Molecular Epidemiology of Hepatitis A Virus in Patients in the Ahwaz Region of Iran

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Hepatitis A virus (HAV) is one of the etiologic agents of acute viral hepatitis, an important public health problem worldwide. The aim of this study was to investigate the genetic diversity of HAV in Southwest Iran (Ahwaz). A total of 59 sera were collected from acutely ill patients with anti-HAV IgM antibodies during 2009 and 2010 were tested also by RT-PCR targeting the 5' NCR for molecular diagnosis and examined in the VP1-2A and VP3-VP1 regions for genotyping. Twelve (20%) patients were detected VP1-2A by RT-PCR and 10 patients had VP3-VP1. The resulting amplicons were sequenced for genotype identification. All HAV strains were identified as subgenotype IB. Phylogenetic analysis revealed an extensive genetic heterogeneity among the strains. Seven hundred sixty-five S→F and 788 K→R amino acid substitutions in IRI49 isolate were found. It is concluded that subgenotype 1b is the sole genotype HAV in this region. J. Med. Virol. 9999:1–5, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: hepatitis A; genotyping; Iran

INTRODUCTION

Hepatitis A virus (HAV) is the major cause of acute hepatitis worldwide [CDC, 1999] and presents a public health problem in developing countries where HAV infection causes 20–25% of clinical hepatitis. Outbreaks continue to be reported worldwide [Robertson et al., 1991; Hussain et al., 2005] despite the increasing use of inactivated hepatitis A vaccine [Kokkinos et al., 2010]. The clinical presentation of acute HAV hepatitis depends on the age at the time of infection. In infants, the infection is often subclinical, with symptomatic infection occurring more frequently in adults.

Because of poor sanitation, higher rates of infection are found in younger age groups in developing countries [Koff, 1998]. HAV is transmitted most often by person-to-person spread by the fecal-oral route either by direct contact or through the consumption of polluted water or contaminated food [Koff, 1998; Cuthbert, 2001]; transmission by sexual activity has also been reported rarely [Bruisten et al., 2001].

HAV belongs to the genus Hepatovirus within the family Picornaviridae. HAV is a positive-stranded RNA virus, 27–32 nm in diameter, with icosahedral symmetry and contains a 7.5 kb genome [Nainan et al., 2006]. The HAV genome contains an open reading frame that encodes a single polyprotein that is organized into three regions, structural (or capsid) region (P1) and nonstructural regions (P2, P3). The P1 region is cleaved subsequently into four capsid proteins designated VP1–VP4 [Lee et al., 2009]. And a short sequence in the VP1-2A region (168 nucleotides) has been used to classify HAV into seven genotypes (I–VII). Thus far, subgenotypes IA, IB, IIIA, and IIIB have been identified but recent studies suggested that genotypes II and IIIV may be one genotype, indicating six likely genotypes. Three of these genotypes are derived from humans (I–III) and three have a simian origin (IV–VI). Genotype I is the most prevalent genotype worldwide, representing about 80% of patients [Robertson et al., 1992; Costa-Mattioli et al., 2003; Lu et al., 2004].

Seroepidemiologic studies have shown that HAV infection is endemic in developing countries, including different areas of Iran. The seroprevalence of HAV in a number of Iranian cities including Tehran, Golestan, and Hormozgan has been reported to be 85, 99, and 96%, respectively [Merat et al., 2010]. Several studies showed that genotype I, subtype IB, is the most prevalent in neighboring Turkey [Normann...
et al., 2008]. The genotypes of HAV circulating in Iran have not been reported previously. Therefore, the aim of this study was to determine the HAV genotypes among patients with acute HAV infection during 2009 and 2010 in the Ahwaz City, Iran.

MATERIALS AND METHODS

Study Subjects and Clinical Specimens

Ahwaz City, the capital of Khoozestan province and home of 1.5 million people, is located in the southwestern region of Iran. Sera from patients with clinical signs and symptoms of acute hepatitis were tested for IgM antibodies directed against HAV (anti-HAV IgM). Serum specimens with IgM antibodies against HAV were collected from patients of different hospitals in Ahwaz from June 2009 to May 2010. The sera were stored at −70°C prior to performing RT-PCR.

Molecular Studies

Viral nucleic acid was extracted from 200 μl of each patient’s serum using the High Pure Viral Nucleic Acid Kit (Roche Applied Science, Germany). Fifty microliters of RNA was eluted and stored at −70°C until use. For cDNA preparation, 6 μl of the extracted RNA was mixed with 25 μl of reaction mixture containing: 2.5 μl 5ґ RTQ PCR buffer, 1.0 μl dNTPs (10 mM), 1.0 μl random primers (1.8 mg/ml), 0.5 μl of RNase inhibitor (10 U), 0.5 μl MgCl2 (25 mM) and 13 μl RNase free water, and the mixture was heated at 42°C for 60 min.

Three nested PCR assays targeting the VP1-2A junction, 5ґ-NCR and VP3-VP1 regions were performed using primers listed in Table I. A 50 μl reaction mixture was prepared containing 10× PCR buffer with MgCl2, dNTPs (10 mM), DNA polymerase (1 U), and 0.5 μM forward and reverse primers. In the first round PCR including, 5 μl of cDNA as template was added to the mixture. The mixture was put in the thermocycler system (Techne TC-512, UK) with the following conditions: 5 min at 94°C, 35 cycles of 1 min at 94°C, 45 sec at 55°C, 45 sec at 72°C, and a final elongation step of 5 min at 72°C. For the second amplification, 5 μl of the first PCR product was added to a mixture containing 10× PCR buffer with MgCl2, dNTPs (10 mM), DNA polymerase (1 U), and 0.5 μM forward and reverse primers with identical cycling conditions as described above.

The PCR products from VP1-2A junction and VP3-VP1 junction were sequenced using the PRISM 377 automated DNA sequencer (Applied Biosystems). The nucleotide sequences of VP3-VP1 and VP1-2A junction were aligned by the CLUSTAL W. The phylogenetic tree analysis of VP3-VP1 and VP1-2A junction regions was obtained by the neighbor-joining method under the Kimura-two parameter distance model. These methods were implemented in the software package of MEGA version 4 (www.mega-software.net). HAV reference sequences used for comparisons with sequences from this study were obtained from GenBank (Table II).

RESULTS

Fifty-nine patients had IgM antibody to HAV detected in Ahwaz region between 2009 and 2010. The age of patients ranged from 2 to 25 years (mean age 8.7 years) and 34 (57.6%) were male. Twelve patients were HAV detected by nested RT-PCR; the characteristics of these patients are shown in Table III. These patients, consisting of five females and seven males, were all from the Ahwaz region and most were children. The mean age was 8.4 years (range, 2–17 years). The source of infection was not identified.

Three different nested RT-PCRs were performed for three different HAV genome regions. First amplified 5ґ NCR with two primer pairs with a 290 bp PCR product and second amplified the VP1/2A region with two primer pairs with a 234 bp PCR product; third region was VP3-VP1 region with a 247 bp PCR product. Overall, HAV RNA was detected in 12 cases using nested RT-PCR for 5ґ NCR and VP1-2A regions. However, nested RT-PCR carried out in the VP3-VP1 only detected specific regions in 10 samples. The VP1-2A junction region was amplified by nested RT-PCR from 12 (20.3%) of the 59 IgM positive sera and successfully sequenced (Table III). The VP1-2A junction region, used mostly for genotyping and

### Table I. Primers Used for HAV RNA Amplification From Clinical Specimens

<table>
<thead>
<tr>
<th>Region</th>
<th>Name of primers</th>
<th>Sequence 5′-3′</th>
<th>Nucleotide number</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>VP1/P2A external</td>
<td>BR-5a</td>
<td>TTGCTGTCACAGAAGAATCAG</td>
<td>2950–2972</td>
<td>361</td>
</tr>
<tr>
<td></td>
<td>BR-9a</td>
<td>AGTCACACTCTCCAGGAAACTT</td>
<td>3310–3286</td>
<td></td>
</tr>
<tr>
<td>Internal</td>
<td>RJ-3b</td>
<td>TCCAGAAGCTTCCATGGA</td>
<td>2984–3002</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td>BR-6a</td>
<td>AGGAGTGGAGAAGCTTCCATTTGA</td>
<td>3217–3193</td>
<td></td>
</tr>
<tr>
<td>5 NTR external</td>
<td>HAV1</td>
<td>TGGGAACGTCACCCTGCAGT</td>
<td>332 ± 352</td>
<td>368</td>
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<tr>
<td></td>
<td>HAV2</td>
<td>CTGAGTACCCTCAGGGAACA</td>
<td>680 ± 700</td>
<td></td>
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<tr>
<td></td>
<td>neHAV1</td>
<td>ATCTCTTTTGATCTTCCACAAG</td>
<td>371 ± 391</td>
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<tr>
<td></td>
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<td>GAAAGTCCACGGTCCGATTGG</td>
<td>641 ± 661</td>
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<tr>
<td>VP3-VP1 external</td>
<td>VP1-4</td>
<td>CGTGGCTTTCCCATGTCAGA</td>
<td>2115–2135</td>
<td>369</td>
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<td></td>
<td>VP1-5</td>
<td>GACCTTCCCAATACTTGAG</td>
<td>2843–2462</td>
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<td>Internal</td>
<td>VP1-2</td>
<td>GTTGTCCTCTCTTTTACATGTATG</td>
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<td>247</td>
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<td></td>
<td>VP1-1</td>
<td>GGAAATGTCTCAGGACTTTCG</td>
<td>2415–2390</td>
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</table>
phylogenetic analysis, was considered for nucleotide BLAST and neighbor-joining phylogenetic method. The results of nucleotide blast revealed that all patients with HAV in Ahwaz City had HAV genotype IB.

A sequence comparison among the clinical samples showed 94–98.8% identity with the control strain in the GenBank. Based on phylogenetic tree the alignment of VP1-2A junction is shown in Figure 1.

The PCR products amplified from VP3-VP1 region of the HAV genome were also subjected to sequence analysis followed by phylogenetic analysis. Analysis of the VP3-VP1 region showed similar results in the comparison with the VP1-2A region (Fig. 2). The sequence of the isolated 10 HAV strains had 91–99% homologies in the VP3-VP1 junction region compared with other strains around the world available in the GenBank. Both regions of the HAV genome were classified as genotype IB.

The predicted amino acid sequences from aa764 to 820 of the VP1-2A junction for the 12 Iranian strains were compared with HM175 strain (Fig. 3). The data indicated two substitutions in region of 765, S → F and 788, K → R position, respectively.

**DISCUSSION**

In this study, sera from 12 of 59 patients with acute hepatitis and HAV anti-IgM antibodies were sequenced. All HAVs were found to belong to genotype IB, suggesting that this was the dominant genotype circulating in Ahwaz City during the study period. HAV genotypes from other Iranian cities were not detected. Previous studies in neighboring countries have also shown that subgenotype IB was the major HAV genotype in these regions [Normann et al., 2008]. Phylogenetic analysis that showed by Mirko et al. [2009] indicated importation of genotype IB strains to Germany isolated from patients who traveled from Turkey and the Middle East. This finding provides evidence that genotype IB is also detected in this area.

Overall, 84.7% of cases with symptomatic hepatitis who had HAV IgM were between the ages of 2–15 years. All patients with HAV were hospitalized children and adolescents over a wide time range. There was no evidence of HAV outbreaks in Ahwaz city but apparently sporadic cases over time, indicating persistent and likely high rates of HAV circulating in this region. This is supported by relatively high rates of seroprevalence in Iran. For example, one recent study showed that the seroprevalence of HAV varied between 85 and 96% and Golestan and Hormozgan reported seroprevalence rates of 85 and 96%, respectively [Merat et al., 2010].

Molecular studies have been very useful for establishing the genetic diversity and evolutionary relationships among HAV strains and allowing investigation of chains of transmission and documenting the source of HAV outbreaks [Bruisten et al., 2001; Costa-Mattioli et al., 2001]. The phylogenetic tree from the different regions of the HAV genome has shown differing homology such as Normann et al. [2008] and Chironna et al. [2003]. In this study, the VP3-VP1 junction region of HAV RNA in these subjects showed similar rates, ranging from 91 to 99% and from 94 to 98% in the VP1-2A junction region. All strains were subgenotype IB and no striking differences were observed between the phylogenetic results from VP1-2A region and VP3-VP1 region.

In the present study, nested RT-PCR assays for both the VP1-2A and 5'-NCR genome regions gave similar results when detecting HAV RNA in serum

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**TABLE II. HAV Control Strains**

<table>
<thead>
<tr>
<th>No.</th>
<th>Strain/year</th>
<th>Country/genotype</th>
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<tr>
<td>1</td>
<td>HM175</td>
<td>Australia/IB</td>
<td>M14707</td>
</tr>
<tr>
<td>2</td>
<td>H-153</td>
<td>Tunisia/IB</td>
<td>L07727</td>
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<td>3</td>
<td>Ag6014</td>
<td>Greece/IB</td>
<td>L07703</td>
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<td>4</td>
<td>Jor88</td>
<td>Jordan/IB</td>
<td>L07728</td>
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<tr>
<td>5</td>
<td>IT-COL-00</td>
<td>Italy/IB</td>
<td>AJ505564</td>
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<tr>
<td>6</td>
<td>HS-21</td>
<td>Spain/IB</td>
<td>AF386910</td>
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<td>7</td>
<td>412991</td>
<td>South Africa/IB</td>
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<td>RJ-004</td>
<td>Brazil/IB</td>
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<td>Iraq/IB</td>
<td>L20554</td>
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<td>MT90</td>
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<tr>
<td>13</td>
<td>India80</td>
<td>India/IA</td>
<td>L07725</td>
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**TABLE III. Patients Results**

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<tr>
<th>Isolate</th>
<th>Gender</th>
<th>Age</th>
<th>5’ NCR</th>
<th>VP1-2A Identity (%)</th>
<th>VP3-VP1 Identity (%)</th>
<th>Genotype</th>
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<td>IRI2</td>
<td>F</td>
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<td></td>
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<tr>
<td>IRI4</td>
<td>M</td>
<td>5</td>
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<td>+</td>
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<td>IRI13</td>
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<td>IRI24</td>
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<tr>
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<td>M</td>
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<td>IRI43</td>
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<td>IB</td>
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<tr>
<td>IRI49</td>
<td>M</td>
<td>5</td>
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<td>+</td>
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<td>IB</td>
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<tr>
<td>IRI54</td>
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<td>9</td>
<td>+</td>
<td>+</td>
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<td>IB</td>
</tr>
<tr>
<td>IRI56</td>
<td>M</td>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
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samples. In contrast, Normann et al. [2008] reported that a 5'-NCR RT-PCR assay was more sensitive than assays targeting the VP1-2A region. Therefore, nested RT-PCR is likely to be better than the one step RT-PCR for detection of HAV RNA genome.

In general, one subgenotype of HAV has been reported in these specific regions. Chironna et al. [2003] evaluated different HAV strains in the southern Italy and found mostly genotype IB. And also the study reported that imported HAVs from endemic regions of Mediterranean were found among patients.

Costa-Mattioli et al. [2001] reported that an analysis of the molecular characteristics of HAV isolates from hepatitis cases and seafood, suggesting that shellfish could be a source of HAV infection.

The main source of drinking water for Ahwaz City is the Karon River. This river is highly contaminated with sewage from the upstream cities of Shoshtar and Molasani before entering Ahwaz City. It is likely that

![Image of phylogenetic tree](image1)

**Fig. 1.** Neighbor-joining phylogenetic tree of the nucleotide sequences in the VP1-2A regions of the strains of acute hepatitis A in Ahwaz-Iran. Numbers in branch are reproducibility after 100 bootstraps. Sequences reported in this study have GenBank Accession number: JN388678- JN388689. Scale bar = 2% and Genotypes are indicated.

![Image of phylogenetic tree](image2)

**Fig. 2.** Neighbor-joining phylogenetic tree of the nucleotide sequences in the VP3-VP1 regions of the strains of acute hepatitis A in Ahwaz-Iran. Numbers in branch are reproducibility after 100 bootstraps. Sequences reported in this study have GenBank Accession number: JN388668- JN388677. Scale bar = 1% and Genotypes are indicated.

![Image of amino acid comparison](image3)

**Fig. 3.** Comparison of the predicted amino acid sequences of the VP1-2A junction region. The consensus amino acid sequence of HM175 is shown in the top. The numbers above the consensus amino acid sequence indicate the predicted amino acid number from the start of HAV full amino acid. Dots show conserved amino acid and amino acid codes indicate different with consensus amino acid.
inadequate purification of the water led to increased risk of HAV infection among Ahwaz City residents.

In conclusion, HAV strains circulating in Ahwaz City in 2009 and 2010, though genetically diverse, constituted only a single genotype, IB. Additional epidemiologic studies on HAV from other Iranian cities is necessary to establish the full genetic diversity of HAV in Iran.

ACKNOWLEDGMENTS

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REFERENCES


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