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Epidemiological Study of Genotypes of Hepatitis B Virus in Northern Portugal

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While the overall prevalence of hepatitis B virus (HBV) infection in Portugal is around 1%, there are no published studies examining HBV genotypes in this country. This study aimed to survey HBV genotypes in the northern Portugal and to examine the possible associations between genotypes and gender, viral transmission routes, viral markers, viral load, and biochemical tests of liver function. The study sample consists of 340 patients with HBV infection of whom 42.9% were women. Tests were carried out for HBV genotypes and biochemical liver function while demographic information, including alcohol intake, was obtained from the patient files. The results indicate the predominance of genotype D (60.3%) and genotype A (31.5%). Intrafamilial transmission was predominant in female patients, while males were infected in equal proportions by perinatal, sexual, and intrafamilial transmission. Absence of HBeAg was found in a significantly smaller proportion of female patients with genotype D as compared to A (56.6% vs. 82.1%, $P = 0.028$). High viral load was associated significantly and independently with genotype D and HBeAg. Both alanine and aspartate aminotransferases (ALT and AST) were associated with gender and HBeAg. Thus, genotypes A and D were found to be the most prevalent in the north of Portugal. Patients infected with genotype D had higher levels of HBV DNA. HBeAg was associated with genotype D, viral load, and ALT and AST. ***J. Med. Virol. 81:1170–1176, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; genotypes; virological markers; viral load; liver enzymes; northern Portugal

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major health problem, with approximately 350–400 million virus carriers worldwide [Alter, 2003]. At present, there is no information about the prevalence of HBV infection in Portugal. According to the most representative research in this field, patients infected chronically with HBV represent approximately 1% of the Portuguese population [Lecour et al., 1984; Santos et al., 2000].

Seven HBV genotypes (A–G) have been identified, with a worldwide geographical distribution [Kao, 2002; Norder et al., 2004; Halfon et al., 2006]. In addition, genotype H was described recently in American Indian patients [Arauz-Ruiz et al., 2002; Halfon et al., 2006]. In Europe, genotypes A and D are reported most frequently. Genotype classification is based on an inter-group divergence of 8% or more in the nucleotide sequence [Okamoto et al., 1988]. In addition to genotypes, stable HBV mutant strains displaying changes in the precore, core, surface, and polymerase gene sequences are found frequently, and mutants unable to achieve synthesis of HBeAg are particularly common [Avellon and Echevarria, 2006].

It was suggested that HBV genotypes may influence the clinical outcome of HBV infection [Kao et al., 2000]. Although the relationship between HBV genotype and pathogenicity remains largely unknown [Bahri et al.,

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2006], current research indicates that an increase in the viral load is associated with liver injury, independent of serum alanine aminotransferase (ALT) levels or the presence of the HBeAg in the serum [Chen et al., 2007b; Wu et al., 2007].

Alcohol consumption in Portugal is 15% higher than in the rest of Europe [WHO, 2008b]. It is estimated that there are approximately 1 million alcoholics or excessive alcohol drinkers in Portugal [WHO, 2008b]. In 2003, a WHO survey showed an annual pure alcohol consumption of 9.49 L per capita [WHO, 2008a]. While this study does not suggest an association between alcohol consumption and severity of liver disease, it may be an important confounding factor for the incidence of hepatitis B.

The north of Portugal is populated by approximately 3.7–4 million inhabitants, with a slight predominance of females (51.62%), making up one-third of the country's total population. The region is characterized by an industrialized, and heavily populated western Atlantic coast, and a rural and sparsely populated interior plateau. In the north of the country, around 30% of the population is less than 25 years old, making this region one of the youngest in Portugal [INE, 2008]. At present, 4% of the population of Portugal consists of immigrants from Africa, Brazil, and Eastern Europe, although the north of the country has relatively fewer immigrants than the country as a whole [MAI, 2008].

The aim of this study was to assess the characteristics and distribution of HBV genotypes in the northern region of Portugal, and to identify the association between HBV genotypes linked with patient demographics, including alcohol intake and viral transmission routes. Viral markers for HBV, HCV (hepatitis C virus), and HIV (human immunodeficiency virus) were also considered, as was alcohol intake.

MATERIALS AND METHODS

Patients

The study population was composed of patients with HBV infection, documented by the presence of the HBsAg in the serum for at least 6 months. These patients were observed in the two hospitals in the city of Oporto: the Hospital de Santo António (HSA), where there is a hepatitis clinic, and the Hospital Joaquim Urbano (HJU), specializing in infectious diseases. Along with the Hospital de São João, these hospitals cover the majority of the patients from the north of Portugal.

The demographics, clinical, and medical history of the patients were retrieved from medical files. Demographic characteristics include age, gender, place of birth and residence, marital status, and alcohol consumption (categorized as less than or greater than 20 g/day). The presumed sources of HBV infection were also identified, with information provided by physicians, based on the clinical history and responses to a questionnaire. Four categories were considered: (1) perinatal transmission, (2) sexual intercourse, (3) intrafamilial sources of infection, and (4) other sources, including at least one incidence of intravenous drug use, blood transfusion, occupational exposure, iatrogenic exposure (e.g., endoscopy or coloscopy examination, history of surgery, acupuncture, or hemodialysis), or unknown. Sexual transmission was recorded for patients with a sexual partner infected with HBV. Intrafamilial transmission was recorded when all other routes of transmission were excluded.

Detection of Viral Markers in Serum

Serum samples were tested for HBsAg, antibody to HBsAg (anti-HBs), antibody to HBV core antigen (anti-HBc), HBeAg, and antibody to HBeAg (anti-HBe). Tests for co-infection by HCV and HIV types 1 and 2 were also performed. All tests were carried out by automated chemiluminiscent assays (Vitros ECi, Ortho-Clinical Diagnostics, Amersham, Buckinghamshire, UK).

HBV DNA Quantitation

HBV DNA levels were determined using the VERSANT® HBV DNA 3.0 assay (bDNA) (Bayer, Tarrytown, New York), a signal amplification nucleic acid probe assay for direct DNA quantitation. The assay quantification range is 357–17,900,000 HBV DNA IU/ml.

HBV Genotyping

HBV genotypes were identified by the TRUGENE® HBV genotyping kit. When used in conjunction with the Open Gene® DNA Sequencing System (Bayer), this kit provides a method for obtaining bi-directional sequences from overlapping surface antigens and domains B through E of the HBV genome reverse transcriptase region. The HBV genotype was identified automatically by comparing the sequence with a library of DNA sequences from the known HBV genotypes.

Biochemical Tests of Liver Function

ALT and AST (aspartate aminotransferase) were determined by enzyme tests without piridoxalphosphate. γ-glutamil transpeptidase (GGT) was determined by an enzyme test with γ-glutamil phosphatase. All tests were performed using the Cobas Integra 800 (Roche, Mannheim, Germany). Levels were considered above the normal range for results higher than 36, 30, and 66 U/L, respectively.

Statistical Analysis

Statistical analysis was performed using SPSS (version 16.0) and STATA (version 9.0) software with two-sided significance set at 5% throughout.

Qualitative variables were described as percentages and quantitative variables as mean, median, or geometric mean where necessary.

Proportions were compared using the Pearson χ^2 test (with continuity correction) as appropriate, or by Fisher's exact test. Continuous variables were compared with the

t-test, after log transformation so that assumptions were met, if necessary.

Statistical analysis of binary outcomes was performed using multivariable logistic regression analysis. Statistical analysis of viral load (a continuous variable) was performed using multiple linear regression analysis, to see which variables were associated with the outcome. Viral load was converted to a natural logarithm to normalize its skewed distribution of residuals and reduce the influence of outlying high values on regression coefficients, and adequately specify the relation of the considered outcome and independent variables. As a first step, factors found to be associated significantly at $P < 0.15$ in univariable regression were considered in a multiple regression analysis. In the second stage, a stepwise approach was applied. Both forward and backward selection methods were used to obtain the smallest number of explanatory variables to provide a well-identified model. The continuous variable modeling was tested by the fractional polynomial method, a procedure that make use of the full information available in the data when a linear relationship is not assumed [Royston and Altman, 1994]. Fractional polynomial modeling confirmed that the association with age was linear. Specific interactions between parameters of interest were also investigated, but none of these were significant. The Hosmer–Lemeshow goodness of fit test was used to check for lack of fit of the final logistic model. The determination coefficient was assessed for the final linear model.

RESULTS

The distribution of HBV genotypes among the 340 patients studied is shown in Table I. Genotypes D (60.3%) and A (31.5%) were predominant, and genotypes C, E, and F were found in minor proportions. Characteristics of patients infected by HBV strains from genotypes A and D are summarized in Table II.

Virological markers found among these latter patients are shown in Table III. Two patients infected by genotype D were negative for anti-HBc, and one of these was positive for HBeAg. Among female patients, absence of HBeAg was significantly more common in patients infected by HBV genotype A strains.

Co-infection with HCV was observed in 16 (5.5%) individuals, co-infection with HIV 1 and 2 was only found in males, three of whom were infected by genotype A and two additional patients with genotype D.

Determination of HBV DNA levels showed that the proportion of undetectable DNA values (as defined in the Materials and Methods Section) was similar for genotypes A and D (8.4% vs. 7.3%). However, the proportion of patients with values higher than the upper limit of the assay (17,900,000 IU/ml) was smaller in genotype A than genotype D (9.3% vs. 22%). When the viral load was examined for values between 357 and 17,900,000, a significantly higher level ($P < 0.001$) of viral load was observed in genotype D (geometric mean 391,954.1; 95% CI 238,828.3–643,321.6) as compared to patients with genotype A (geometric mean 95,463.6; 95% CI 54,268.54–167,929.6).

Only gender, HBV genotypes and HBeAg were found to be associated significantly with HBV DNA levels in the univariable analysis: females, patients with genotype D and HBeAg had a higher viral load (Table IV). Multiple regression analysis showed that the viral load was associated significantly with genotype D and HBeAg, with gender being excluded from the equation.

Biochemical tests of liver function (Table V) were performed at the same time as genotyping. In order to investigate these parameters, the percentage of upper reference values was calculated, and the median and the range were estimated only for those values.

ALT was elevated in a smaller proportion of female patients with genotype A (27%), as compared to those with genotype D (40.7%). However, in male patients elevated ALT levels were observed in a similar proportion in the two genotypes, but in much higher proportion than in females (around 60%).

The proportion of elevated values of AST in female patients was the same as for ALT in both genotypes; however, they did not correspond to the same patients. Again, a larger proportion of male patients with increased AST values were observed.

Elevated levels of GGT seem to be higher in patients with genotype A than in genotype D, regardless of gender.

In order to identify any association between increased levels of ALT and gender, age, genotype, HBeAg, and the presumed source of HBV infection, a univariable analysis by logistic regression was carried out (Table VI).

TABLE I. Genotypes Distribution and Origin

	Portuguese		Non-Portuguese		
Genotype	n	%	n	%	Origin
A	103	32.1	4	21.1	Two from Central Europe and two from Africa
C	0	0.0	2	10.5	China
D	201	62.6	4	21.1	Two from Oriental Europe and two from Africa
E	3	0.9	9	47.4	Africa
F	13	4.0	0	0.0	
D and F	1	0.3	0	0.0	
Total	321	100	19	100	

TABLE II. Demographic Characteristics of Individuals With Genotypes A and D*

	Genotype A (n = 107)		Genotype D (n = 205)	
	n	%	n	%
The presumed source of HBV infection				
Perinatal transmission				
Female	9/38	23.7	18/92	19.6
Male	13/69	18.8	15/113	13.3
Sexual transmission				
Female	5/38	13.2	14/92	15.2
Male	13/69	18.8	19/113	16.8
Intrafamilial transmission				
Female	15/38	39.5	37/92	40.2
Male	13/69	18.8	27/113	23.9
Others[a]				
Female	9/38	23.7	23/92	25.0
Male	30/69	43.5	52/113	46.0
Alcohol intake >20 g/day				
Female	6/33	18.2	19/76	25.0
Male	27/58	46.6	50/96	52.1
Median age ± SD				
Female	48.82 ± 11.97		41.41 ± 14.55	
Male	43.03 ± 13.73		43.88 ± 15.80	

*Total for each variable may not sum to the sample size due to missing data.
[a]Other sources, including at least one incidence of intravenous drug use, blood transfusion, occupational exposure, iatrogenic exposure (e.g., endoscopy or coloscopy, history of surgery, acupuncture, or hemodialysis), or unknown.

For patients with high ALT serum levels, three factors were found to be significant in the univariable analysis: gender, age, and HBeAg. After adjusting for the effects of other variables, multiple regression analysis showed that only gender and HBeAg was associated significantly with ALT. The multiple logistic regression analysis showed males having a significantly higher probability of increased ALT levels compared to females (OR = 3.835; 95% CI 2.234–6.586; $P < 0.001$) and that patients with HBeAg were more likely to have increased ALT levels than patients without HBeAg (OR = 3.020; 95% CI 1.695–5.381; $P < 0.001$).

TABLE III. Detection of Virological Markers in Serum

	Genotype A (n = 107)		Genotype D (n = 205)	
	n	%	n	%
Anti-HBc positive				
Female	28/28	100.0	84/84	100.0
Male	58/58	100.0	97/99	98.0
HBeAg negative				
Female*	23/28	82.1	47/83	56.6
Male	42/58	72.4	68/99	68.7
Anti-HBe positive				
Female	23/38	82.1	43/83	51.8
Male	39/69	67.2	62/99	62.6
Anti HCV positive				
Female	1/34	2.9	4/84	4.8
Male	4/66	6.0	7/105	6.7
Anti-HIV 1 and 2 positive				
Female	0/65	0.0	0/105	0.0
Male	3/65	4.6	2/105	1.9

*$P < 0.05$.

In addition, males were also more likely to have increased AST levels than females (OR = 2.086; 95% CI 1.245–3.496; $P = 0.005$), after adjusting for the effects of the other variables in the model. Furthermore, patients with HBeAg had a significantly higher probability of an increased level of AST (OR = 2.414; 95% CI 1.412–4.127; $P = 0.001$).

After adjusting for the effects of the other variables in the model, increased GGT serum levels were associated marginally with being male (OR = 2.009; 95% CI 1.005–4.016; $P = 0.048$) and with increasing age of the patient (OR = 1.021; 95% CI 0.999–1.044; $P = 0.060$).

DISCUSSION

The results of the study show that in the north of Portugal, HBV genotype D was predominant (60.3%), followed by genotype A (31.5%). These results are consistent with research that shows that genotype D is the most common genotype in Mediterranean countries [Echevarria et al., 2005; Ezzikouri et al., 2008]. Genotypes C, E, F, and dual infection by genotype D and F were also observed in the population studied. All patients with genotype F were born in mainland Portugal. The patients with genotype E (n = 12) included nine cases from Angola and one patient each from Mozambique, S. Tome, and Guinea-Bissau. The small prevalence of genotype E can be explained by the low immigration levels in this area, but may suggest a special category for people originating from or traveling frequently to the former Portuguese colonies.

The two most representative genotypes, A and D, were the focus of this study. With both genotypes, males

TABLE IV. Significant Predictors of Viral Load* by Linear Regression**

	Univariable analysis				Multivariable analysis[a]				
	Unstandardized coefficients	CI 95%		*P*	Unstandardized coefficients	CI 95%		Standardized coefficients	*P*
Gender (female as reference)	−0.826	−1.594	−0.058	0.035					
Age	−0.023	−0.049	0.004	0.096					
Genotype (genotype A as reference)	0.471	0.214	0.727	<0.001	0.379	0.109	0.649	0.181	0.006
HBeAg (negative as reference)	2.668	1.743	3.593	<0.001	2.539	1.624	3.453	0.358	<0.001

*ln viral load.
**Two hundred thirty-three individuals with viral load of between 357 and 17,900,000 IU/ml (quantification limits of VERSANT HBV DNA 3.0 assay (bDNA)).
[a]The model accounts for 17% variability of the viral load.

represented an increased prevalence of cases. The unequal gender distribution (57.1% male) observed, is in accordance with most published research. The median patient age was identical for patients with genotypes D and A, irrespective of gender.

Intrafamilial transmission appears to be the predominant presumed source of HBV infection in female patients (about 40% for both genotypes), while in male patients this was reduced to about 20%. Sexual transmission as the presumed source of infection was increased slightly in males, while perinatal transmission was increased in females.

Similar European studies carried out in the Netherlands [Toy et al., 2008] and France [Grandjacques et al., 2000] examining the transmission routes of HBV have shown that genotype A was found most often in patients considered with infection transmitted by the sexual route. In contrast, genotype D was most often found in patients with perinatal transmission.

An examination of alcohol intake revealed that males consumed more alcohol than females, irrespective of HBV genotypes. Increased alcohol consumption in males is consistent with the findings in the WHOSIS report [WHO, 2008b].

Anti-HBc was not found only in two male cases with genotype D, one with HBeAg and the other without HBeAg. The absence of anti-HBc may be explained by the existence of a precore stop *codon* mutation in HBeAg seroconversion, but the existence of this mutation was not sought [Chen et al., 2007a]. This situation must be taken into consideration and investigated routinely, since this atypical pattern can also reflect unusual HBV infection [Echevarria and Leon, 2004].

In female patients, absence of HBeAg was observed in a significantly smaller percentage in genotype D than in genotype A patients (56.6% vs. 82.1%, $P = 0.028$). In male patients, a similar proportion (about 70%) was observed with both genotypes. In contrast with this result, another Spanish study [Sanchez-Tapias et al., 2002] found that absence of HBeAg was more prevalent in patients with genotype D.

Co-infection with HCV or HIV 1 and 2 was observed in only a small proportion of patients.

Increased levels of viral load were associated significantly and independently with genotype D and with HBeAg.

Some investigators found that HBV infection remained active after seroconversion to anti-HBe in nearly 40% of patients with genotype D. These findings may explain the high prevalence of genotype D found in patients with chronic hepatitis B and anti-HBe in Mediterranean countries [Rodriguez-Frias et al., 1995; Grandjacques et al., 2000].

Many biochemical tests that detect abnormal liver function can indicate liver disease, but do not define the cause. The most common blood tests that assess liver

TABLE V. Biochemical Tests of Liver Function Greater Than Reference Values*

	Genotype A (n = 107)				Genotype D (n = 205)			
	n	%	Median	Range	n	%	Median	Range
ALT								
Female	10/37	27.0	71.5	40.0; 396.0	35/86	40.7	75.0	37.0; 1,044.0
Male	38/64	59.4	64.5	37.0; 608.0	69/107	64.5	57.0	37.0; 272.0
AST								
Female	10/37	27.0	56.0	32.0; 208.0	35/86	40.7	51.0	31.00; 871.0
Male	36/64	56.3	52.0	37.0; 702.0	52/107	48.6	45.0	31.0; 1,000.0
GGT								
Female	4/35	11.4	163.50	97.0; 256.0	7/81	8.6	76.0	68.0; 149.0
Male	16/64	25.0	150.00	69.0; 1,400.0	15/104	14.4	114.0	72.0; 210.0

*Parameters were evaluated only for a fraction of patients.

TABLE VI. Factors Associated With Increased Levels of ALT, AST, and GGT by Logistic Regression

	Univariable analysis				Multivariable analysis[a]			
	Unadj. OR	CI 95%		*P*	Adj. OR	CI 95%		*P*
ALT								
Gender (female as reference)	2.898	1.793	4.684	<0.001	3.835	2.234	6.586	<0.001
Age	0.982	0.966	0.998	0.026				
Genotype (genotype A as reference)	1.290	0.797	2.090	0.300				
HBeAg (negative as reference)	2.420	1.418	4.128	0.001	3.020	1.695	5.381	<0.001
Infection[b]				0.602				
Sexual	1.461	0.679	3.141	0.332				
Intrafamiliar	1.174	0.600	2.297	0.640				
Others	1.507	0.775	2.930	0.227				
AST								
Gender (female as reference)	1.838	1.144	2.952	0.012	2.086	1.245	3.496	0.005
Age	1.005	0.990	1.022	0.496				
Genotype (genotype A as reference)	0.981	0.605	1.591	0.939				
HBeAg (negative as reference)	2.206	1.309	3.717	0.003	2.414	1.412	4.127	0.001
Infection[b]				0.393				
Sexual	1.687	0.781	3.646	0.183				
Intrafamiliar	1.028	0.519	2.034	0.937				
Others	1.410	0.721	2.757	0.315				
GGT								
Gender (female as reference)	1.981	0.995	3.943	0.052	2.009	1.005	4.016	0.048
Age	1.021	0.999	1.043	0.065	1.021	0.999	1.044	0.060
Genotype (genotype A as reference)	0.561	0.298	1.057	0.074				
HBeAg (negative as reference)	1.129	0.546	2.333	0.744				
Infection[b]				0.133				
Sexual	2.293	0.724	7.258	0.158				
Intrafamiliar	1.270	0.409	3.948	0.679				
Others	2.686	0.953	7.573	0.062				

[a]Hosmer–Lemeshow test (ALT: $\chi^2 = 0.003$, $P = 0.999$; AST: $\chi^2 = 0.012$, $P = 0.994$; GGT: $\chi^2 = 9.566$, $P = 0.297$).
[b]The presumed source of HBV infection (perinatal as reference).

function include ALT, AST, and GGT. Blood levels of these enzymes are elevated roughly according to the degree of liver damage. ALT, AST, and GGT enzymes were also evaluated and associated with gender, age, genotype, HBeAg, and presumed transmission routes using multivariable analysis, which adjusts for the differences in the distribution of, and associations among, the other variables in the model. Two factors were found to be associated significantly and independently with increased levels of ALT, male gender (OR = 3.835; 95% CI 2.234–6.586; $P < 0.001$), and HBeAg-positivity (OR = 3.020; 95% CI 1.695–5.381; $P < 0.001$). A study conducted in San Francisco (USA) reported higher mean levels of ALT with infection with genotype A than with genotype D [Kato et al., 2004]. However, in this study, after controlling for gender and the presence of HBeAg, no significant differences were found between genotypes A and D.

Similarly, with regards to AST, only gender and HBeAg were associated significantly and independently. After adjusting for the effects of the other variables in the model, men were more likely to have increased levels of AST than women (OR = 2.086; 95% CI 1.245–3.496; $P = 0.005$). Patients with HBeAg had a significantly higher probability of having an increased level of AST (OR = 2.414; 95% CI 1.412–4.127; $P = 0.001$). In Spain, [Sanchez-Tapias et al., 2002] a comparison of AST values in patients with genotypes A and D did not reveal any significant differences; however, the investigators did not control for possible confounding variables. In this sample, elevated levels of GGT were associated marginally with being male and with increasing age.

In summary, this study examined a sample of people infected with HBV living in the north of Portugal, where genotypes A and D are predominant. Men and women differed in the distribution of presumed routes of transmission. While the most frequent transmission in female patients was intrafamilial, male patients were infected equally by perinatal, sexual, and intrafamilial paths. Alcohol intake was predominant among men. In female patients, absence of HBeAg was observed in a significantly smaller proportion of females with D genotype, as compared to those with A genotype, while in males statistically significant differences were not observed. HBeAg is associated with more severe liver damage.

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