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Ana Mota[I,II]

Fátima Guedes[III]

Jorge Areias[IV,V]

Luciana Pinho[II,IV]

Margarida Fonseca Cardoso[IV,VI,VII]

Epidemiological and genotypic profile of Hepatitis B virus infection in Northern Portugal

ABSTRACT

OBJECTIVE: To describe the epidemiological and genotypic profile of chronic hepatitis B infection in Northern Portugal.

METHODS: This survey comprised 358 subjects with positive serology for hepatitis B antigen for at least six months, recruited from specialist appointments in two hospitals in Northern Portugal between 2008 and 2009. Data were obtained from patient files, laboratory tests performed at the time of viral genotyping, echograms and/or ultrasonogram results, and liver biopsies. Demographic characteristics, viral markers, viral load and genotype, and severity of liver disease were evaluated and compared between sexes.

RESULTS: Genotypes A and D were predominant in both sexes. Intrafamilial transmission occurred mostly among female patients. One-third of females and 58,9% of males showed alcohol intake above 20 g/day. Absence of AgHBe was similar in both sexes (p = 0.662). Elevated biochemical parameters and presence of necroinflammatory activity and steatosis in liver biopsies were more frequent among male patients (p=0.003).

CONCLUSIONS: Differences in terms of route of HBV infection between men and women may be a consequence of gender-associated risk behaviors. Excessive alcohol intake is more frequent among males than females, as is more severe liver disease.

DESCRIPTORS: Hepatitis B, epidemiology. Hepatitis B virus, isolation & purification. Risk Factors. Epidemiology, Descriptive.

INTRODUCTION

Infection with the hepatitis B virus (HBV) is of concern to public health in Portugal, despite the relatively low prevalence of infection (1%).[12,20] On the other hand, a nation-wide serological survey in 2004 showed that 0.36% of the population is chronically infected by HBV. Epidemiological studies suggest that between 100 and 120 thousand individuals are chronically positive for the Hepatitis B surface antigen (HBsAg).[12,20]

In Portugal, viral hepatitis is the second most important cause of liver disease.[4] Roughly 2.5 thousand deaths occur every year as a consequence of hepatic cirrhosis. According to hospital statistics, between 15% and 20% of liver cirrhosis patients are HBV-positive.[6]

The isolation of HBV allowed for its classification into nine subtypes according to antigenic determinants of HBsAg. Recent studies suggest that the different HBV genotypes can have distinct effects in terms of the severity of the resulting liver disease, including progression to cirrhosis and hepatocellular carcinoma, as

I Programa de Doutoramento em Ciências Biomédicas. Instituto de Ciências Biomédicas Abel Salazar (ICBAS). Universidade do Porto. Porto, Portugal

II Serviço de Hematologia Clínica. Centro Hospitalar do Porto (CHP). Hospital de Santo António (HSA). Porto, Portugal

III Faculdade de Ciências da Saúde. Universidade Fernando Pessoa. Porto, Portugal.

IV ICBAS. Universidade do Porto. Porto, Portugal

V Serviço de Gastrenterologia. CHP-HSA. Porto, Portugal

VI Centro Interdisciplinar de Investigação Marinha e Ambiental. Porto, Portugal

VII Instituto de Saúde Pública da Universidade do Porto. Porto, Portugal

Correspondência | Correspondence:
Margarida Fonseca Cardoso
Instituto de Ciências Biomédicas Abel Salazar
Universidade do Porto
Largo Abel Salazar, 2
4099-003 Porto, Portugal
E-mail: mcard@icbas.up.pt

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well as in terms of the patient's response to treatment.[21] However, it is still unclear which HVB genotypes are associated with greater risk of severe liver disease.

Epidemiological studies suggest that certain genotypes are predominant in certain regions of the world.[1,5] Verschuere et al[23] report that the predominant subtypes in Europe are genotypes A and D. This is true also for Portugal, especially in the Northern region of the country.[14,15] In countries where endemicity is low, and in age groups not covered by mandatory vaccination, sexual transmission is more common among males, whereas intrafamilial transmission seems to predominate among females.[14,22] Vaccination is mandatory for all newborns in Portugal since 2000.

Knowledge of the characteristics of affected individuals may help to define and implement measures aiming to reduce the negative impact of HBV infection in diseased individuals as well as the number of new infections. The present study aimed to describe the epidemiological and genotypic profiles of chronic HBV infection.

METHODS

Subjects included in the study were recruited from among gastroenterology appointments at the Hospital Santo António and general appointments at the Hospital Joaquim Urbano, taken place between 2008 and 2009. The inclusion criteria was presence of HBsAg for at least six months, which led to a total of 358 chronically infected individuals.

Viral genotypes were determined by molecular biology. Histological evaluation was carried out on 292 subjects upon request from their clinicians.

Data on sex, age, means of HBV infection, lifestyle variables, and alcohol intake were collected by the subjects' clinicians, using a pre-defined spreadsheet.

Means of transmission were described as sexual, mother-to-child, and intrafamilial. The latter is defined as having occurred within the family, not necessarily by sexual contact, and in the absence of evidence for other forms of transmission.

Alcohol intake was categorized as less than 20 g/day or more than 20 g/day. This classification was chosen based on data from the World Health Organization (WHO) and from other relevant publications in the field.[7,13]

Serum samples were tested for presence of HBsAg and its respective antibody (anti-HBs), HBe antigen (HBeAg) and its respective antibody (anti-HBe), and anti-HBc antibody. Tests were performed using a Vitros ECI instrument, which detects positivity based on chemiluminescence (Ortho-Clinical Diagnostics, Amersham, Buckinghamshire, UK).

The VERSANT®HBV DNA 3.0 Assay (bDNA) (Bayer, New York, USA) is a procedure for the direct quantification of HBV DNA by amplification of the signal emitted by the nucleic acid. This assay is optimized to detect the presence of virus in the range of 2 thousand to 100 million copies/ml. Levels lower than 2 thousand copies/ml are considered as "undetectable."

Genotypes were identified using the TRUGENE ® HBV genotyping kit, along with the Open Gene ®DNA Sequencing System (Bayer, New York, USA), which allow for bidirectional sequencing of the surface antigen and of domains B and E of HVB reverse transcriptase. The sequenced HBV DNA is compared to a library of known genotypes and mutants, and a final report on the unknown genotype is then elaborated.

The various laboratory tests that detect alterations in hepatic function are not capable of defining the cause of these alterations. In the present study, it was evaluated the following parameters: alanine aminotransferase (ALT) (reference values: 10 to 36 U/L at 37ºC); aspartate aminotransferase (AST) (10 to 30 U/L at 37ºC); gamma-glutamyl transferase (γ-GT) (10 to 66 U/L at 37ºC); alkaline phosphatase (ALP) (45 to 122 U/L at 37ºC); alpha-fetoprotein (AFP) (below 10.9 ng/ml). Elevated levels of one or more of these parameters are indicative of inflammation or liver disease. The parameters were measured by kinetic enzyme assay using a Cobas Integra 800 instrument (Roche, Mannheim, Germany).

Protrombin time (PT) was determined using an ACL TOP coagulation analyzer (Instrumentation Laboratory Company, Lexington, USA).

Subjects underwent echography and/or ultrasonography for evaluation of liver disease.

In the group of subjects from which a liver biopsy was obtained, results were classified as bearing signs of necro-inflammatory activity, fibrosis, and/or steatosis.

Severity of liver disease was determined using the Child-Pugh score.[17] This system classifies liver disease into three levels of severity, from A to C, in which level C corresponds to 88% mortality in five years, level B, to 38% mortality, and level A, to 29% mortality.

We adapted this scale to provide a more rigorous description of our subjects, as follows: (1) level C, (2) level B, and (3) "others," which included not only subjects classified as level A, but also others with no known liver disease.

Data were analyzed using SPSS for Windows v. 16.0. We used descriptive analysis for sample characterization. Qualitative variables were described as percentages and quantitative variables as means or geometric means, depending on their distributions. The total number of subjects varied across the different variables

investigated due to the inclusion of patient files with missing data. All statistical tests were two-tailed. We adopted a significance level of 0.05.

Proportions were compared using Pearson's chi-squared test with continuity correction, whenever the test's assumptions were met. Otherwise, Fisher's exact test was used. Quantitative variables were compared using Student's *t* test for independent samples after transforming variables, if necessary, in order to meet the test's assumptions.

The present study was approved by the Research Coordinating Office of the Hospital de Santo António in July 2007. Written consent was obtained from all subjects.

RESULTS

Of the 358 subjects chronically infected with HBV, 43.3% (n = 155) were females (Table 1). Almost all subjects were Portuguese (93.6%), followed by subjects of African origin (Table 2). There was a predominance among both sexes of viral genotypes D (61.9% among females; 58.6% among males) and A (26.5% among females; 34.5% among males). Although genotypes C, E, and F could be identified in subjects of both sexes, such cases were rare. Double-infection by genotypes D and F was found only among males.

Mean age of male and female subjects was similar (mean: 44 years; standard-deviation (SD): 15 years). Whereas sexual transmission occurred with similar frequency in both sexes (approximately 16%), intrafamilial transmission was predominant among females, in which this type of transmission was more frequent (36.1%; n = 56) than in males (20.7%; n = 42) ($p < 0.001$).

Men and women differed significantly in terms of alcohol intake ($p < 0.001$). One-third of women (33.3%) ingested more than 20 g of alcohol per day. This proportion was higher among men (58.9%).

HBeAg was absent from 73.5% of subjects.

The proportion of subjects with elevated biochemical parameters was significantly higher among males than among females ($p < 0.038$), with the exception of alpha-fetoprotein.

Liver biopsy evaluation showed a prevalence of "necro-inflammatory activity" and "steatosis" among males. Females displayed a higher proportion of "fibrosis" ($p = 0.003$).

More males were included in levels C and B of the adapted Child-Pugh scale. Though significance was marginal ($p = 0.060$), males showed higher severity of liver disease.

DISCUSSION

As reported in other European studies,[2,10] genotypes A and D were predominant among both sexes. This genotypic distribution seems not to be dependent on sex ($p = 0.173$), and corresponds to the so-called "Mediterranean pattern." [2,8,19]

Almost all subjects were born in Portugal (93.6%), followed by males originating from Portuguese-speaking countries such as Angola, Mozambique, São Tomé e Príncipe, and Guinea-Bissau, all former Portuguese colonies in Africa. Immigration from Asia seems to have introduced genotype C among the Portuguese population, and the only two cases of infection with this genotype were of Chinese origin.

An Italian study evaluated the profile of HBV infection among immigrants (< six months), with similar results: a predominance of males, originating mostly from Africa, followed by Eastern Europe and Asia, and with a genotypic distribution similar to that found in the present sample. Migratory flow seems to introduce new genotypic profiles, and eventually to alter incidence rates among the autochthonous population.[16] In face of these results, we suggest that HBsAg screening be provided to immigrants upon entry into Portugal. Immigrants with negative results should be vaccinated against HBV, and those with positive results should receive the appropriate treatment.[18]

The predominant route of infection seems to differ between the men and women studied. Intrafamilial transmission was more frequent among females (36.1%), whereas frequency of sexual transmission was similar in both sexes. This form of transmission is the most frequent in countries with low endemicity, as is the case of most European countries, as confirmed in a survey carried out in Holland.[22]

Excessive alcohol intake is predominant among males (58.9%; $p < 0.001$). In other studies, especially from Holland, it was concluded that males showed greater prevalence of alcoholism than females (9.5% and 3.6%, respectively).[3] An Australian population-based study also showed higher frequency of alcohol ingestion among males (3.2%) than among females (1.3%).[11] The rate of absorption in women in relation to men for a same amount of ingested alcohol ranges from 30% to 50%, which results in higher alcoholemia in women.

In the present study, a large proportion of subjects was negative for HBeAg, similarly to what was found in a study carried out in France.[24] These findings support the probable absence of antigen, and raise the possibility of mutations in the pre-core or core promoter regions.

Liver biopsies from male subjects were more likely to show necroinflammatory activity and steatosis ($p =$

Table 1. Characteristics of chronically HVB infected subjects according to sex. Portugal, 2008-2009.[a]

Variable	Females (N = 155)		Males (N = 203)		p-value
	n	%	n	%	
Genotypes					
A	41	26.5	70	34.5	0.173[b]
C	1	0.6	1	0.5	
D	96	61.9	119	58.6	
E	7	4.5	7	3.4	
F	10	6.5	5	2.5	
D and F	0	0.0	1	0.5	
Mean age (SD)	43.61	(14.3)	43.8	(15.0)	0.918
Place of birth					0.661
Portugal	147	94.8	188	92.6	
Foreign	8	5.2	15	7.4	
Transmission route					0.001
Vertical	38	24.5	45	22.2	
Sexual	24	15.5	32	15.8	
Intrafamilial	56	36.1	42	20.7	
Other[c]	37	23.9	84	41.4	
Alcohol intake					<0.001
< 20 g/day	86	66.7	72	41.1	
> 20 g/day	43	33.3	103	58.9	
Viral markers					
AgHBe negative	110	71.0	153	75.4	0.662
Geometric mean viral load 95%CI	760.172.0	(403.325.8; 1.432.884.4)	590.072.1	(348.676.6; 998.690.3)	0.541
Elevated biochemical parameters					
Alanine aminotransferase	48	41.7	121	59.6	<0.001
Aspartate aminotransferase	48	41.7	100	49.2	<0.001
Alkaline phosphatase	7	4.5	22	10.8	0.038
Gamma glutamyl transferase	15	5.1	37	18.2	0.032
Alpha-feto protein	8	16.7	12	5.3	0.720
Hepatic biopsy					0.003
Necroinflammatory activity	20	20.8	42	31.1	
Fibrosis	60	62.5	54	40.0	
Steatosis	16	16.7	39	28.9	
Adapted Child-Pugh classification					0.060
Level 1 (C)	1	0.8	4	2.3	
Level 2 (B)	4	3.3	16	9.4	
Level 3 (other)[d]	116	95.9	151	88.3	

[a] Totals for each variable may not correspond to the total number of subjects due to missing data.
[b] Only the most important genotypes – A and D – were evaluated.
[c] Other forms of transmission include: at least one contact with intravenous drugs, transfusion, occupational exposure, iatrogenic exposure (e.g., endoscopy, colonoscopy, history of surgery, acupuncture, hemodialysis or unknown).
[d] Includes subjects classified as Child-Pugh level A, as well as those with no known liver disease.

0.003). Steatosis may be associated with alcohol intake, which is also higher among men.

One of the limitations of the present survey is the procedure by which the study sample was obtained. However, this sample is likely to satisfactorily approximate the scenario of HBV infection in Northern Portugal. The studied hospitals include a regional reference hospital for Gastroenterology and a hospital specializing in the treatment of infectious diseases, which provide care to the majority of HBV patients in Northern Portugal. The present sample refers to all subjects seeking specialist

Table 2. Genotype and place of birth, among females (F) and males (M). Portugal, 2008-2009.

Genotype	Portuguese (N = 335)		Non-Portuguese (N = 23)		Origin
	n F / M	% F / M	n F / M	% F / M	
A	39 / 65	25.1 / 32.0	2 / 5	1.3 / 2.5	Europe and Africa
C	0 / 0	0.0 / 0.0	1 / 1	0.65 / 0.5	China
D	94 / 117	60.6 / 57.6	2 / 2	1.3 / 1.0	Angola, Moldova, and Ukraine
E	4 / 0	2.6 / 0.0	3 / 7	1.9 / 3.4	Africa
F	10 / 5	6.5 / 2.5	0 / 0	0.0 / 0.0	
D and F	0 / 1	0.0 / 0.5	0 / 0	0.0 / 0.0	
Total	147 / 188	94.8 / 92.6	8 / 15	5.2 / 7.4	

appointments at these institutions in 2008 and 2009. It is possible that there is a bias towards the exclusion of individuals that did not seek medical appointments. However, there is nothing to indicate that this group would differ from the study population with respect to the variables investigated. Moreover, the relative weight of these individuals in the sample was small, since HBV-infected patients were in principle evaluated on a semiannual basis and followed-up for a period of two years, even if they were absent to one or more appointments.

Another potential bias relates to the presence of immigrants in Portugal, some of which are infected with HBV and originate from areas of high HBV endemicity. Failure to include these patients may have altered the epidemiological profile of HBV infection. HBV infection status in these situations would be unknown, resulting in underreporting – a potential bias which should also be taken into consideration. The impact of this phenomenon is likely to be small, however, insofar as the current Portuguese National Health Care System provides universal and practically free access to medical care, even to the immigrant population. Furthermore, according to the Ministry of Internal Administration, immigration in the studied area is low, being lower than the 4% recorded for the entire Portuguese population.

There seems to be no direct relationship between diet and liver disease. However, excess alcohol intake is a known risk factor for cirrhosis. This is especially important in this group of patients, which, in addition to HBV infection, show high prevalence of alcohol intake. This is a common habit in Portugal, where there are approximately 1.7 million alcoholics or excessive drinkers.[a]

According to a study published in 2009,[9] liver disease is fifth in the list of the ten most important diseases in Portugal in terms of years of life lost by age 70. For these reasons, it would be important to conduct a nationwide survey aimed at determining the prevalence of HBV and characterizing the virus in detail, especially in terms of genotype. This data, along with the follow-up of infected patients, would allow for the development of studies that would further our knowledge of the disease and of the response of patients to treatment. It would also allow for an investigation of the association between different genotypes and the severity of liver disease, which could considerably reduce the negative impact of HBV infection in Portugal.

In terms of public health, the clinical follow-up of chronic HBV patients should be a major concern. Since 2000, HBV vaccination is mandatory for all newborns. However, it will be necessary to implement continued health education measures focusing on prevention, especially among age groups that were not reached by vaccination, such as sexually active adults. Chronically infected individuals should be given detailed information aimed at promoting changes in lifestyle, especially with respect to prevention of HBV infection at the intrafamilial level and safe sex practices. Screening immigrants for infection will also be essential to prevent the propagation of HBV. We expect that these measures will lead to a significant decrease in HBV infection among future generations.

[a] World Health Organization. Global Status Report on Alcohol 2004. Geneva; 2004. [cited 2010 Apr 1] Available from: www.who.int/entity/substance_abuse/publications/global_status_report_2004_overview.pdf

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