ORIGINAL ARTICLE 10.1111/j.1469-0691.2004.00871.x

Acute hepatitis B virus infection in Turkey: epidemiology and genotype distribution

H. Leblebicioglu, C. Eroglu and members of the Hepatitis Study Group

Department of Infectious Diseases and Clinical Microbiology, Ondokuz Mayis University Medical School, Samsun, Turkey

ABSTRACT

The aim of this study was to investigate the prevalence of hepatitis B virus (HBV) genotypes in Turkey. Epidemiological and clinical data for 158 patients with acute HBV infection from 22 medical centres in the period February 2001 to February 2002 were collected prospectively. HBV genotyping was based on analysis of restriction fragment length polymorphisms and nested PCR. There were 59 female and 99 male patients, with a mean age of 34.2 ± 15.6 years. The most common probable transmission route was blood contact in 63 (41.1%) cases, but was unknown in 78 (49.4%) cases. The mean alanine aminotransferase level was 1718 ± 1089 IU/L. Four of the 158 patients (2.5%) died because of fulminant hepatitis. One year after discharge, 11 (10.6%) of 103 cases were positive for hepatitis B surface antigen (HBsAg) and 80 (77.7%) were positive for anti-HBsAg. Genotype determination was unsuccessful in 11 cases because of a negative PCR; genotype D was found in the remaining 147 cases. The results suggested that acute HBV infection constitutes a significant health problem in Turkey and that genotype D is predominant.

Keywords Acute infection, genotype, hepatitis B virus, hepatitis D virus, viral hepatitis

Original Submission: 5 May 2003; **Revised Submission:** 25 July 2003; **Accepted:** 20 August 2003

Clin Microbiol Infect 2004; 10: 537–541

INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem, with an estimated 400 million being chronic carriers of the virus [1]. HBV is associated with acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [2]. HBV strains were classified originally into six different genotypes, namely HBV genotypes A–F [3], but two new genotypes, G and H, have been reported recently [4,5]. Previous studies have shown characteristic geographical distributions of different HBV genotypes [6–8]; however, few data are available regarding the distribution of HBV genotypes in acute hepatitis B patients [9], particularly in the Turkish population. The aim of this study was, therefore, to investigate the prevalence of HBV genotypes in Turkey, as well as the epidemiology, clinical course and outcome for patients with acute HBV infections.

Corresponding author and reprint requests: H. Leblebicioglu, Department of Infectious Diseases and Clinical Microbiology, Ondokuz Mayis University Medical School, Samsun, Turkey
E-mail: hakanomu@omu.edu.tr

MATERIALS AND METHODS

Patients

Consecutive adult patients ($n = 158$) with acute HBV infection who were admitted to 22 medical centres in Turkey between February 2001 and February 2002 were included prospectively in the study. The medical centres were representative of all the geographical regions of the country. Acute HBV infection was defined by the presence of positive hepatitis B surface antigen (HBsAg) and anti-Hepatitis B core (HBc) IgM in patients with an acute onset of alanine aminotransferase (ALT) elevation (more than twice the normal upper limit; see below). None of the patients had a previous history of liver disease, or reported any other causes of liver injury (e.g., alcohol-, metabolite- or drug-induced injury) or heart failure. Epidemiological and clinical data were obtained prospectively during the first week after the onset of viral hepatitis (initial visit) and after 6 and 12 months. The possible transmission route of HBV, with regard to blood contact, sexual, or horizontal transfer (e.g., the presence of HBV in close contacts), was investigated at the initial examination. If the probable method of transmission could not be established, it was recorded as unknown.

Biochemistry and serology

Liver biochemical tests, including albumin (normal range, 3.5–5 g/dL), ALT (7–46 IU/L), aspartate aminotransferase (8–46 IU/L), alkaline phosphatase (95–280 IU/L), gamma-glutamyltranspeptidase (7–49 IU/L) and total bilirubin (0.1–1.5 mg/dL) levels, were done for all patients at initial examination and during the follow-up at each institution. Serological markers for hepatitis viruses were tested at each institution during first evaluation with commercially available kits (Axsym; Abbott Laboratories, North Chicago, IL, USA) for HBsAg, Hepatitis B early antigen (HBeAg), anti-HBeAg, anti-HBc IgM, anti-HBc IgG, anti-HBsAg, anti-hepatitis A virus (anti-HAV) (IgM and IgG), anti-hepatitis C virus (anti-HCV), and anti-hepatitis D virus (anti-HDV) (IgM + IgG).

Genotyping of HBV

Serum samples from each subject were taken at first examination and stored at −70 °C until they were studied. HBV DNA was isolated with a High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. In brief, 200 μL of serum were used for DNA extraction, and 50 μL of elution buffer were used to elute the DNA. An aliquot (5 μL) of HBV DNA eluate was then added to 45 μL of reaction mix (containing 200 mM of each nucleotide triphosphate, 0.4 mM of primers HBV-1 and HBV-2 [10] (Table 1), 1.5 mM $MgCl_2$, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, and 1.25 U *Taq* polymerase). PCR amplification comprised 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. PCR amplicons were separated by electrophoresis on an agarose 1.5% w/v gel, stained with ethidium bromide, and visualised under UV light. Standard procedures for reducing contamination were followed strictly.

The six major genotypes of HBV, i.e., A, B, C, D, E and F, were recognised by analysis of the restriction fragment length polymorphisms (RFLPs) created by digestion with *Ava*II and *Dpn*II of an amplified segment of the pre-S region as described previously [10]. If genotype determination was not successful by this method, a second method [11] was used in which the HBV genome was amplified by nested PCR, first with the universal primers HBV-1 and S1-2, followed by amplification with mixtures containing type-specific inner primers (BD1: type D-specific sense; BE1: type E-specific sense; BF1: type F-specific sense; B2R: types D- to F-specific antisense). PCR products were separated by electrophoresis on an agarose 3% w/v gel, stained with ethidium bromide, and visualised under UV light. HBV genotypes were determined by identifying the genotype-specific DNA bands (*c*. 119 bp, 167 bp and 97 bp for genotypes D, E and F, respectively). This method did not allow subtypes of genotype D to be identified.

Data in the text and tables are expressed as means ± standard deviation (SD). Differences between groups were examined by χ^2 and Fisher's exact test. A p value of < 0.05 was considered to be significant.

Table 1. Primer sequences used for HBV genotyping by PCR-RFLP and nested PCR

HBV-1	5′-TCACCATATTCTTGGGAACAAGA-3′ (nt 2823–2845, sense)
HBV-2	5′-TTCCTGAACTGGAGCCACCA-3′ (nt 80–61, antisense)
S1-2	5′-CGAACCACTGAACAAATGGC-3′ (nt 685–704, antisense)
BD1	5′-GCCAACAAGGTAGGAGCT-3′ (nt 2979–2996, type D-specific, sense)
BE1	5′-CACCAGAAATCCAGATTGGGACCA-3′ (nt 2955–2978, type E-specific, sense)
BF1	5′-GYTACGGTCCAGGGTTACCA-3′ (nt 3032–3051, type F-specific, sense)
B2R	5′-GGAGGCGGATYTGCTGGCAA-3′ (nt 3078–3097, types D- to F-specific, antisense)

Y represents a nucleotide that could be either a C or a T.
nt, nucleotide.

RESULTS

Of 158 patients with acute HBV infection, 59 were females and 99 were males (female/male = 0.59) with a mean age of 34.2 ± 15.6 years (Table 2). All patients were native residents of Turkey. Although the HBV transmission route remained unidentified in 49.4% of the patients, the most common probable transmission route was blood contact (41.1%), following recent surgery, dental treatment, tooth extraction or body piercing, followed by heterosexual transmission (Table 2). Neither homosexual transmission nor a history of intravenous drug use was documented for any patient. The vast majority (154/158) of patients had symptomatic and icteric infection. All patients had elevated ALT levels (i.e., more than twice the normal upper limit). The mean ALT, aspartate aminotransferase and total bilirubin levels were 1718 ± 1089 IU/L, 1266 ± 806 IU/L and 11.3 ± 7.8 mg/dL, respectively (Table 2).

Of the 158 patients, 74 were positive for HBeAg and 60 were positive for anti-HBeAg on first examination. One (0.6%) patient was serologically positive for HDV, and one patient (0.6%) had HCV infection. Of 109 patients investigated, 102 (93.6%) were positive for anti-HAV IgG.

Four (2.5%) of the patients died because of fulminant hepatitis. Six months after the initial evaluation, 103 patients came for re-examination; of these, only patients who were positive for anti-HBc IgG, with or without HBsAg ($n = 31$), were invited for follow-up after a further 6 months. Overall, HBsAg, isolated anti-HBc IgG, and anti-HBs positive rates were 10.6%, 11.7% and 77.7%, respectively, in control patients. There was no significant difference in the spontaneous HBsAg clearance rate according to the HBeAg status (HBeAg-positive/anti-HBe-negative) determined at the first diagnosis of HBV infection (90.2% vs. 83.3%). All HBsAg carriers, except one, had normal ALT values at final follow-up.

Genotype determination was unsuccessful in 11 cases because of a negative PCR; therefore, the HBV genotypes were investigated for

Table 2. Characteristics of patients with acute HBV infection

Characteristics	Patients		
	Total $n = 158$	**HBeAg-positive** $n = 74$	**Anti-HBeAg-negative** $n = 60$
Age (years)	34.2 ± 15.6	33.9 ± 15.4	32.9 ± 15.4
Female/male	59:99 (0.59)	29:45 (0.64)	19:41 (0.46)
Probable transmission route			
Unknown	78 (49.4%)	36 (48.7%)	32 (53.3%)
Blood contact	65 (41.1%)	28 (37.8%)	25 (41.7%)
Heterosexual	13 (8.2%)	9 (12.2%)	2 (3.3%)
Horizontal	2 (1.3%)	1 (1.3%)	1 (1.7%)
ALT (IU/L)	1718 ± 1089	1772.7 ± 1082.2	1681.8 ± 113.4
AST (IU/L)	1266 ± 806	1337 ± 798.3	1171.3 ± 751.7
Total bilirubin (mg/dL)	11.3 ± 7.8	11.3 ± 7.7	10.6 ± 7.7
Died	4	2	2

There was no significant difference for all parameters between HBeAg-positive and anti-HBeAg-negative patients ($p > 0.05$).
ALT, alanine aminotransferase; AST, aspartate aminotransferase.

147 patients. Genotype D was recognised in 129 serum samples on the basis of RFLP patterns. There was no amplicon in 13 samples, while five samples yielded inconclusive RFLP patterns. Fig. 1 shows the RFLP patterns observed following analysis of the pre-S region of HBV from serum samples. Subtype D2 was found in 122 (94.6%) of 129 patients with genotype D, with subtype D1 in five (3.9%) and D2 + deletion in two (1.5%) cases. For the remaining 18 patients, the genotypes were determined by nested PCR, with genotype D being found in all cases.

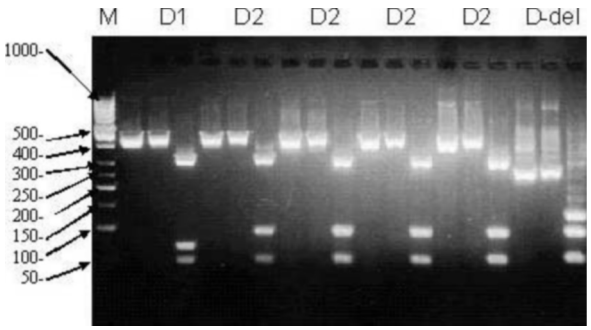

Fig. 1. Examples of RFLP patterns obtained by analysis of the pre-S region of HBV from serum samples.

DISCUSSION

The present study showed that blood exposure is still the major means of acquiring HBV infection in Turkey. Heterosexual contact was the second most important transmission route (8.2% of patients), but this percentage was below the reported rates for other countries in Europe [9]. It is possible that sexual transmission was underreported because of social pressure. Intravenous drug use is an important risk factor for transmission of HBV and HCV, but no drug use was identified in the present study. Acute HBV infection is generally a self-limited disease, but four patients in the study had fulminant hepatitis characterised by encephalopathy and coagulopathy, accompanied by deterioration in liver function, including elevated bilirubin levels. All four of these patients died because of complications. The overall mortality rate was 2.5%.

In Turkey, an area of intermediate endemicity, the prevalence of HBV is highly variable in different geographical regions within the country, but HBV carriage is estimated at *c.* 6% in western parts of Turkey, rising to 12.5–14.3% in eastern and southeastern Turkey. High rates of HBsAg at 6 and 12 months after acute infection were detected in this study, possibly reflecting this geographical difference. However, the number of follow-up patients was small. Discharged patients who feel well do not usually present for follow-up, which may affect the apparent carriage rate of HBsAg. These patients should be monitored, as the spontaneous clearance of HBsAg can be seen later. There was no significant difference in the spontaneous HBsAg clearance rate according to HBeAg status (HBeAg-positive/HBeAg-negative) determined at the first diagnosis of HBV infection.

This study also demonstrated some characteristics of infections with other hepatitis viruses, such as HAV, HCV and HDV. Most of the adult patients were immune to HAV infection. In early studies, the prevalence of HDV infection was reported as 23.9% in chronic HBV carriers, and the delta coinfection was detected in 20 (8.4%) of 237 patients [12], but in the present study, the rate of delta coinfection was only 0.6%. In Turkey, the

incidence of delta hepatitis is decreasing, especially in the western parts of the country, but it is common in eastern and southeastern Turkey [13]. Interestingly, patients with delta coinfection lived in eastern parts of Turkey. The prevalence of chronic HCV infection was reported to be 1.5% in Turkey in 1994 [14], but was found to be 0.6% in the present study; the difference may be associated with regular screening of blood donations since 1996, or may reflect the small size of the study population used to determine HCV prevalence.

Eight genotypes (A–H) of HBV have now been described [3–5]. In brief, genotypes B and C are prevalent in Asia and the Far East, while genotype A is prevalent in northwestern Europe, North America and Africa. Genotype D predominates in the Mediterranean area and India [15], while genotype E circulates in sub-Saharan Africa [8]. Genotype F is found in Central and South America. Genotype G has been reported from France and North America [5]. Genotype H has been described only recently, and its distribution is not yet understood [4]. The distribution of HBV genotypes in patients with acute HBV infection in Turkey has not been reported previously, but in this study, the most common genotype was genotype D, in agreement with previous data derived from patients with chronic HBV infection in the same geographical region [6,8,15].

Previous studies have reported a correlation between HBV genotypes and HBeAg clearance [16–18], cirrhosis [18,19], hepatocellular carcinoma [18–21], and response to antiviral therapy [22–24]. Mayerat *et al.* [25] found that genotype A was more common among patients with chronic HBV infection, whereas genotype D was more prevalent among patients with resolving acute HBV infection, suggesting that HBV genotype D was associated with a lower rate of chronic HBV infection; however, Thakur *et al.* [26] concluded that genotype D was associated with more severe liver disease and may predict the occurrence of hepatocellular carcinoma in young Indian patients. Erhardt *et al.* [23] reported that the rate of interferon-induced HBeAg seroconversion was lower among patients with genotype D than among those with genotype A (6% vs. 37%). In the present study, the relationship between HBV genotypes and the outcome of acute HBV infection was inconclusive because only genotype D was detected.

The present study used RFLP analysis to classify HBV genotypes. RFLP is a simple method that can be applied easily in clinical diagnostic laboratories. In 18 serum samples, the genotype could not be detected by PCR-RFLP analysis, but could be determined by nested PCR, reflecting the fact that nested PCR is *c.* 1000-fold more sensitive than PCR-RFLP. There was no detectable relationship between subtypes and the outcome of HBV infection. However, it is clear that acute HBV infection constitutes a significant health problem in Turkey. The Turkish goverment implemented a universal HBV immunisation programme in 2000 [27], and it is hoped that routine vaccination may protect adults from acute HBV infection.

HEPATITIS STUDY GROUP

E. Aribas (Konya); F. Sirmatel (Gaziantep); C. Ayaz (Diyarbakir); M. F. Ozsoy, S. Cavuslu (Istanbul); R. Turkyilmaz, E. Tutuncu (Ankara); M. Sunbul, S. Esen, D. Turan (Samsun); A. Akbulut, M. Cihangiroglu (Elazig); B. Aygen, O. Yildiz (Kayseri); G. Usluer, E. D. Kartal (Eskisehir); H. Irmak, O. Evirgen (Van); N. Tulek, M. A. Yetkin (Ankara); I Dokmetas, M. Bakir (Sivas); O. Ozgenc (Izmir); M. Parlak, E. Parlak (Erzurum); I. Koksal, R. Caylan (Trabzon); I. Sencan (Duzce); T. Yamazhan, S. Ulusoy (Izmir); Z. Akcam, G. Yayli (Isparta); G. Ersoz, A. Kaya (Mersin); N. Saltoglu, Y. Tasova (Adana); Y. Ersoy (Malatya); D. Kilic, S. Kaygusuz (Kirikkale).

ACKNOWLEDGEMENTS

This work was supported by a grant from Roche Pharmaceuticals, Turkey. This study was presented in part at the 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, USA.

REFERENCES

1. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733–1745.
2. Chen DS. From hepatitis to hepatoma: lessons from type B viral hepatitis. *Science* 1993; **262**: 369–370.
3. Okamoto H, Tsuda F, Sakugawa H *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; **69**: 2575–2583.
4. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; **83**: 2059–2073.
5. Stuyver L, De Gendt S, Van Geyt C *et al.* A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; **81**: 67–74.
6. Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. *Hepatology* 2002; **35**: 1274–1276.
7. Norder H, Hammas B, Lee SD *et al.* Genetic relatedness of hepatitis B viral strains of diverse geographical origin and

natural variations in the primary structure of the surface antigen. *J Gen Virol* 1993; **74**: 1341–1348.
8. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus—large-scale analysis using a new genotyping method. *J Infect Dis* 1997; **175**: 1285–1293.
9. Lindh M, Horal P, Norkrans G. Acute hepatitis B in Western Sweden—genotypes and transmission routes. *Infection* 2000; **28**: 161–163.
10. Lindh M, Gonzalez JE, Norkrans G, Horal P. Genotyping of hepatitis B virus by restriction pattern analysis of a pre-S amplicon. *J Virol Meth* 1998; **72**: 163–174.
11. Naito H, Hayashi S, Abe K. Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. *J Clin Microbiol* 2001; **39**: 362–364.
12. Balik I, Onul M, Tekeli E, Caredda F. Epidemiology and clinical outcome of hepatitis D virus infection in Turkey. *Eur J Epidemiol* 1991; **7**: 48–54.
13. Yurdaydin C, Bozkaya H, Gurel S *et al.* Famciclovir treatment of chronic delta hepatitis. *J Hepatol* 2002; **37**: 266–271.
14. Thomas DL, Mahley RW, Badur S, Palaoglu E, Quinn TC. The epidemiology of hepatitis C in Turkey. *Infection* 1994; **22**: 411–414.
15. Kao JH. Hepatitis B viral genotypes: clinical relevance and molecular characteristics. *J Gastroenterol Hepatol* 2002; **17**: 643–650.
16. Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. *J Infect Dis* 1999; **179**: 775–782.
17. Orito E, Mizokami M, Sakugawa H *et al.* A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001; **33**: 218–223.
18. Ding X, Mizokami M, Yao G *et al.* Hepatitis B virus genotype distribution among chronic hepatitis B virus carriers in Shanghai, China. *Intervirology* 2001; **44**: 43–47.
19. Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000; **118**: 554–559.
20. Fujie H, Moriya K, Shintani Y *et al.* Hepatitis B virus genotypes and hepatocellular carcinoma in Japan. *Gastroenterology* 2001; **120**: 1564–1565.
21. Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34**: 590–594.
22. Kao JH, Wu NH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes and the response to interferon therapy. *J Hepatol* 2000; **33**: 998–1002.
23. Erhardt A, Reineke U, Blondin D *et al.* Mutations of the core promoter and response to interferon treatment in chronic replicative hepatitis B. *Hepatology* 2000; **31**: 716–725.
24. Buti M, Cotrina M, Valdes A *et al.* Is hepatitis B virus subtype testing useful in predicting virological response and resistance to lamivudine? *J Hepatol* 2002; **36**: 445–446.
25. Mayerat C, Mantegani A, Frei PC. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepatol* 1999; **6**: 299–304.
26. Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *J Gastroenterol Hepatol* 2002; **17**: 165–170.
27. Van Damme P. Hepatitis B: vaccination programmes in Europe—an update. *Vaccine* 2001; **19**: 2375–2379.