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Hepatitis in Albanian Children: Molecular Analysis of Hepatitis A Virus Isolates

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Hepatitis A is a common disease in developing countries and Albania has a high prevalence of this disease associated to young age. In spite of the occurrence of a unique serotype there are different genotypes classified from I to VII. Genotype characterisation of HAV isolates circulating in Albania has been undertaken, as well as the study of the occurrence of antigenic variants in the proteins VP3 and VP1. To evaluate the genetic variability of the Albanian hepatitis A virus (HAV) isolates, samples were collected from 12 different cities, and the VP1/2A junction amplified and sequenced. These sequences were aligned and a phylogenetic analysis performed. Additionally, the amino half sequence of the protein VP3 and the complete sequence of the VP1 was determined. Anti-HAV IgM were present in 66.2% of all the sera. Fifty HAV isolates were amplified and the analysis revealed that all the isolates were sub-genotype IA with only limited mutations. When the deduced amino acid sequences were obtained, the alignment showed only two amino acids substitutions at positions 22 and 34 of the 2A protein. A higher genomic stability of the VP1/2A region, in contrast with what occurs in other parts of the world could be observed, indicating high endemicity of HAV in Albania. In addition, two potential antigenic variants were detected. The first at position 46 of VP3 in seven isolates and the second at position 23 of VP1 in six isolates. ***J. Med. Virol. 72:533–537, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: hepatitis A virus; genetic variability; molecular epidemiology

INTRODUCTION

Human hepatitis A virus (HAV) is classified in the *Hepatovirus* genus in the Picornaviridae family [Minor, 1991] and it is world-wide present in an unique serotype [Lemon and Binn, 1983]. However, genetic variability between HAV isolates in the world has permitted the classification of HAV strains in seven different genotypes with the genotypes I, II, III and VII including human isolates. The most frequent human genotype is genotype I (more than 80%), that has been divided into two sub-genotypes: IA and IB. This classification has been based on a 168 nucleotide sequence at the VP1/2A junction [Robertson et al., 1992; Normann et al., 1995].

HAV is transmitted by person-to-person contact within close communities, and the faecal-oral route through contaminated food or water [Divizia et al., 1993; De Serres et al., 1999; Massoudi et al., 1999]. In the industrialised countries, due to improvements of the public health and social-economic conditions, there has been a shift towards a higher age with an increase of the hospitalised and severe cases associated to outbreaks, whereas in the developing countries the disease is largely endemic with a high incidence rate in the young people [Shapiro and Margolis, 1993].

Albania is considered a poor country in the Balkans with serious socio-economical problems. The migratory movements, with a strong tendency towards urbanisation, specially in the capital city, Tirana, have contributed to the increase of environmental pollution and the contamination of water resources [Palombi et al., 2001].

In Albania, in 1998, more than 3,200 cases of hepatitis A disease were recorded, of which 62% were reported in the 0–9 group age and 38% in the group over 10-year-old

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[Schick et al., 1999]. However, no data exist on the HAV genotypes circulating in Albania. In the present study, serum samples selected at random from hepatitis serum collection of the Public Health Institute in Tirana were undertaken from 12 Albanian cities, and HAV isolates were analysed by sequencing the VP1/2A junction region, the amino region of VP3 and the complete VP1 region which included most HAV epitopes so far described [Emini et al., 1985; Nainan et al., 1992; Ping and Lemon, 1992; Bosch et al., 1998].

MATERIALS AND METHODS

Serum Samples

Overall 202 serum samples selected at random were involved in the present study. In particular, serum samples were collected from 12 different cities in Albania and from several suburban areas.

In the study was also included six samples from an intra-familiar outbreak of Kosovo refugees. The sera were collected, with the aim to cover the country, from individuals with a suspected hepatitis infection.

The samples were kept frozen at −20°C with limited freeze-thawing for serological tests, and keeping the ice-chain carried to Italy and Spain for the molecular analysis of HAV. The serological test for IgM anti-HAV were carried out directly in Albania using a commercial Elisa kit from Abbott Diagnostic (Rome, Italy).

RNA Extraction and RT-PCR Test

Total viral RNA was extracted only from anti-HAV IgM positive samples, all the extractions were carried out using a commercial TRIzol kit (Life Technologies, Milan, Italy) based on guanidinium isotiocyanate method [Boom et al., 1990]. Table I shows the primers employed in this study. For each sample, 100 μl of sera were extracted and the final RNA was kept in ethanol until the RT-PCR test.

For reverse transcription, one fourth of the RNA volume was pelleted at 10,000 rpm at 4°C for 10 min and dried in a vacuum centrifuge pump (Eppendorf, concentrator 5301) for 5 min.The dried pellet was resuspended in 10 μl of RNAse-free-water and converted to complementary DNA. For VP1/2A both RT, first and second PCR test were carried out as described previously [Divizia et al., 1999].

For the amplification of the amino half VP3 coding region and the entire VP1 region of HAV, the previously described procedures [Sánchez et al., 2003a] were employed in a RT-PCR method.

Ten microlitre of the second round PCR was analysed on 2% agarose (Amersham, Milan, Italy) gel electrophoresis and stained with ethidium bromide (Sigma, Milan, Italy).

Sequence Analysis of the VP1/2A Junction Region the VP3 Amino Half Region and the Complete VP1 Region of HAV

The amplified products were purified according to QIAgen PCR purification Kit and sequenced using 0,8 ng/bp, with the Big Dye Terminator Cycle Sequencing Ready Reaction version 2.0, and the reading was performed using an ABI Prism DNA Sequencer (Perkin Elmer Italy SpA, Monza, Italy).

Nucleotide Sequence Accession Numbers

The nucleotide sequences corresponding to the VP1/2A, VP3 amino and VP1 regions determined in this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov), and have been assigned accession numbers as reported in Table II.

RESULTS

The IgM anti-HAV positive sera were distributed depending on the different cities and randomly two or more sera were examined by RT-PCR, amplifying an overlapping region of 168 bp of the putative VP1/2A junction. Overall, 50 sera were collected from 12 different cities and the sequence alignment shows only 11 point mutations among them, in particular 7 out 50 are identical with no mutations among them (Table II). The sequences of the Albanian isolates differed between each other by around 4%, and all were genotype IA (Table III). When the phylogenetic tree was generated with the Phylip program (Department of Genetics, University of Washington), all the isolates were dis-

TABLE I. Used Primers for Hepatitis A Virus

Target region	Sequence	Position	$[Mg^{2+}]$ (mM)	Primer annealing temperature (°C)
	HAV primers			
VP1/2A	AGTCACACCTCTCCAGGAAAACTT	3285–3308	2.0	45
VP1/2A	TTGTCTTTTAGTTGTTATTTGTCTGT	2935–2959	2.0	45
Inner VP1/2A	CATTATTTCATGCTCCTCAG	3283–3264	2.0	45
Inner VP1/2A	TATTTGTCTGTCACAGAACAATCAG	2949–2973	2.0	45
NH_2-VP3	GGGACAGGAACTTCAGCTTATAC	1380–1402	2.0	50
NH_2-VP3	TCTACCTGAATGATATTTGG	1859–1840	2.0	50
NH_2-VP1	AATGTTTATCTTTCAGCAAT	2136–2155	2.0	53
NH_2-VP1	ACAGCTCCAAGAGCAGTTTT	2751–2770	2.0	53
COOH-VP1	ATGGCCTGGTTTACTCCAG	2673–2691	2.5	45
COOH-VP1	CCCTTCATTTTCCTAGG	3229–3213	2.5	45

TABLE II. Accession Number for VP1/2A, VP1 and VP3

Accession no. for VPI/2A		Accession no. for VP1		Accession no. for VP3	
Alb 12	AY332637	Alb 3	AY 334032	Alb 7	AY 334063
Alb 17	AY332639	Alb 4	AY 334028	Alb 8	AY 334045
Alb 31	AY332636	Alb 6	AY 334023	Alb 9	AY 334064
Alb 36	AY332642	Alb 9	AY 334021	Alb 17	AY 334059
Alb 37	AY332641	Alb 10	AY 334025	Alb 18	AY 334045
Alb 47	AY332640	Alb 13	AY 334027	Alb 19	AY 334049
Alb 50	AY332638	Alb 14	AY 334035	Alb 23	AY 334055
		Alb 17	AY 334031	Alb 42	AY 334071
		Alb 18	AY 334040	Alb 51	AY 334066
		Alb 19	AY 334026	Alb 55	AY 334070
		Alb 21	AY 334039		
		Alb 23	AY 334040		
		Alb 42	AY 334030		
		Alb 51	AY 334022		
		Alb 55	AY 334036		

tributed in seven different clusters, including two groups with only one strain (Fig. 1).

When the deduced amino acid sequences were compared, only two amino acid substitutions were identified. One (Arg → Lys) in the strain Alb17 determined by a nucleotide change (G → A) at position 3124 on the second base of the codon 34 of the 2A region; the second amino acid modification (Glu → Asp) was determined by a point mutation at position 3089 on the third base of codon 22 at the cluster group with Alb47. The sequences from a family of Kosovo refugees clustered together and presented the same sequence with the GBM strain, with the sole exception of the Alb 51 sample which exhibited a nucleotide change in the position 3056 (C → T).

Twenty one of the 50 genotyped samples could be amplified for the analysis of the VP3 and VP1 regions. Two conservative aminoacid substitutions occurred at positions 46 of VP3 (Ile → Val) and 23 of VP1 (Ile → Val) in 5 and 6 out of the 21 analysed sequences respectively. All samples containing the VP3 aminoacid substitution clustered together, and all presented the VP1 substitution too. The sixth VP1-bearing aminoacid substitution samples, clustered in a different, but closely related branch and also contained the substitution at position 22 of 2A protein above mentioned (Fig. 1).

DISCUSSION

Little information are available on hepatitis A in Albania [Schick et al., 1999] and until now almost all the data were obtained from refugees in different countries of Western Europe.

TABLE III. Nucleotide Mutations in Albanian Isolates

Position	Substitution	Position	Substitution
3029	T → C	3089	G → T
3041	A → G	3124	G → A
3056	C → T	3134	C → T
3062	G → A	3158	G → C
3068	A → G	3173	A → G
3080	T → C		

The nucleotide modifications 3089 and 3124 are responsible of two amino acid substitutions respectively in the codon 22 and 34.

HAV has a classic faecal-oral route, associated to poor hygienic level and environmental pollution. Our sera were randomly collected to obtain representative samples from different cities and no epidemic outbreaks samples were included, with the sole exception of a Kosovar family.

Albania is one of the poorest countries in the Balkan area and the recent years the migratory movements, the collapse of the social structure and the rapid urbanisation, mostly in Tirana, which represents one sixth of the entire population of the country, has caused the degradation of the environment. Hepatitis A is largely endemic in Albania with an overall prevalence higher than 95% in the younger age. In autumn 1994, a cholera epidemic outbreak [Greco et al., 1995] appeared in the Southern-Central region, and in 1996 an outbreak of poliomyelitis [Divizia et al., 1999a] involved the entire country. In a survey study on drinking water quality in Tirana, Palombi et al. [2001] has clearly demonstrated the high pollution of the potable water in Tirana and, indirectly, the possibility to transmit enteric viruses by contaminated water. In fact, a probable rotavirus water-borne gastroenteritis outbreak affecting two thousand children has recently been reported [Villena et al., 2003].

Genomic classification of HAV strains allows the elucidation of their geographic origin and their transmission patterns. Despite the limited amino acid heterogeneity of HAV, a significant degree of nucleic acid variability has been observed among different isolates from different regions of the world [Taylor, 1997; Arauz-Ruiz et al., 2001; Costa-Mattioli et al., 2001]. Robertson et al. [1992] has previously shown that sequence analysis of the VPI/2A junction region enables to distribute HAV strains into seven genotypes (Fig. 1). In this study, the analysis of the 50 amplified regions revealed that all the isolates belonged to genotype IA, suggesting the circulation of a unique strain in this country, mostly related to GBM strain. Genotype I is largely endemic in the Mediterranean area [Normann et al., 1995; Costa-Mattioli et al., 2001a]. Normally, samples collected during an outbreak show a single genotype [Robertson et al., 2000; Arauz-Ruiz et al.,

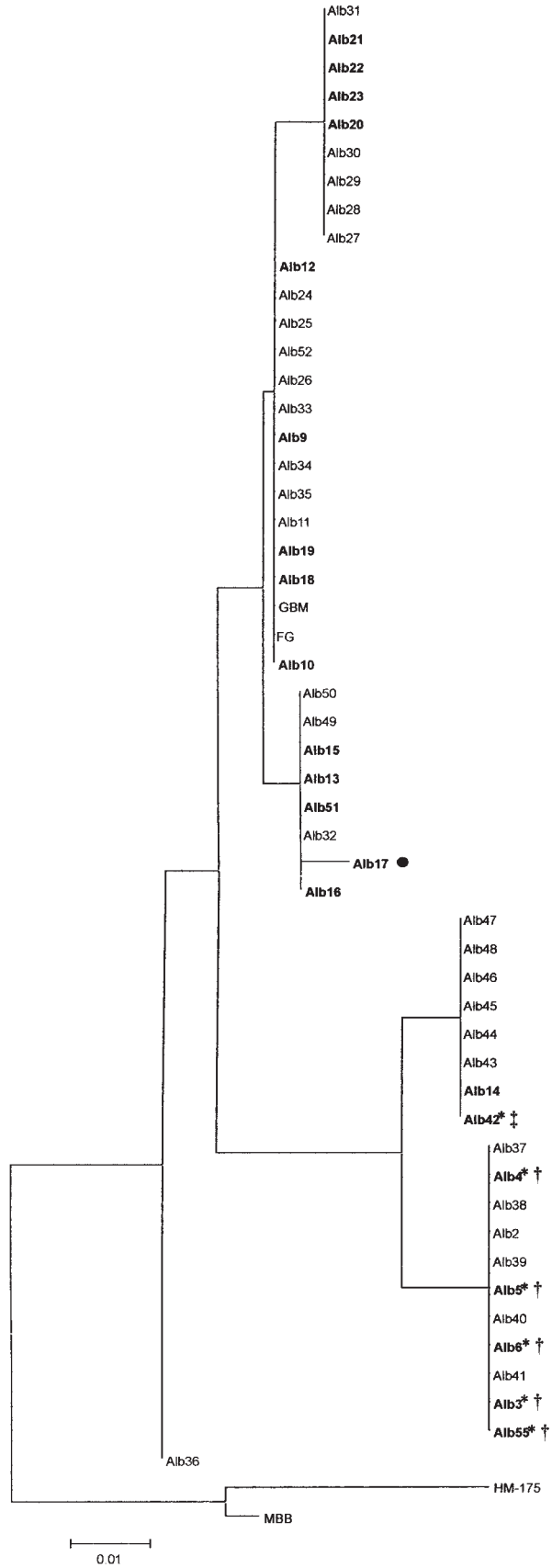

Fig. 1. Phylogenetic tree based on the nucleotide sequence of the VP1/2A region (nt 3,024–3,191). †Samples with an isoleucine to valine change at position 46 of protein VP3. *Samples with an isoleucine to valine change at position 23 of protein VP1. ‡Samples with a glutamic acid to aspartic acid change at position 22 of protein 2A. ●Samples with an arginine to lysine change at position 34 of protein 2A.

2001], whereas samples collected from different sources in the same area can reflect the circulation of different genotypes [Diaz et al., 2001; De Paula et al., 2002]. In the present study, the analysis of samples which were randomly collected from 12 different cities and several suburban areas in Albania, show the occurrence of a unique genotype IA.

A limited number of point mutations in the VP1/2A junction are present (Table III), and 50 isolates can cluster into just seven groups with a maximum distance of about 4%, confirming that the HAV genome is more stable with respect of the high genomic variability observed in other RNA viruses [Sánchez et al., 2003].

The observed Kosovar family shows a unique intra-familiar strain with just one point mutation (strain Alb51) and 100% homology with other strain isolates from different cities, excluding the import of these strains from Kosovo. Interestingly, the occurrence of a mutant in a small intra-familiar outbreak of a common origin, suggested the existence of a quasiespecies dynamics of replication of HAV as has been recently reported [Sánchez et al., 2003a].

When the amino acid sequences were compared with the other isolates, all the nucleotide modifications were silent with the exception of two substitutions identified in the codon 22 and 34 of the protein 2A. One of these mutations, position 3089, G → T, had only been previously reported in some Cuban isolates [Diaz et al., 2001]. More recently, Chironna et al. [2003] identified, in the region of Puglia, a strain of HAV classified IT-BIA-01 (acc. no. AJ505561) with the same point mutation and amino acid substitution. The great stability of Albanian isolates confirms the solely circulation of autochthonous strains.

Interestingly, two potential new antigenic variants were detected in several samples, the first at position 46 of VP3 and the second at position 23 of VP1. This last position is located in the middle of a previously identified continuous epitope [Emini et al., 1985], representing an antigenic variant of HAV. It is remarkable that several variants in residues contained in or near this epitope have been previously reported, such as the Met → Val, at residue 28 [Sánchez et al., 2002] or Lys → Arg at residue 29 [Arauz-Ruiz et al., 2001], indicating that the amino terminus of VP1 is a surface exposed region with a high tolerance to aminoacid substitutions. Residue 46 of VP3, although not associated with any of the described epitopes of HAV, aligns with an epitope of the Theiler's murine encephalomyelitis virus located in the VP3 amino terminus [Usherwood and Nash, 1995]. Although all sera from these variant samples were IgM positive, a partial lost of recognition of these isolates by monoclonal anti-HAV antibodies may occur as reported elsewhere [Sanchez et al., 2002]. However, no faecal

samples were available, in order to ascertain this possibility.

Information on the prevalence and circulation of HAV variants in different parts of the world is epidemiologically relevant. The social isolation of Albania until the beginning of the 1990s and the extremely high prevalence of hepatitis A infection in this country has restricted the introduction and diffusion of new strains, and explains the great genetic stability of the isolates characterised in the present study.

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