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Molecular characterization of hepatitis A virus isolated from acute infections in Turkey

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Background/aims: *Hepatitis A virus is a global public health problem, especially in developing countries, and the most common cause of hepatitis in childhood. Hepatitis A virus is a single- stranded positive RNA virus subdivided to 6 genotypes (3 human, 3 simian). The aim of this study was to determine the prevalent genotype in Turkey using sera of acute hepatitis A virus-infected patients from different geographical regions of the country.* ***Materials and Methods:*** *Sera of 137 patients with acute hepatitis A virus from different geographical regions were collected for phylogenetic analysis. The VP1-2A region of the hepatitis A virus genome was amplified by real-time-polymerase chain reaction in 76 patients where possible. Amplified polymerase chain reaction fragments were sequenced, and phylogenetic analysis was done together with other reference hepatitis A virus sequences obtained from GenBank database.* ***Results:*** *Sequencing and phylogenetic analysis of the VP1-2A junction of hepatitis A virus showed that the most prevalent genotype in Turkey is IB (100%). Comparison of Turkish isolates and reference sequences of genotype IB showed a similarity of 94.9%. The same comparison was done between Turkish isolates and reference hepatitis A virus genotype IB and HM175, and it was found that similarity between them ranged from 93.0-95.9%. When Turkish isolates were compared according to Mean Percentage Nucleotide Distance analysis, similarity ranged between 95.3%-100%.* ***Conclusions:*** *Phylogenetic analysis pointed out that all Turkish isolates belong to genotype IB. Sequence analysis is a useful tool in revealing hepatitis A outbreaks, and allows us to detect and distinguish the presence of epidemic and small outbreaks.*

Key words: Hepatitis A virus, genotyping, phylogenetic analysis

Türkiye'de akut enfeksiyonlu hastalardan elde edilen hepatit A virüsünün moleküler karakterizasyonu

Giriş ve Amaç: *Hepatit A virüsü özellikle gelişmekte olan ülkelerde yaygın olmak üzere küresel bir sağlık problemidir. Özellikle çocukluk döneminde meydana gelen hepatitin de en yaygın sebebidir. Hepatit A virüsü pozitif tek zincirli bir RNA virüsü olup, 3 insan 3 maymunumsu genotipi olmak üzere toplam 6 genotipi vardır. Bu çalışmanın amacı ise, Türkiye'nin farklı coğrafi bölgelerinden hepatit A virüsü ile enfekte olmuş hasta serumlarının analizlerine bağlı olarak en yaygın hepatit A virüsü genotipini saptamaktır.* ***Gereç ve Yöntem:*** *Bu çalışmaya Türkiye'nin farklı coğrafi bölgelerinden hepatit A virüsü ile akut infeksiyonlu 137 hasta dahil edilmiştir. Alınan serum örneklerinden elde edilen hepatit A virüsü genomunun VP1-2A bölgesi gerçek zamanlı polimeraz zincir reaksiyonu yöntemi ile çoğaltılmış ve bu örneklerin dizi analizi sonucunda filogenetik analiz yapılmıştır.* ***Bulgular:*** *Hepatit A virüsü genomunun VP1-2A bölgelerinin sekans ve filogenetik analizleri sonucunda Türkiye'de ki en yaygın genotip IB olarak bulundu (%100). Türk izolatları ile genotip IB'nin referans sekansı arasında yapılan karşılaştırmada benzerlik %94,9 oranında bulundu. Aynı karşılaştırma Türk izolatları ve referans hepatit A virüsü genotip 1B, HM175 ile yapıldığında aralarındaki benzerlik %95,3 ile %100 arasında değişkenlik göstermiştir. Türk izolatlar kendi aralarında "Mean Percentage Nucleotide Distance" analizi ile karşılaştırıldıklarında ise aradaki benzerlik %95.3 ile %100 arasında değişkenlik gösterdi.* ***Sonuç:*** *Yapılan filogenetik analizler sonucunda Türkiye'de ki en yaygın genotipin IB olduğu gözlemlendi. Yapılan sekans analizi sonucunda elde edilen veriler, lokal veya büyük bir salgının var olup olmadığının anlaşılmasında önemli bir veri kaynağı oluşturarak, yapılacak olan müdahelelerin doğru ve zamanında olmasına yardımcı olmaktadır.*

Anahtar kelimeler: Hepatit A virüsü, genotipleme, filogenetik analiz

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INTRODUCTION

Hepatitis A virus (HAV) is the causative agent of infectious hepatitis and widely spread throughout the world (1,2). The mortality rate changes according to the age, while severity of the infection is higher in adults than children (3). The primary transmission of HAV is via personal contact with an infected person or household transmission. Intravenous drug addiction, traveling to endemic areas, hemophiliacs, and contaminated food and drink usage are the other main routes of transmission (3).

Hepatitis A virus (HAV) is the only member of the genus Hepatovirus that belongs to the Picornaviridae family (4-6). HAV is an RNA virus with positive polarity having no envelope. Its viral genome is 7.5 kb in length (1) and composed of a 5' nontranslated region, a single open reading frame (ORF) and a 3' untranslated region with poly-A tail (2,7). After cleavage by proteases, the single polyprotein of HAV yields three major protein groups. While P1 encodes for capsid proteins VP1-VP4, P2 and P3 encode for non-structural proteins related to viral replication, including proteases and polymerase (8,9).

Several regions on the HAV genome have been used for genotyping analysis. The most common ones are the C terminus of the VP3 region, N terminus of the VP1 region, VP1-2A junction region, VP1-2B region, entire VP1 region, VP3-2B region, and 5'UTR region (3,10). Complete genome sequence analysis reveals that genotypes I, II and III are human genotypes. Genotypes I–III are divided into sub-genotypes A and B. The other three genotypes, IV-VI, are classified as simian genotypes (11,12). The most prevalent genotype in humans throughout the world is genotype I.

Hepatitis A virus (HAV) is highly conserved throughout its genome according to nucleotide and also amino acid level. The estimated mutation rate of HAV is $1x10^{-3}$ to $1x10^{-4}$ substitution per site, which is very low when compared to other RNA viruses (13). It does not significantly accumulate genetic changes on its genome. However, HAV has enough diversity on its particular region to define its genotypes and sub-genotypes.

The aim of this study was to determine the prevalent genotype in Turkey using the sera of acute HAV-infected patients from different geographical regions of the country.

MATERIALS AND METHODS

Samples

Immunoglobulin (Ig)M anti-HAV-positive serum samples, taken from 137 patients during the acute phase of infection, were collected from different regions of Turkey, namely İzmir, Şanlıurfa, Van, and Kayseri, representing West, Southeast, East, and Central Turkey. Out of 137, HAV RNA could be amplified in 76 patients (female: 36, male: 39). The number of adults (>20 years of age) was less than of non-adults (<20 years of age) (Table 1). The most variable region of HAV genome, the VP2-2A junction, was used for analysis.

Determination of Anti-HAV IgM

Hepatitis A IgM antibodies (anti-HAV IgM) were detected using Architect i2000 SR (Abbott, Germany) system and Centaur XP Immunoassay System (Siemens, USA) in four different locations, according to the manufacturer's instructions.

Real-Time-Polymerase Chain Reaction (RT–PCR) and Sequencing

Viral RNA was extracted from 200 µL serum by a commercial kit (Viral RNA Extraction Kit, Roche Diagnostics, GmbH, Manheim, Germany) according to the manufacturer's instructions. A total volume of 20 µL reaction was used for cDNA synthesis from purified RNA consisting of 1.25 pmol reverse primer of first round PCR, 5 µL RNA, 4.3 µL distilled water, 0.4 mM dNTP, 4 U AMV (Roche Diagnostics GmbH, Manheim, Germany), and 4 µL from 5X AMV RT buffer. PCR amplifications were carried out using the following steps: 95°C for 3' and 42°C for 94.5'. For first-round PCR, 0.4 pmol 1F-1R primers, 5 µL cDNA, 20.6 µL distilled water, 5 µL of 10X Taq Poly buffer, 2 U Taq polymerase, 0.1 g/mL gelatin, and 2.5 mM $MgCl_2$ were used, for a total volume of 50 µL. For nested PCR, a nested primer set was used and only the $MgCl_2$ (2 mM) concentration and template (3 µL) volume differed from that of first-round PCR. The same amplification durations were car-

Table 1. General and clinical information about VP1-2A-positive patients

Age	Numbers of patients (male/female)	IgM (Mean±SD)
0-5	40 (23/17)	7.8±2.5
6-10	18 (8/10)	8.7±2.7
11-20	11 (5/6)	10.4±4.4
>20	7 (3/4)	8.2±3.7

ried out for both PCR rounds and were as follows: 94°C for 5', 30 cycles at 94°C for 30", 55°C for 30", 72°C for 30", and after 30 cycles 72°C for 7' (5). Amplicons were run at 1% agarose gel electrophoresis for confirmation of the results. Both strands of amplicons were sequenced by primers used for PCR on a 310 ABI PRISM Genetic Analyzer using Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA).

Phylogenetic Analysis

Sequences were aligned and phylogenetic comparison was done by distance matrix analysis using Kimura two-parameter distance method followed by ClustalW-Neighbor Joining by using the MEGA 4.1 program.

Nucleotide Sequences and Accession Numbers

The following strain sequences from GenBank were used: AF485328 (IA), AB020568 (IA), M20273 (IB), AF268396 (IB), AY644676 (IIA), AY644670 (IIB), FJ360735 (IIIA), AJ299464 (IIIA), AB279735 (IIIB), and M59810 (HM175) (IB).

RESULTS

Seventy-six of 137 specimens were detectable after PCR amplification via agarose gel electrophoresis. The fragments were ~350 bp long as expected.

After sequencing of PCR amplicons, the phylogenetic analysis of 76 VP1-2A-positive patients was done. Turkish isolates were aligned with reference sequences obtained from GenBank database. Only human HAV reference sequences were used for phylogenetic analysis. Comparison between reference HAV sequences and Turkish HAV isolates demonstrated that all of the 76 isolates were branched as genotype IB (Figure 1).

According to comparison with reference sequences of HAV, the mean percentage inter-genotypic nucleotide distances calculated by the Kimura two-parameter algorithm using MEGA software revealed a distance of 5.1% between Turkish HAV isolates and HAV genotype IB isolates. The comparison of this rate with genotypes I-III is shown in Table 2.

The comparison between Turkish isolates and HAV sequences showed that the closest relation was between isolate 1 and genotype IB (HAF203), which revealed 95.9% sequence homogeneity. The similarity between Turkish HAV isolates with reference sequence HM175 ranged from 92.9-95.9%.

Table 2. Mean percentage nucleotide distance based on the analysis of the VP1-2A region of Turkish Isolate (TR), wild type HM175 and human HAV genotypes (HAV I-III), the corresponding sequence of which were retrieved from GenBank

	IIIA	IIIB	IIA	IIB	IA	IB
IIIB	13.1					
IIA	33.1	28.9				
IIB	26.7	26.8	8.5			
IA	28.6	31.2	21.8	19.9		
IB	29.2	30.8	22.1	18.7	9.2	
TR	29.8	31.7	21.4	18.3	8.6	5.1

The most distant relation was between isolates 7, 8, 9, 15, 17, 21, 23, 34, 35, 74, 133 and genotype II-IB (HAJ85-1), which was 67.0%.

When all 76 Turkish isolates were compared, the most distant relation was between isolate 19 and isolates 105, 111, 113, 124, 129, 132, 135, 136, and 137, which was 95.3%. Most of the isolates showed 100% identity. Isolates from the same region were analyzed within each other and results showed that isolates from Şanlıurfa mainly clustered in three groups, and each group member showed almost complete similarity, with a few exceptions. Isolates from Kayseri and Van also clustered into two main groups when compared (data not shown). Those data can be subtracted from Figure 1, although differences and similarities are better recognized when clustered separately.

Amino acid sequences alignment was also done with reference sequence of HM175. Mainly four amino acid substitutions were detected. At the 768th amino acid position, in seven isolates, a serine to asparagine substitution was observed. In a further seven isolates, a cysteine to leucine substitution was observed at codon 819. Isolate 133 displayed substitution from glutamic acid to glycine at the 786th codon. A unique substitution emerged at amino acid 812 from leucine to isoleucine in isolate 40. Substitutions are summarized in Table 3.

DISCUSSION

Molecular structure of HAV seems to be more stable when compared to other picornaviruses, allowing the virus to persist on environmental surfaces (14). In addition to the stability of the virus, the route of transmission, mainly fecal-oral, also accounts for its high prevalence in many parts of the world.

The VP1-2A junction, as known to be one of the

most variable regions of the HAV genome, is generally chosen for phylogenetic analysis. In the present study, the VP1-2A junction of HAV was amplified and sequenced in order to determine the prevalent genotype in Turkey. The phylogenetic analysis with sequences derived from 76 Turkish HAV isolates revealed that all Turkish HAV isolates were clustered on genotype IB (Figure 1).

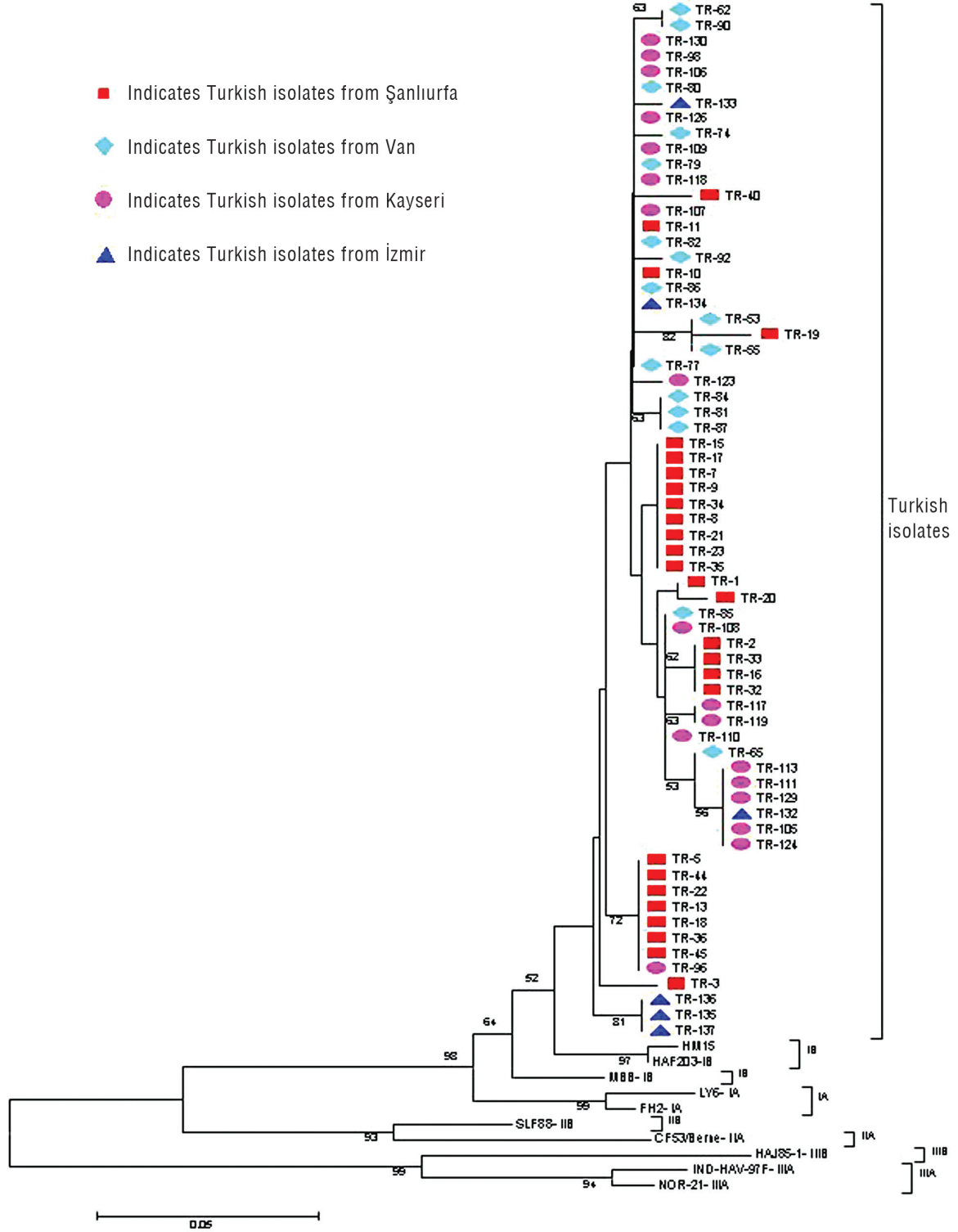

Figure 1. Phylogenetic analysis of HAV based on a VP1-2A junction sequence using NJ Method performed with 76 Turkish HAV isolates and 10 HAV sequences of genotype I-III and HM175, the corresponding sequence of which were retrieved from GenBank.

Table 3. Amino acid substitutions in Turkish isolates compared to genotype IB and reference sequences

Isolate No	Amino Acid Position			
	S768	E786	L812	L819
TR-65, 105, 111, 113, 124, 129, 132	N			
TR-17, 32, 33, 34, 35, 36, 37				C
TR-40			I	
TR-133		G		

The genotypes of HAV vary in different regions of the world. Genotype I is the most common genotype, and sub-genotype IA is more frequently detected than sub-genotype IB (15). Co-circulation of genotypes and sub-genotypes is also possible as IA and IB (16). Mediterranean basin countries generally have genotype I. Italy, France, Tunisia, and Greece have genotype IA (15,17), while genotype IB seems to be present in Spain, Jordan and Egypt. Our results also support a previous study done in Germany reporting that the most common genotype in Turkey is IB (18).

Hepatitis A virus (HAV) genotypes seem to show less diversity when compared to HBV and HCV genotypes in the same geographical settings. This may be explained by the low accumulation of the genetic changes in HAV RNA. Since Turkey is situated between continents and served as a crossroads of civilizations throughout history, dominance of just one genotype in HAV (genotype IB , 100%) in addition to HBV (genotype D, 100%), HCV (genotype IB, 91%) and HDV (Genotype I, 100%) (19) is remarkable, when compared to island countries, where more diversity in genotypes of hepatitis viruses is observed.

In outbreaks, isolates are mainly the isolates of outbreak origin, because of the low accumulation of genetic changes in HAV RNA (3). In the present study, some isolates showed 100% sequence similarity, indicating that they originated from the same source. When isolates of Şanlıurfa were compared within each other, they mainly clustered in three groups. Some of the isolates showed unique sequence in these groups. Other than these exceptions, it can be assumed that patients in the different groups originated separately from small local outbreaks. Isolates from Kayseri and Van were compared as well and were clustered mainly in two groups, again representing possibly local outbreaks. However, some patients showed 100% similarity, despite being from different regions. In such cases, sequence of short HAV segments may not be sufficient to clarify the origin of the infection. Longer segments or full length sequences of the same isolates can be used for more accurate identification. Sequence analysis is a useful tool in revealing hepatitis A outbreaks, and allows us to detect and distinguish the presence of epidemic and small outbreaks. Early detection of an outbreak may result in preventive measurements to limit the epidemic at an early stage.

When the amino acid sequences of Turkish HAV isolates were compared to HM175 reference HAV amino acid sequences, Turkish isolates showed complete similarity except for four distinct amino acid substitutions in 16 isolates: 7 with S768N, 1 with E756G, 1 with L812I, and 7 with L819C substitutions, respectively.

Turkey is a developing country, and is considered an intermediate endemic region for HAV. In Turkey, the occurrence rate of HAV infection is common in non-adults (<20 years of age, 90.8%), as was the case in the present study. A successful vaccine prepared against genotype IB is protective against all kinds of HAV human genotypes because of its simple antigenicity (20). When taken in two doses, vaccination provides lifelong protection (21). It should be clarified whether vaccination of newborns would be a cost-effective procedure in Turkey.

In conclusion, genotype IB was found to be the prevalent genotype in Turkey. Despite the low diversity of HAV sequences, application of sequence analysis methodology is also important in countries lacking HAV genotype diversity, such as Turkey, to identify HAV outbreaks and to provide preventive healthcare measures for those in need.

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