Journal of Bacteriology and Virology 2009. Vol. 39, No. 3 p.165 – 171
DOI 10.4167/jbv.2009.39.3.165

Original Article

Genetic Analysis of Hepatitis A Virus Isolated from Korea

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Hepatitis A virus (HAV) is one of the most important causes of acute infectious hepatitis. The aim of this study was to determine the genotypes of HAV that have been circulating in Koreans. A total of 76 sera referred to our institute for HAV genotyping from 11 Korean provinces were used for this study. Those samples were diagnosed by positive of IgM anti-HAV. HAV RNA was extracted from 150 μl of serum, and reverse transcription PCR-sequencing was used to detect and characterize HAV RNA. Primer pairs from the VP1/2A region of the HAV were used for amplification and sequencing. HAV RNA was found in 64.5% (n = 49) of the 76 patient sera with acute hepatitis A. Forty-seven strains were genotype IIIA in a total of 49 isolated strains (95.9%, 47/49); only two strains belonged to genotype IA (4.1%, 2/49). Thirty eight genotype IIIA isolates were 100% identical to consensus amino acid sequences of the reference strain AJ299467. The amino acid change of L772F was found in two IIIA strains; other IIIA isolates showed one amino acid change. Amino acid of genotype IA was compared to reference strain L20541. K801R was found in 1 strain and Q810S in both strains. The amino acid change of K801R was the first report in Koreans. Until recently HAV genotype IA has been reported as a major circulating HAV genotype in Koreans. In the present study, the predominant HAV strain in Koreans seemed to be HAV genotype IIIA.

Key Words: Hepatitis A, Genotype

INTRODUCTION

Hepatitis A virus (HAV) infection is the leading cause of clinically apparent viral hepatitis in many parts of the world, including both developed and developing countries. HAV is a hepatotropic member of the *Picornaviridae* family and has been classified in the genus hepatovirus. Its genome is a linear, positive-sense, single-stranded RNA, about 7.5 kb in length (1). Good sanitation practices and a clean water supply are essential to the prevention of HAV infection. In developing countries experiencing a high incidence of HAV, the main transmission route of HAV is the fecal-oral route caused by poor sanitation, which increases the chance of ingesting contaminated food or water (2). In a number of Asian and Latin American countries, the epidemiology is presently changing from high to intermediate endemicity, due to improvements in sanitary conditions (3, 4). In Korea, due to sanitation-related improvements, epidemic outbreaks of HAV infection have become less frequent (5). However, recently HAV infection is rapidly increasing nationwide, especially in young people who have a weak immunity to HAV.

HAV strains have maintained low rates of mutation accumulation over extended periods of time (6). However recent nucleotide sequencing of selected genome regions that encode the putative VP1/2A junction region of wild-type HAV strains present in human specimens has demonstrated substantial sequence heterogeneity. Using this approach, the

Received: June 16, 2009/ Revised: July 15, 2009
Accepted: July 21, 2009
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isolates of HAV have been categorized into seven genotypes (7). The genomic characterization of HAV has been carried out mainly by genotyping strains from different geographic regions worldwide (8). De Paular *et al*. (9) reported that genotype IA constitutes the major HAV population in North America, China, the former USSR, and Thailand, whereas genotype IB contains strains from Europe, North Africa, the Middle East, and Australia. Co-circulation of IA and IB of HAV has been reported in Italy (10); and in Brazil (11). HAV genotype III has been reported to be linked with intravenous drug use in Sweden during the 1980s (7); it has also been found in India, Sri Lanka, Nepal, Malaysia, and the USA (12); in Norway (13); in Germany (14); and in Russia (15). Studies from Korea have previously shown the HAV genotype I to be predominant, and some changes therein have been recently reported (16~19). In this study, the molecular epidemiology of HAV viral strains were determined by reverse transcription polymerase chain reaction (RT-PCR) and sequencing in the VP1/2A region of HAV isolates from Korean patients.

MATERIALS AND METHODS

RNA extraction from patient sera

Seventy-six sera of HAV patients from 11 Korean provinces of Korea during 2007~2008 were tested (Table 1). The diagnosis of hepatitis A was based on a high titer serum IgM anti-HAV level. HAV RNA was extracted from 150 μl of serum using a Viral Gene spin kit (Intron, Sungnam, Korea). Viral gene-spin lysis buffer (250 μl) was added to serum and incubated at room temperature for 10 min and 350 μl of binding buffer was added. Lysate was loaded on a spin column and centrifuged at 13,000 rpm for 1 min. After discarding the solution, 500 μl of washing buffer A was added to column and centrifuged for 1 min at 13,000 rpm. After discarding the solution, 500 μl of washing buffer B was added to the column and centrifuged for 1 min at 13,000 rpm. After discarding the solution, the spin column was placed in a RNase-free 1.5 ml microcentrifuge tube, and elution buffer (30 μl) was directly added onto the membrane and incubated at room temperature for 1 min. Eluted solution (2~5 μl) was used for PCR.

HAV genome amplification

For reverse transcription, 1 μl of RNA solution was heat-denaturated at 68℃ for 10 min. It was chilled rapidly on ice and mixed with 4 μl of 1.5 mM $MgCl_2$ solution, 2 μl of RNA PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 8.5 μl of RNase-free distilled water, 2 μl of dNTP mixture (10 mM dATP, dCTP, dGTP, dTTP), 1 μl of random 9-mers (5'-NNNNNNNNN-3'), 0.5 μl of RNase inhibitor (Takara-Shuzo, Kyoto, Japan), and 1 μl of reverse transcriptase (Takara-Shuzo). After incubation at 30℃ for 10 min, reverse transcription reaction was carried out at 42℃ for 30 min, followed by inactivation at 95℃ for 5 min. Primers for HAV RT-PCR reported by Takahashi *et al*. (20) were used in the present study. The first PCR was performed in 20 μl of reaction mixture containing 1.0 μM each of outer sense primer (5'-GGT TTC TAT TCA GAT TGC AAA TTA-3': nt. 2891-2914) and antisense primer (5'-AGT AAA AAC TCC AGC ATC CAT TTC-3': nt. 3398-3375), 5 μl of cDNA solution, 200 μM of each dNTP, 2.0 μl of 10 × PCR buffer [100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin], and 2.5 U of *Ex Taq* polymerase (Takara-Shuzo) with proofreading activity. The amplification condition were 94℃ for 16 min, followed by 40 cycles of 94℃ for 1 min, 50℃ for 1 min, 72℃ for 1 min, and an additional 10 min at 72℃ in the last cycle. The second PCR was carried out with 1.5 μl of the first PCR product. The reaction mixture contained 1.0 μM each of inner sense primer (5'-TTG CAA ATT ACA ATC ATT CTG-3': nt. 2905-2925) and inner antisense primer (5'-TTC AAG AGT CCA CAC ACT TCT-3': nt. 3377-3367), 2 μl of 10 × PCR buffer and 15.1 μl of RNase free dH_2O, 1 μl of dNTP mixture (2 mM dATP, dCTP, dGTP, dTTP), and 0.2 μl of amplitaq gold (Roche Diagnostics, Branchburg, NJ, USA). The amplification conditions for the second PCR were the same as those of the first PCR. Takara TP-100 PCR machine (Takara Bio Inc., Shiga, Japan) was used for HAV PCR. The PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination.

Table 1. Molecular characters and nucleic acid sequences homologies of 49 strains at the VP1/2A region of hepatitis A Virus (HAV) in Korean HAV isolates compared to reference strains, AJ299467 and L20541 for genotype IIIA and IA, respectively

Isolate	Identity (%)	Genotype	Province	Year
ND91610	99.22	3A	Chonan	2007
ND91611	98.83	3A	Inchon	2007
ND91612	99.22	3A	Seoul	2007
ND91613	99.61	3A	Seoul	2007
ND91614	99.22	3A	Suwon	2007
ND91616	99.61	3A	Anyang	2007
ND91618	99.61	3A	Inchon	2007
ND91620	98.44	3A	Seoul	2007
ND91623	96.50	3A	Kwangju	2007
ND91624	98.83	3A	Jonju	2007
ND92401	98.83	3A	Anyang	2008
ND92403	98.44	3A	Seoul	2008
ND92404	98.83	3A	Suwon	2008
ND92406	98.83	3A	Anyang	2008
ND92407	99.61	3A	Wonju	2008
ND92408	99.61	3A	Seoul	2008
ND92427	99.61	3A	Anyang	2008
ND92428	98.83	3A	Seoul	2008
ND92430	99.22	3A	Seoul	2008
ND92432	99.22	3A	Kwangju	2008
ND92433	99.22	3A	Anyang	2008
ND92435	99.61	3A	Seoul	2008
ND92437	99.61	3A	Kwangju	2008
ND92439	99.61	3A	Seoul	2008
ND92443	100.00	3A	Inchon	2008
ND92444	99.22	3A	Anyang	2008
ND72600	99.22	3A	Chonan	2008
ND72800	98.05	3A	Suwon	2008
ND81200	98.83	3A	Inchon	2008
ND81800	99.61	3A	Seoul	2008
ND82600	98.83	3A	Anyang	2008
ND90400	98.05	3A	Inchon	2008
ND92301	99.22	3A	Inchon	2008
ND92303	100.00	3A	Seoul	2008
ND92305	97.28	3A	Inchon	2008
ND10141	98.83	3A	Inchon	2008
ND10143	99.22	3A	Anyang	2008
ND10144	99.22	3A	Inchon	2008
ND10211	99.61	3A	Anyang	2008
ND91201	99.22	3A	Seoul	2008
ND91203	99.61	3A	Anyang	2008
ND91205	99.61	3A	Seoul	2008
ND91206	99.22	3A	Seoul	2008
ND91207	99.61	3A	Kangleung	2008
ND12312	98.83	3A	Seoul	2008
ND12314	99.22	3A	Wulsan	2008
ND12315	98.44	3A	Seoul	2008
ND91622	96.43	1A	Suwon	2007
ND93000	96.43	1A	Seoul	2008

Sequencing of PCR products

Amplification products were purified on Wizard PCR Preps DNA purification resin (Promega, Madison, WI, USA), and sequenced bidirectionally with Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) using the above PCR primers. Sequencing was performed on an automated DNA sequencer ABI 3730 (PE Applied Biosystems). The nucleotide sequences of HAV isolates from the patients were compared with those of reference HAV strains retrieved from the GenBank databases. Nucleotide sequences of HAV isolates were compared to published HAV sequences from different genotypes. The reference sequences of HAV, listed here with the NCBI accession number of the HAV VP1/P2A junction region, belong to genotypes IA (L20541, L07676, L20553, L07722, and L07717), IB (L07703), IIB (L07729), IIIA (L07725, L07668, and AJ299467), IIIB (L20532), IV (L07732) and VI (L07731). The relatedness of the HAV RNA nucleic acid sequence was assessed through multiple sequence alignment, using the Clustal X program (21). The calculation of nucleotide and amino acid identity, the determination of genetic distances between sequences, and the construction of phylogenetic trees were

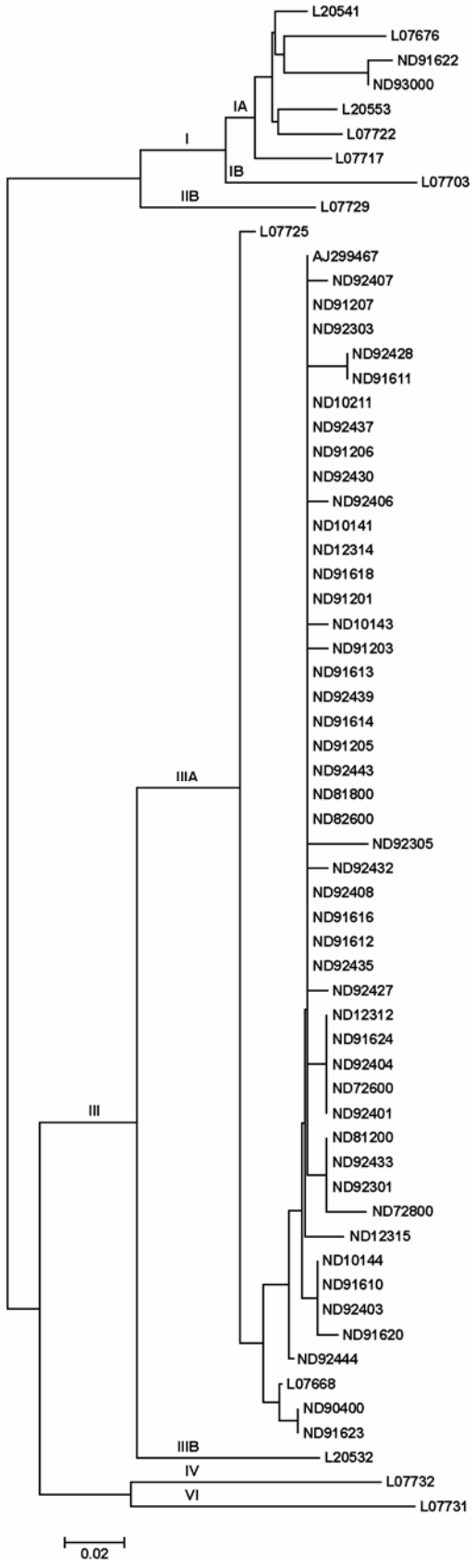

Figure 1. Neighbor-joining phylogenetic tree nucleotide sequences in the VP1/P2A region of HAV isolates in Korea. The tree includes reference strains L20541, L07676, L20553, L07722 and L07717 in genotype IA, L07703 in genotype IB, L07729 in genotype IIB, L07725, L0766 and AJ2994678 in genotype IIIA, L205328 in genotype IIIB, L077328 in genotype IV, L07731 in genotype VI. The horizontal bar at the bottom represents a genetic distance of 0.02.

performed through the use of MEGA software, version 4.0 for Windows. Phylogenetic trees were generated via the neighbor-joining (N-J) method (1).

RESULTS

Genetic analysis of HAV isolates

HAV RNA was found in 49 samples in a total of 76 sera of HAV patients (64.5%) collected from the 11 provinces of Korea (Table 1). The sequence between nucleotides 3024 and 3191 of the VP1/2A region was determined for the 49 patients. In the present study, HAV genotype IIIA was predominant (95.9%, 47/49); only two strains belonged to genotype IA (4.1%, 2/49). Two HAV genotype 1A strains were found in Suwon area in 2007 and Seoul area in 2008. Nucleic acid sequences homologies at the VP1/2A junction of HAV genome in HAV isolates compared to reference strains were listed in Table 1. The nucleotide sequence identities of the VP1/P2A junction region with genotype IIIA ranged from 96.5% to 100%, compared to the reference strain, AJ299467. While genotype IA showed 96.4% identity with regards to nucleotide sequence identity, compared to the reference strain, L20541 (Table 1). We then performed a phylogenetic analysis of the region between nucleotides 3024 and 3191 and classified the virus strains (Fig. 1). Most strains belonged to genotype IIIA, except two strains that belonged to genotype IA.

Changes in amino acid sequences among HAV isolates

A comparison of the amino acid sequences from position 758 to 842 of the VP1/2A region of HAV strains are presented in Fig. 2. Amino acid substitutions were found 12 and 2 different sites in genotype IIIA and IA, respectively (Table 2). Within genotype IIIA, the amino acid sequence was conserved with a few exceptions, while greater differences

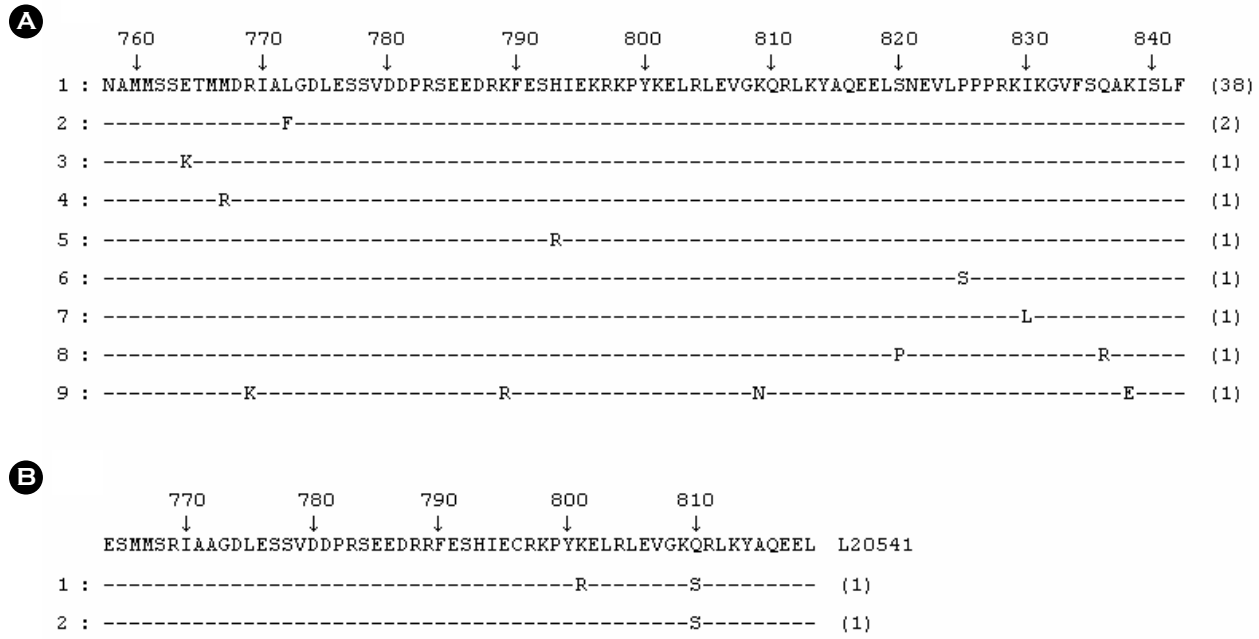

Figure 2. Comparison of the predicted amino acid sequences of the VP1/VP2 junction region of HAV. The consensus amino acid sequences of AJ299467 for HAV genotype IIIA and L20541 for HAV genotype IA are shown on the top line of **(A)** and **(B)**, respectively. "–" indicates conserved amino acid; differences are shown by the appropriate single letter amino acid code. The numbers above the consensus amino acid sequence indicate the predicted amino acid number from the start of HAV full amino acid. The numbers of the right ends indicate the number of HAV strains we found in the present study.

Table 2. Amino acid changes of Korean HAV strains in VP1/2A region of HAV from 2007 to 2008

Amino acid position	Genotype	Prototype	Substitution	Number
764	3A	E	K	1
767	3A	M	R	1
769	3A	R	K	1
772	3A	L	F	2
789	3A	K	R	1
793	3A	H	R	1
809	3A	K	N	1
820	3A	S	P	1
825	3A	P	S	1
830	3A	I	L	1
836	3A	Q	R	1
838	3A	K	E	1
801	1A	K	R	1
810	1A	Q	S	2

were evident in the amino acid sequences between genotype IIIA and IA of HAV. Among the genotype IIIA strains, 38 isolates showed 100% identity in terms of consensus amino acid sequences with the reference strain AJ299467, a representative HAV genotype IIIA strain. The amino acid change of L772F was found in two IIIA strains. Amino acid changes of E764K, M767R, R769K, K789R, H793R, K809N, S820P, P825S, I830L, Q836R, and K838E were found in each of the different genotype IIIA strains (Table 2). These changes in amino acid sequence of genotype IIIA have never been reported in Koreans. Two HAV genotype IA strains were compared to reference strain L20541. The amino acid change of K801R was found in one strain, and that of Q810S was found in both strains (Table 2).

DISCUSSION

More recently, HAV infection has increased in Korean peoples. Vaccination of HAV is recommended to persons who don't have anti-HAV IgG. In the present study, we examined HAV genotypes in the HAV patients who showed positive reaction for anti-HAV IgM. Compared to other RNA viruses, the amino acid and nucleotide sequences of

HAV are highly conserved (10). In recent years, studies on the nucleic acid heterogeneity of HAV isolates have allowed the characterization of strains and groupings into different genotypes and sub-genotypes. Studies of genotypes and changes in the nucleotide and amino acid sequences of HAV may provide valuable information with regards to the epidemiological aspects of a particular region (7).

Among several genome regions of HAV, including the C terminus of the VP3 protein (22), the N terminus of the VP1 protein (12), the junction region of VP1/2A proteins (23, 24) and the entire VP1 protein (25) have been used for HAV genotyping. The VP1/2A junction region selected in our study has been most frequently used for HAV genotype analysis in Korea (17~19). Among HAV isolates from Korean patients with acute hepatitis A from 1994 to 1998, all 18 isolates were clustered within genotype IA, irrespective of the patients' geographical location (26). Moreover outbreak cases of HAV caused by HAV genotype IA have been reported in Korea (16, 17). Yun *et al.* (19) reported the prevalence of IA and IIIA to be 83.3% and 16.7%, respectively; these data showed that the prevalent HAV genotype was IA, however the newly identified IIIA strain was co-circulating with the genotype IA strain during 2005~2006. They suggested that new IIIA strains might have been imported from high-endemic countries into Korea.

In the present study, a phylogenetic analysis of the sequences obtained from distinct HAV isolates revealed that most of the strains were genotype IIIA (95.9%); IA was found in only 4.1% of the specimens (Fig. 1). Executing a comparison of the partial nucleotide sequences of the HAV genome is a useful approach to defining the differences among HAV isolates (27). The molecular characters and amino acid sequences homologies of HAV isolates at the VP1/2A region of HAV genome were compared to reference strains, AJ299467 for genotype IIIA and L20541 for genotype IA, which were used in a previous study (19). HAV strains detected in this study showed over 96.0% identity compared to reference strains. Most genotype IIIA isolates showed the same amino acid sequences compared to reference strain; other isolates were conserved, with one or two amino acid changes (Fig. 2). This is the first report of amino acid changes of genotype IIIA in the VP1/2A region of HAV in Korean population. In two genotype IA strains, one strain was changed from K to R in position 801, while the other strain was changed from Q to S in position 810 (Table 2). The amino acid change of K801R of genotype 1A strain is the first reported in this study, whereas Q810S was frequently found in a previous study (19). The present study indicates that HAV genotype in Korea seemed to be changed from genotype IA to IIIA. These changes suggest that there is a need for continual surveillance that focuses on strain variation, and that it is important for understanding the epidemiology and development of a strategy for disease control and prevention.

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