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Variability of the PreS1/PreS2/S regions of hepatitis B virus in Hungary

K. N. Szomor[1], **Á. Dencs**[1], **G. Tóth**[2], **G. M. Kovács**[3], **Y. Saleh Ali**[1], **G. Berencsi**[1], and **M. Takács**[1]

[1] Division of Virology, National Center for Epidemiology, Budapest, Hungary
[2] Bioinformatics Group, Agricultural Biotechnology Center, Gödöllö, Hungary
[3] Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

Summary

Infection with the hepatitis B virus can occur perinatally, parenterally, or sexually, and it can cause acute or chronic liver diseases. Phylogenetic analysis of the virus has led to its classification into eight genotypes (A–H), which show a characteristic worldwide distribution. The aim of this study was to reveal the HBV genotypes present in Hungary and to investigate a nosocomial and an intrafamilial outbreak.

The collected samples were tested by nested PCR, and a 650-nucleotide-long segment of the preS1/preS2/S region was sequenced. As no previous genotype data were available from Hungary, sera of 24 HBsAg-positive patients were collected from different regions of the country. They also served as control samples for the molecular epidemiologic study. Nineteen of them carried genotype D of hepatitis B virus, and five of them carried genotype A. Twenty-nine patients from a haemato-oncology unit were affected in a nosocomial outbreak. The patients had haematological and/or oncological diseases, most of them were immunosuppressed. In twenty-eight cases, based on phylogenetic analysis of the viruses, there was presumably a common source of infection, and an epidemiological investigation showed that the infections seemed to be hospital-acquired. In the intrafamilial outbreak, two asymptomatic carrier children infected their foster mother. The three sequences were totally identical.

Introduction

Hepatitis B virus (HBV) infection is one of the most common chronic infections in the world. More than 400 million people worldwide are chronically infected by hepatitis B virus. More than the half of liver cancer cases are associated with hepatitis B [11]. HBV (family *Hepadnaviridae*, genus *Orthohepadnavirus*) can be transmitted sexually, parenterally, or perinatally. Since donor blood has been screened in Hungary since 1982 for HBV markers, the rate of parenteral transmission has decreased dramatically. HBsAg screening of pregnant women started in Hungary in 1995. Babies of HBsAg-positive mothers receive passive and active immunisation on the day after birth. The proportion of children who become HBsAg positive despite immunisation is below 1%. Vaccination has proved to be an effective way of preventing hepatitis B virus infection. Nowadays, most of the transmission occurs sexually. To prevent sexual transmission of the virus, vaccination of teenagers was begun in 1998.

Author's address: Dr. Mária Takács, Division of Virology, National Center for Epidemiology, Gyáli u. 2-6, 1097 Budapest, Hungary. e-mail: takmar@freemail.hu

Identification of the HBV genotype may be epidemiologically and clinically important [8, 11, 25]. The ability of HBV to induce hepatitis depends on the clinical data of the patient (e.g. age at the time of infection, his/her immune response), and the type of virus strain (e.g. genotype, vaccine escape or therapy-resistant mutants) infecting the patient. Different genotypes of the virus are assumed to be associated with the severity of the disease and response to antiviral therapy [5, 11, 23]. Based on nucleotide sequence differences, hepatitis B virus genomes have been classified into eight genotypes (A–H), and more than 20 subgenotypes [16]. The genotypes show a characteristic geographical distribution [7, 8, 27]. Genotype A is pandemic and divided into three subtypes: A1–A3. A1 is most prevalent in India and in Sub-Saharan Africa, A2 in Northern and Central Europe, and in North America, A3, found in Cameroon, is a recombinant of genotypes A and E [10]. Genotypes B and C are present mainly in the Far East. The four subgroups of genotype B (B1–B4) are prevalent in Indonesia, China and Vietnam, while the four subgroups of genotype C (C1–C4) can be found in Korea, China, Japan, Polynesia, Australia, and Vietnam. Genotype D is also a widespread strain and is predominant in the Mediterranean region, Southern and Eastern Europe, North Africa, and the Middle East, and it is divided into four subgenotypes (D1–D4) [2, 16, 21]. Type E is present mainly in West and Sub-Saharan Africa. F (F1, F2) and the H strains are found among Amerindian natives [1] and in Nicaragua, Venezuela, Mexico and even in California, USA. Genotype G can be found in the USA (Georgia), Mexico and Europe (France, Germany) [19, 26].

As no previous Hungarian data were available, we wanted to determine the dominant HBV genotypes and subgenotypes in the Hungarian population. Our further aim was to investigate the source of infection in a nosocomial and in an intrafamilial outbreak, both of which occurred in Hungary. To clarify intrafamilial HBV transmission, genotyping as well as phylogenetic analysis were performed in a similar study [12].

The amplified PCR products were sequenced, and the genotypes and phylogenetic relatedness of the sequences were determined. In addition, subgenotypes were identified within the genotypes. In previous studies, subgenotype identification for strains from genotypes A [4, 9, 11, 20] and D [2] was carried out for describing their epidemiological distribution.

Materials and methods

Serum samples

Sera of 24 HBsAg-positive individuals from different regions of the country and sera of 33 HBsAg/HBV-DNA-positive patients from the two outbreaks were tested. The 24 samples also served as controls for the epidemiological survey. All samples were stored at $-20\,^{\circ}\mathrm{C}$.

Detection of serological markers of hepatitis B

For the detection of HBsAg, Hepanostika HBsAg Uni-FormII (BIOMERIEUX); for detection of HBc IgG antibodies, Hepanostika anti-HBc Uniform (BIOMERIEUX); for HBc IgM; HBc IgM "capture" ELISA (DiaPro); for detection of HBe Ab/Ag, Hepanostika HBe Microelisa (BIOMERIEUX); for HAV IgG antibodies, the Bioelisa HAV (BIOKIT); for IgG antibodies against HDV, the HDV Ab Elisa (DiaPro); and for anti-HCV IgG, Bioelisa HCV (BIOKIT) Microelisa system were used according to the technical protocols of the manufacturers.

DNA purification

Viral DNA was extracted from $160\,\mu l$ serum by treatment with $4\,\mu l$ $20\,mg/ml$ proteinase K and $395\,\mu l$ proteinase digestion buffer (25 mM EDTA, 0.2 M Tris–HCl pH = 7.5, 0.3 M NaCl, 2% SDS) at $37\,^{\circ}\mathrm{C}$ for one hour, followed by deproteinization with phenol/chloroform. The DNA was precipitated with isopropanol (12 h at $-20\,^{\circ}\mathrm{C}$), washed with ethanol (each step was followed by centrifugation), then resuspended in $8\,\mu l$ RNase/DNase-free, double-distilled, sterile water.

Nested PCR for sequencing

PCR primers (Table 1) located in the surface-protein-coding region of the genome were used as described previously [3, 15]. First-round PCR was carried out in a 50-μl volume using $2\,\mu l$ of DNA and 40 pmol of "outer1" and "outer2" primers. The PCR conditions were $94\,^{\circ}\mathrm{C}$, 3 min; $94\,^{\circ}\mathrm{C}$, 30 sec/$55\,^{\circ}\mathrm{C}$, 30 sec/$72\,^{\circ}\mathrm{C}$, 30 sec, 40 cycles; and a final extension at $72\,^{\circ}\mathrm{C}$, 7 min.

Three microliters of the first PCR product, diluted tenfold, and 25 pmol of "inner1" and "inner2" primers were used for the second PCR. The PCR conditions were $94\,^{\circ}\mathrm{C}$, 3 min;

Table 1. PCR primers used to amplify a segment of the surface protein coding region of HBV

Name	Sequence (5′–3′)	nt position
Outer1	TCA CCA TAT TCT TGG GAA CAA GA	2823–2845
Outer2	CGA ACC ACT GAA CAA ATG GC	704–685
Inner1	AAT CCA GAT TGG GAC TTC AAC C	2965–2986
Inner2	GAG GAC AAA CGG GCA ACA TAC	479–459

94 °C, 30 sec/57 °C, 30 sec/72 °C, 30 sec, 35 cycles; and a final extension at 72 °C, 7 min.

To assess the specificity and sensitivity of the nucleic acid amplification method for detection of HBV, a proficiency panel designed by the Quality Control for Molecular Diagnostics was tested. The sensitivity of the PCR method was less than 200 copies/ml according to the proficiency panel of QCMD.

Sequencing

For direct sequencing, PCR products were purified using a PCR Clean up-M Kit (Viogene). An ABI PRISM 3.1 BigDye Terminator Kit (Perkin Elmer) was used according to the instructions of the manufacturer. Electrophoresis was carried out on an ABI PRISM 3100 Genetic Analyzer.

Phylogenetic analysis

DNA fragments (650 bp) of ORF *preS1*, *preS2* and *S* were used to study the phylogenetic relationship of the hepatitis B virus isolates. The variability of this region allows genotyping and phylogenetic analysis of the sequenced PCR products [24, 25]. Previously published representative sequences of genotypes A–H were included in the analysis to determine the similarity and genotype distribution of our sequences. DNA sequences were aligned using the Clustal W program [22]. A rooted phylogenetic tree was constructed by the neighbor-joining method using the Kimura two-parameter substitution model; to estimate the reliability of the tree topology, a bootstrap analysis of 1000 replicates was performed, using MEGA software (version 3.1) [13].

Hybridization

To confirm the genotype data of nosocomial samples obtained by sequencing, hybridization was performed using an Inno-Lipa HBV (Innogenetics) kit according to the instructions of the manufacturer.

Results

Sera of 24 HBsAg-positive carriers from different parts of the country were taken to identify
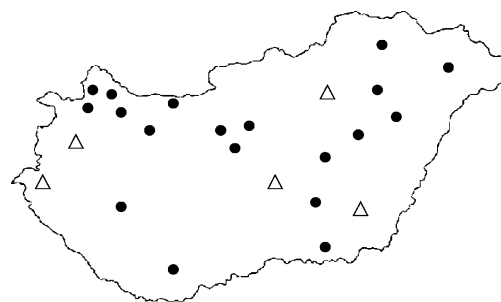

Fig. 1. Geographical distribution of HBV genotypes in Hungary. Dots indicate the origin of genotype D, triangles show the source of genotype A samples

the prevalent genotypes of hepatitis B virus in Hungary (EMBL/GenBank accession numbers: AM040684–AM040705). The origin and the genotypes of those sera are shown on a map of Hungary (Fig. 1). Phylogenetic relatedness of the virus sequences is shown on the rooted phylogenetic tree (Fig. 2).

The nosocomial outbreak occurred in an onco-haematology unit; 29 patients treated there were affected. The unit had 18 beds, with approximately 500 admissions per year. Since 1996, routine testing of serological markers of hepatitis B and C has been done at the first admission of the patients. Most of the patients were admitted to the unit on multiple occasions. The patients' mean age at the presumed time of infection was 11.0 (range 4–20), and 16 (55%) of them were males. The presumed period of infection was between January of 2001 and March of 2003. The patients had primarily haematological and/or oncological malignances, most of them were in an immunsuppressed/immun-deficient state, some of them had received blood and/or blood products during their medication. The blood products were tested for serologic markers of hepatitis B and C viruses. Only three of the 29 HBsAg-positive patients showed symptoms of
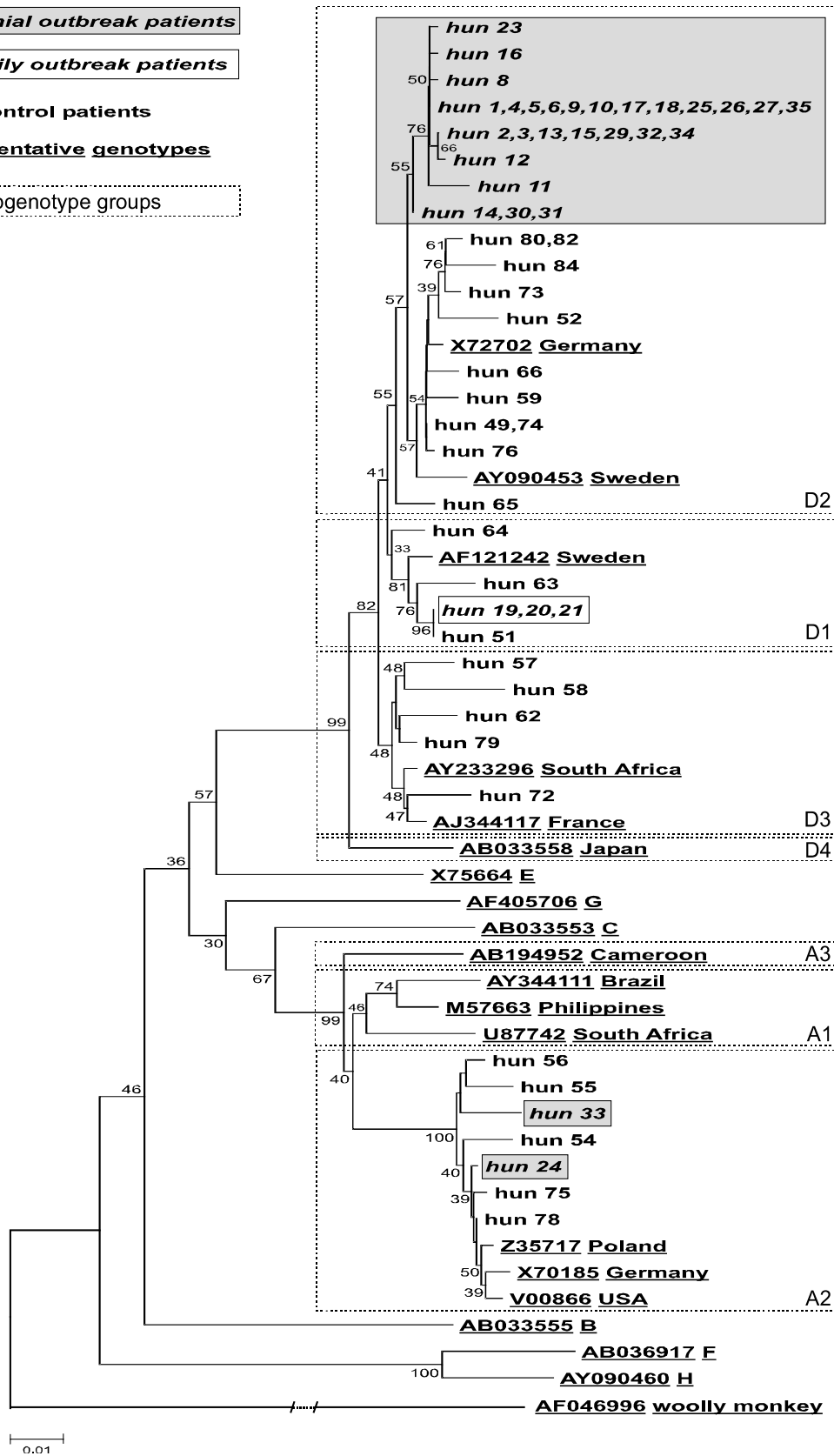
acute hepatitis. Two patients with acute hepatitis died, one of them because of the cancer (astrocytoma malignum), the other because of the hepatitis. The third one has recovered from hepatitis. The other patients remained symptomless, but the information on how many of them became chronic carriers was not available. Epidemiological investigations were carried out after infection was verified. The possible source of nosocomial infections might have been an HBV-positive patient previously treated in the unit. The spread of the infection was likely due to inappropriate infection control measures and the impact of overcrowding of the hospital unit. Simultaneously, the sera of all available patients during that two-and-a-half-year period, health care workers, and administrators were tested by ELISA for hepatitis A, B, C and D serological markers.

The patients had no antibodies against HAV, HCV and HDV. Neither the doctors nor the nurses had positive results for hepatitis viruses, although one of the administrators of the unit was HBsAg positive. Sera obtained from HBV-infected patients and the administrator were also tested for HBV DNA, and all HBsAg-positive samples proved to be positive by PCR. Three of the control patients had negative results for HBsAg by ELISA (presumably because of the presence of immune complexes), but were positive for both HBV DNA and some other HBV markers. The DNA-positive samples were further analysed by sequence and phylogenetic analysis to establish the relationship between isolated viral strains (EMBL/Genbank accession numbers: AM040674–AM040683). In the case of nosocomial samples, Inno-Lipa tests were carried out parallel to

Table 2. Serological findings and PCR results from hospital outbreak patients and control patients selected for the study

	Number of patients	HBsAg	HBeAg	HbeAb	aHBc (total)	aHBc IgM	DNA
Control patients	1	+	+	negative	+	negative	+
	10	+	negative	+	+	+	+
	8	+	negative	+	+	negative	+
	2	negative	negative	+	+	negative	+
	1	negative	N.A.	N.A.	+	negative	+
	1	+	negative	+	N.A.	N.A.	+
	1	+	N.A.	N.A.	N.A.	N.A.	+
Total	24						
Nosocomial outbreak patients (29) + Administrator (1)	10	+	+	negative	+	+	+
	3	+	negative	+	+	negative	+
	1	+	+	negative	N.A.	+	+
	3	+	+	negative	negative	negative	+
	11	+	+	negative	+	negative	+
	1	+	N.A.	N.A.	+	+	+
	1	+	N.A.	N.A.	+	negative	+
Total	30						

N.A. No data were available or not tested.

◄

Fig. 2. Phylogram depicting the phylogenetic relationship of the sequences obtained from the nosocomial and intrafamily outbreaks and from the control samples collected in Hungary. Previously published sequences representing HBV genotypes are included for reference (GenBank accession numbers: *A* – A1 subgenotype: U87742 (South Africa); AY344111 (Brazil), M57663 (Philippines) – A2 subgenotype: Z35717 (Poland), X70185 (Germany), V00866 (USA); – A3 subgenotype: AB194952 (Cameroon) *B*: AB033555; *C*: AB033553; *D* – D1 subgenotype: AF121242 (Sweden) – D2 subgenotype: AY090453 (Sweden), X72702 (Germany), – D3 subgenotype: AJ344117 (France), AY233296 (South Africa); – D4 subgenotype: AB033558 (Japan) *E*: X75664; *F*: AB036917; *G*: AF405706; *H*: AY090460). Percentage bootstrap values greater than 30% are shown at the respective nodes. The scale bar at the bottom indicates the genetic distance

Table 3. Comparison of A2 subgenotype amino acid sequences (including Hungarian isolates) with A1 and A3 subgenotype isolates. Hyphens indicate amino acid identity within the subgenotype groups. Amino acid differences unique to subgenotype A2 are shaded in grey. Neither deletions nor insertions were found in the Hungarian isolates

A	region	preS1						preS2				S											
	aa codon	54	67	74	89	90	91	13	22	36	47	7	8	29	40	45	46	47	49	51	57	68	74
1	AY344111 – Brazil	Q	F	V	P	A	V	Q	F	P	S	G	F	Q	N	S	P	V	L	Q	T	I	W
	M57663 – Philippines	-	-	-	-	-	M	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
	U87742 – South Africa	-	-	-	-	T	A	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Z35717 – Poland	A	L	I	S	T	I	Q	F	P	A	G	F	Q	N	S	P	V	L	Q	T	I	W
	X70185 – Germany	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	V00866 – USA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AM040682 – hun 24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AM040683 – hun 33	-	F	V	-	-	-	-	-	-	-	-	-	-	S	P	L	E	-	L	-	-	L
	AM040701 – hun 54	-	-	-	-	-	-	-	-	-	-	-	L	-	-	P	-	-	P	-	I	-	-
	AM040702 – hun 75	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-
	AM040703 – hun 78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AM040704 – hun 55	-	F	V	-	-	-	-	L	Q	-	R	-	-	-	-	-	-	-	-	-	-	N	-
	AM040705 – hun 56	-	F	V	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	AB194952 – Cameroon	Q	F	V	P	A	V	L	F	P	S	G	F	Q	N	A	P	V	L	Q	T	I	W

sequencing, confirming the results. The varying serological profiles of control and nosocomial outbreak patients are shown in Table 2.

As a result of the epidemiological investigations, strict infection control measures were introduced to prevent new infections, the number of beds in the unit was reduced to 16, and one of the wards was converted into a treatment room. As a consequence of these arrangements, new infections have not occurred since March of 2003.

The other outbreak occurred in a family, affecting two children and their foster mother. The children presumably had been infected perinatally by their natural mother, who had been known to be a symptomless virus carrier for years. The children became carriers in spite of passive and active immunization. The foster mother showed symptoms of acute hepatitis a few months after adopting the children. The results of the serological tests confirmed a recent infection with hepatitis B virus. To confirm the source of the virus infecting the mother, sequence analysis was performed. (EMBL/GenBank accession number: AM040693).

The nucleotide sequences of Hungarian isolates belonging to the A genotype group were translated to amino acid sequences and compared to the representative amino acid sequences. Several point mutations – both silent and missense – were found, but neither deletions nor insertions could be detect-

ed in the Hungarian isolates. The missense mutations are shown in Table 3.

Discussion

Nested PCR and sequencing methods were used to characterize the hepatitis B viruses circulating in Hungary. The second-round PCR primers were designed to include the variable part of the genome, which is suitable for genotyping and subtyping the viruses.

The viruses of Hungarian carriers and patients from the two outbreaks could be clustered into two main groups, which were genotypes A and D. All of the sequences derived from control patients suffering from hepatitis were found to be different from each other, indicating the genetic heterogeneity of the viruses circulating in Hungary.

Twenty-eight of the 30 sequences obtained from the nosocomial outbreak (hun1–6, 8–18, 22, 23, 25–27, 29–32, 34, 35) were clustered into genotype D. The viruses in this group were genetically very close to each other. This suggests that the source of infection was common in this group. The possible route of infection could not be determined. The viruses of two carriers (hun24: a patient, and hun33: the administrator) proved to be genotype A. The hun24 patient had probably been infected perinatally. The administrator was a symp-

tomless carrier, but as an office worker, she had no contact with the patients. Based on the phylogenetic distance of the hun 24 and hun 33 sequences, a common source of infection of these patients could be excluded.

The viruses isolated from the three persons belonging to the same family (hun19–21) were genetically completely identical in the sequenced region. The sequences could be clustered into genotype D. The source of the infection of the foster mother was probably one of the children. Interestingly, one of the control patients carried almost the same virus sequence, although this patient had never met the family. HBV is not a highly variable virus, but the region sequenced in this study is the most variable part of the genome. However, there is a possibility that two independent patients have a very similar virus sequence. All genotyped Hungarian hepatitis B isolates belonged to either group A or D. These two types seem to be predominant in Hungary.

As other studies suggest [9, 10, 16, 20], genotype A subgenotypes (A1, A2 and A3) show a geographically characteristic spread. All seven Hungarian sequences belonging to the A genotype group (hun24, 33 samples from hospital and hun54, 55, 56, 75, 78, control samples) were clustered into the A2 subgenotype group, which corresponds to its expected geographical distribution. Comparison of representative A1 and A2 subgenotype amino acid sequences with translated Hungarian isolates was performed. Amino acid positions in the sequenced segment of the genome that distinguish A2 from A1 subgenotype are as follows: Ala[54], Ser[89], Thr[90] (the A1 subgenotype U87742 South African isolate has Thr at this position, instead of Ala), Ile[91] in the preS1 and Ala[47] in the preS2 region [9]. All of the amino acid residues typical for subgenotype A2 were identical in the Hungarian isolates and the representative sequences (Table 3).

Similarly, Hungarian D genotype isolate sequences belonging to the D genotype group are delineated on a tree together with other type D (D1–D4 subgenotype) sequences from all over the world. Hungarian strains clustered into subgenotypes D1–D3, but they did not show a characteristic distribution as subgenotypes A1–A3 (Fig. 2).

Our genotyping results correspond well to data published previously from other countries such as Latvia [18], Estonia [21], Spain [4, 6], Turkey [2], Belgium [14], Japan, USA [20], South Africa [9], and the island of Gran Canaria [17]. Our phylogenetic analysis shows that comparing the nucleotide sequence of the pre-S/S region is an adequate method for the molecular epidemiological investigation of nosocomial outbreaks.

Acknowlegdements

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