11 (20–82), 43 ± 17 (22–65), and 53 ± 14 (13–81) years, respectively. While the age distributions of patients infected with types 1 and 4 were similar, those infected with type 2 were significantly lower than in the former group ($P = 0.001$).

There was a significant difference in viral load between genotypes. According to the Bonferroni test, the difference was significant between types 1 and 4. The viral load of patients infected with HCV genotype 4 was significantly lower than those infected with genotype 1 ($P = 0.009$) (Table 3).

3.2. Phylogenetic analysis

Kayseri type 4 isolates were closely related with type 4d reference sequences but formed a separate cluster, a pattern seen in both maximum likelihood and neighbor-joining analyses as shown in the Figure. We repeated the

Table 2. Sex distribution of patients infected with HCV genotypes 1, 2, and 4.

	Genotype 1 n (%)	Genotype 2* n (%)	Genotype 4 n (%)	Total n (%)
Male	45 (33.1)	8 (80)	23 (31.9)	76 (34.9)
Female	91 (66.9)	2 (20)	49 (68.1)	142 (65.1)
Total	136 (100)	10 (100)	72 (100)	218 (100)

*: $P = 0.009$.

Table 3. The comparison of age and viral loads of patients infected with genotypes 1 and 4.

Feature	HCV genotype		P-value*
	1	4	
Age (mean \pm SD)	57.0 ± 11.3	53.3 ± 13.9	0.140
Viral load n (%)	<800,000 IU/mL 67 (50)	48 (66.7)	0.009
	>800,000 IU/mL 67 (50)	24 (33.3)	

*: P-values were calculated by one-way ANOVA.

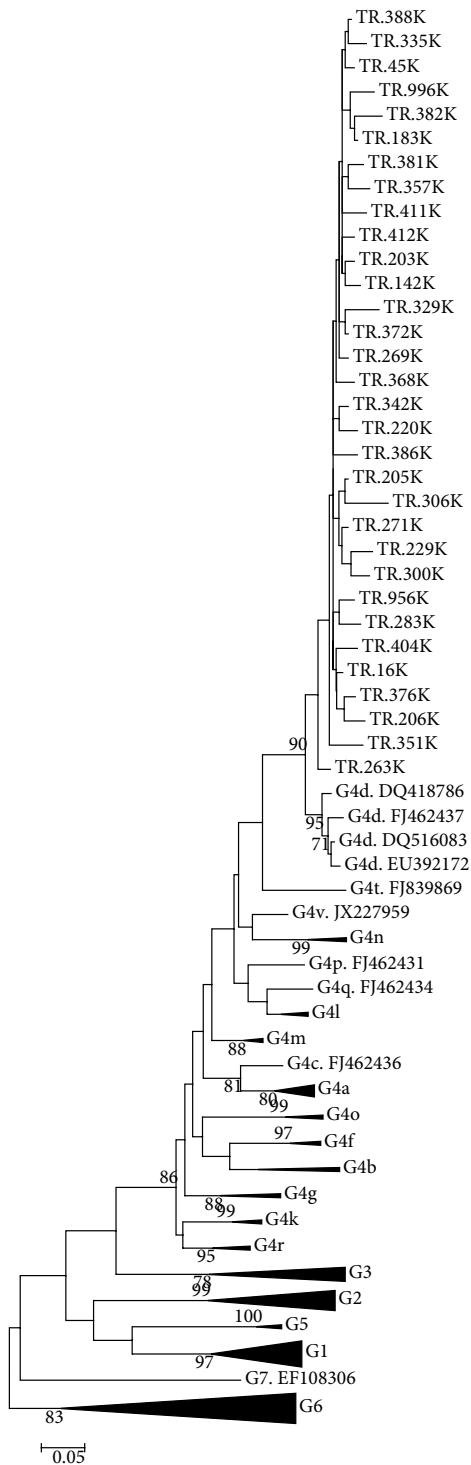


Figure. The phylogenetic tree of Kayseri HCV isolates (n = 32) constructed using the Jukes–Cantor model of substitution and maximum likelihood method. The reference sequences (n = 52) used in the analysis are shown in the text. Only bootstrap values of $\geq 70\%$ are indicated. Branches containing more than one sequence, excluding Kayseri and reference genotype 4 sequences, were clustered and are represented with black triangles to facilitate viewing. G: Genotype.

phylogenetic analysis with a dataset of type 4 sequences containing an increased number of type 4d and other type 4 sequences and confirmed the separate clustering of Kayseri isolates from other type 4d sequences (data not shown).

We dated the time of entry of the 4d HCV isolates to Kayseri Province to be sometime between 30 and 75 years ago by molecular clock analysis.

4. Discussion

In Turkey, genotype 1, and specifically 1b, accounts for most HCV infections. Genotype 2, 3, and 4 infections are found at low frequencies. This pattern is consistent with relatively recent and limited introductions from endemic areas (18). The introduction of HCV type 1b to Turkey has been estimated to have taken place within the first decade of the 20th century through Greece (19). Unsafe medical procedures, including blood transfusions and surgeries, were probably instrumental in the dissemination of the virus between 1940 and 1999 in Turkey (19,20). The spread of epidemics seems to have reached a plateau after 1999, suggesting a partial success of improved transfusion policies and safe medical procedures (19).

The prevalence of genotype 4 HCV infections in our study group is 33%. This figure is similar to the reported prevalence of 35.6% in a recent study conducted at the University Hospital in Kayseri (13). These rates are significantly higher than the average prevalence of 1.4% reported for type 4 HCV infections in Turkey (12).

Comparable age and sex distribution of patients infected with types 1 and 4 viruses in this study suggests that these patients may have been subjected to similar risk factors, i.e. unsafe medical procedures, for acquiring hepatitis C. However, age and sex distributions of type 2 patients were different from those infected with types 1 and 4, suggesting that the epidemiology of type 2 HCV infections is different from the latter in this cohort. In Europe, type 1b and 2 infections are commonly linked to blood transfusions or nosocomial transmission (21). In our case, we do not know the risk factors for infection in these patients, but younger age and male predominance may indicate a cohort effect, which requires further investigation. The relatively small number of type 2 patients, on the other hand, is a limitation, even though the P-values are significant.

Unsafe medical procedures have also been defined as a main risk factor for HCV spread in the Middle East and North African countries where genotype 4 infections are common (5). Turkey has a long history of close relations with some of these countries, including the Hajj and labor migration. Labor migration from Turkey to Middle Eastern and North African countries started in 1967,

peaked in a 10-year period between 1980 and 1990, and reached a total of around 530,000 and 270,000 in Saudi Arabia and Libya, respectively, between 1967 and 2011 (22). HCV type 4 infections are common in Saudi Arabia, Libya, and neighboring countries like Syria and Iraq (7,23–25). A recent report from Turkey identified two patients infected with HCV genotype 4: one had received a blood transfusion in Saudi Arabia and the other had a history of living in Iraq (26).

In recent years, the frequency of genotype 4 HCV infections has increased in Europe, especially in countries around the Mediterranean Basin. Prevalence of between 10% and 24% has been reported in some regions of Italy, Greece, Spain, and France (27–30). Most of these infections are prevalent among intravenous drug users, patients coinfecting with HCV and HIV, and immigrants from North and sub-Saharan Africa (5,27–30). However, Ciccozzi et al. provided evidence that in Calabria, Italy, HCV 4d infections had been maintained in a steady, nonexpanding phase until the 1970s by sporadically acquired infections (31,32). Molecular clock analysis showed that the HCV type 4d virus entered Kayseri Province sometime between 1936 and 1981, a finding that points to the endemic nature of the virus in the province and is also in line with the dates of labor migration from Turkey. Therefore, it is plausible to think that the virus may have been introduced into the province by people infected

in the Middle East or North African countries where type 4d viruses were in circulation and had been maintained in the population by local transmission events.

In this study, viral loads of patients infected with genotype 1 were found to be significantly higher than those of patients infected with genotype 4. This could be attributed to the genotype dependence of the HCV RNA assay (COBAS AmpliPrep/COBAS Taqman HCV (CAP/CTM v1.0)) used to measure the viral load in these patients. This assay has been reported to be sensitive to polymorphisms at the 145th and 165th nucleotides in the 5'-UTR region (33), a problem that has been solved in the newer versions of this assay (34).

This is the first study that shows the presence of HCV type 4d in Turkey. We have also shown that type 4d viruses have existed in Kayseri Province for at least 30 years, indicating the endemic nature of these infections. To define the exact nature of these infections, however, field epidemiological investigation is required, which at this moment is in the planning stage. Analysis of the sequences with Bayesian coalescent method will contribute to timing the origin with precision and identifying the dynamics of the epidemic (32).

Acknowledgment

We thank Dr Varalakshmi Elango for critical review of the manuscript.

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