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ORIGINAL PAPER

Clinical, epidemiological and virological features of acute hepatitis B in Italy

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Abstract
Purpose To evaluate the association of hepatitis B virus (HBV) genotypes, basal core promoter (BCP)/precore (PC) and S gene mutations with the clinical-epidemiological characteristics of acute hepatitis B (AHB) in Italy.
Methods During July 2005–January 2007, 103 symptomatic AHB patients were enrolled and prospectively followed up at 15 national hospitals. HBV genotypes, BCP/PC and S gene variants were determined by nested-PCR and direct sequence analysis.
Results Genotype D, A and F were detected in 49, 45 and 6 % of patients, respectively. BCP, PC, and BCP plus PC variants were found in 3.1, 11.3 and 7.2 % of patients, respectively. At enrollment, 68.3 % of patients were hepatitis B e antigen (HBeAg)-positive and 31.7 % HBeAg-negative. BCP/PC mutations were more common in HBeAg-negative than in HBeAg-positive patients ($p < 0.0001$). Compared to genotype D patients, those harboring non-D genotypes were more frequently males ($p = 0.023$), HBeAg-positive ($p < 0.001$), had higher bilirubin ($p = 0.014$) and viremia ($p = 0.034$) levels and less frequently carried BCP/PC mutations ($p < 0.001$). Non-D genotype patients more often were from Central Italy ($p = 0.001$) and reported risky sexual exposure ($p = 0.021$). Two patients had received vaccination before AHB: one harbored genotype F; the other showed a S gene mutation. Four patients developed fulminant AHB; mutations were found in 2 of 3 patients who underwent BCP/PC sequencing. After a 6-month follow-up, only 2 (2.8 %) patients developed persistent infection.
Conclusion AHB by non-D genotypes is increasing in Italy and is associated with risky sexual exposure. The ability of some genotypes to cause persistent and/or severe infection in Italy warrants larger studies for clarification.

Keywords Acute infection · Epidemiology · Genotypes · Hepatitis B · Mutations · Risk factors

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Members of the Study Group are listed in the Appendix.

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Introduction

Hepatitis B virus (HBV) infection is a major public health problem worldwide [1]. More than 240 million people have chronic infection and about 600,000 people die every year due to the consequences of acute or chronic hepatitis B [2].

The epidemiology of HBV infection in Italy has changed substantially over the last few decades and many cross-sectional and incidence studies have shown that Italy has reached a low level of endemicity [3–6]. Due to implementation of universal vaccination in 1991, all Italians aged 0 through 32 years were virtually protected against HBV infection in 2013. High-risk sexual behavior, cohabitation with a hepatitis B surface antigen (HBsAg)-positive carrier, healthcare-related exposures and several beauty treatments (including tattooing and piercing) remain the most frequent and important risk factors for acute hepatitis B (AHB) in Italy [6, 7]. However, it is conceivable that in Italy the clinical and epidemiological picture of HBV infection, as well as its current level of HBV endemicity, could change because of the increasing migration waves from highly endemic countries [8, 9].

The HBV genome is characterized by genetic heterogeneity. Ten genotypes (A to J) and a number of subtypes have been identified so far [10–12]. Interestingly, geographic distribution, clinical features, disease outcome, and response to therapy have been shown to differ according to HBV genotypes and subtypes in patients with acute and chronic hepatitis B (CHB) [1, 10–12]. Besides, different genotypes have also a distinct pattern of mutations in some HBV genomic regions [PreS and S, basal core promoter (BCP) and precore (PC), polymerase], which, in turn, are correlated with the clinical characteristics and outcome of both AHB and CHB [10, 12]. BCP/PC mutations affecting hepatitis B e antigen (HBeAg) production have been widely investigated and have been found to be associated with a more severe and progressive form of chronic liver disease in patients with genotype C and D and with a fulminant course of AHB [10–13].

Genotype D, which is dominant in the Mediterranean basin, has been responsible for the vast majority of cases of acute and chronic hepatitis B in Italy for decades [14]. However, only a limited number of Italian studies have focused on the relevance of different HBV genotypes and mutations for the natural history of both AHB and CHB [14–20]. Particularly as regards the natural history of AHB, so far only three Italian studies have investigated the influence of HBV genotypes [17–19], and only one has taken into consideration the role of genome mutations deemed able to influence the clinical disease course [19].

In this prospective study we enrolled a cohort of patients with AHB and evaluated the natural course of the infection by analyzing epidemiological, clinical, serological and virological characteristics, including HBV genotype and mutations in S gene and BPC/PC regions. This investigation was also useful to evaluate the impact of immigration on the introduction and spread in Italy of unusual and clinically relevant genotypes and mutations [8, 9].

Patients and methods

Participants and setting

This prospective cohort study was conducted at 15 Infectious Disease Units scattered throughout Italy. Between July 2005 and January 2007, all consecutive patients with symptomatic AHB were enrolled in the study after obtaining written informed consent.

Mandatory inclusion criteria were: high titer [sample/cutoff (S/CO) ratio $\geq$10] of immunoglobulin M (IgM) antibodies to hepatitis B core antigen (anti-HBc) [21]; significant increase in serum alanine aminotransferase (ALT) levels (>10 times the upper limit of normal); acute illness compatible with viral hepatitis. Patients positive for IgM antibodies to hepatitis A virus (HAV) or with any other possible cause of acute liver damage were excluded. All available medical records of patients enrolled in the study were reviewed to ascertain the absence of positive HBsAg or anti-HBc tests before hospital admission. Immunization records of patients who reported previous HBV vaccination were checked to verify the completeness and correctness of vaccination schedule.

A questionnaire recording information on demographic characteristics and risk factors for AHB was administered to all patients. All patients underwent clinical, biochemical and virological assessment at enrolment, then weekly during the first month and, in the event of persistence of HBsAg in serum, every 3 months thereafter for a total of 12 months or until the patients withdrew their participation. Patients were considered to have recovered from AHB, if steady normalization of serum aspartate aminotransferase (AST) and ALT levels together with HBsAg clearance occurred within 6 months after disease onset. Persistence of HBsAg in serum for more than 6 months was considered evidence of chronic disease evolution. Patients were considered to have developed fulminant hepatitis if they showed porto-systemic encephalopathy and coagulopathy (reduction in prothrombin activity below 35 %) within 8 weeks after disease onset.

All patients gave written informed consent before entering the study. The study was approved by the Ethics Committee of the Istituto Superiore di Sanità.

Serological tests

All patients were tested for HBsAg, anti-HBc IgM, anti-HAV IgM, anti-hepatitis C virus (HCV), anti-hepatitis

delta virus (HDV) and anti-human immunodeficiency virus (HIV) antibodies by commercial immunoassays at the enrolling clinical sites. Further serological characterization of HBV infection was performed at the reference laboratory by commercial chemiluminescent assays on the ARCHITECT platform (Abbott Diagnostics, Wiesbaden, Germany). Briefly, quantitative methods were used for HBsAg (sensitivity of 0.05 IU/mL) and anti-HBs (upper quantitation limit of 1000 mIU/mL); total anti-HBc, HBeAg, anti-HBe were determined by qualitative assays, while anti-HBc IgM was examined by a semiquantitative assay.

HBV DNA detection and characterization

HBV DNA was assayed in serum by quantitative PCR (Cobas TaqMan HBV Test, Roche Molecular Systems, USA) at enrolment, a week later and then—in case of positivity—until two consecutive samples collected according to the follow-up schedule tested negative. HBV genotypes, BCP/PC and S gene variants were determined on the first HBV DNA-positive sample by nested-PCR in the core/precore and S regions, followed by direct sequence analysis of the amplicons, as previously reported [22, 23].

Statistical analysis

Mann–Whitney test was used for continuous variables to assess differences between distributions. Pearson's Chi-squared test or Fisher's exact test was used for comparison of frequencies between groups. A p value of 0.05 was considered significant. All statistical analyses were performed using STATA, version 11.2 (StataCorp LP, College Station, TX).

Results

General clinical-epidemiological and virological characteristics of AHB patients at enrolment

A total of 103 consecutive AHB patients were enrolled in the study (Table 1). All patients were symptomatic and 97 (94.2 %) of them had jaundice. All patients showed increased ALT serum levels at least ten times the upper limit of normal and had high-titer anti-HBc IgM antibodies [average sample/cutoff (S/CO) ratio 32.09 ± 9.24]; all but two patients were HBsAg-positive. HBeAg and anti-HBe were tested in 101 patients. Of them, 52 (51.5 %) were HBeAg-positive/anti-HBe-negative, 29 (28.7 %) HBeAg-negative/anti-HBe-positive, 17 (16.8 %) HBeAg-positive/anti-HBe-positive and 3 (2.9 %) HBeAg-negative/anti-HBe-negative (Table 1; Fig. 1a). High-risk sexual behavior and parenteral exposures unrelated to drug use (e.g., nosocomial exposure, cosmetic treatment with percutaneous exposure and cohabitation with a HBsAg-positive carrier) were the most frequently reported risk factors (Table 1).

A concomitant infection with HCV, HDV and HIV was detected in 4/102 (3.9 %), 1/74 (1.4 %) and 4/74 (5.4 %) patients who were tested for these viruses (Table 1).

Serum HBV DNA was examined at disease onset in 102 patients, of whom 101 tested positive (99 %); one was already negative. HBV genotype was determined in 100 of the 101 viremic patients: 49 harbored genotype D, 45 genotype A and six genotype F (Table 1; Fig. 1b).

BCP/PC variants could be determined in 97 of the 101 HBV DNA-positive patients. Either BCP (A1762T/G1764A) or PC (G1896A) variants were found in 3 (3.1 %) and 11 (11.3 %) of these patients, respectively; the simultaneous presence of BCP and PC mutations was observed in 7 (7.2 %) patients while the remaining 76 (78.4 %) patients harbored BCP/PC wild-type sequences (Table 1; Fig. 1c).

HBeAg/anti-HBe status and BCP/PC mutations were tested in 96 patients (Table 2). A significantly higher prevalence of BCP/PC variants was observed in HBeAg-negative patients compared to HBeAg-positive patients (19/29 or 65.5 vs 2/67 or 2.9 %; $p < 0.001$).

The analysis of S gene region mutations was performed on the "a" determinant sequences from 91 patients, seven of whom (7.7 %) showed mutated sequences, for a total of 12 mutations: T126I, S132F, S143L; N131T ($n = 2$), F134Y/N ($n = 2$ each) and T143 M/S/F.

Clinical-epidemiological and virological characteristics of AHB patients at enrolment according to HBV genotype

Data on both infecting genotype and HBe/anti-HBe status were available for 99 patients (Tables 2, 3). Patients infected with non-D genotypes were more often HBeAg-positive (66.2 vs 33.8 %) and less frequently HBeAg-negative (16.1 vs 83.9 %) than patients harboring genotype D (Table 2; $p < 0.001$). The association between HBV genotype and HBeAg status was still statistically significant, even excluding from the analysis patients with BCP/PC mutations (20 HBeAg+/7 HBeAg− in genotype D patients versus 45 HBeAg+/3 HBeAg− in genotype non-D patients, $p = 0.030$). Patients infected with non-D genotypes were more often male ($p = 0.023$), had higher total bilirubin level ($p = 0.014$) and HBV viral load ($p = 0.034$) at enrolment than patients harboring genotype D (Table 3). Data on HBV genotype and mutations in the PC and BCP regions were available for 97 patients: the overall prevalence of BCP/PC mutations was significantly higher in patients infected with genotype D than in those harboring non-D genotypes (19/46 or 41.3 vs 2/51 or 3.9 %; $p < 0.001$) (Table 3).

Table 1 Baseline demographic and clinical characteristics in 103 patients with acute hepatitis B diagnosed at 15 Italian Infectious Disease Units (2005–2007)

Gender (No. %)	103
Males	70 (68.0)
Females	33 (32.0)
Age, median years (range)	37 (16–80)
Geographic area of residence (No. %)	103
North Italy	23 (22.3)
Central Italy	35 (34.0)
Southern Italy and islands	45 (43.7)
Birth country (No. %)	103
Italy	89 (86.4)
Abroad	14 (13.6)
Years of schooling (No. %)	89
≤8	45 (50.6)
≥9	44 (49.4)
Risk factors (No. %)[a]	103
Intravenous drug use	6 (5.8)
Sexual exposure[b]	40 (38.8)
Other[c]	25 (24.3)
Undetermined	22 (21.3)
Jaundice (No. %)	97 (94.8)
Hospitalization (No. %)	102 (99.0)
AST (IU/ml), median (range)	1836 (321–5558)
ALT (IU/ml), median (range)	2431 (851–6324)
Total bilirubin (mg/dL), median (range)	15 (1.1–48.9)
Fulminant hepatitis (No. %)	4 (3.9)
Deaths (No. %)	1 (1.0)
Transplantations (No. %)	2 (2.2)
HBV DNA-positive (No. %)	101 (98.0)
HBV DNA (copies/mL), median (range)	110,683 (7–150,000,000)
anti-HBc IgM-positive	103 (100.0)
HBsAg-positive (No. %)	101 (98.0)
HBeAg/anti-HBe status (No. %)	101
HBeAg+/anti-HBe−	52 (51.5)
HBeAg−/anti-HBe+	29 (28.7)
HBeAg+/anti-HBe+	17 (16.8)
HBeAg−/anti-HBe−	3 (2.9)
Coinfection with HCV (No. %)	4 (3.9)
Coinfection with HDV (No. %)	1 (1.4)
Coinfection with HIV (No. %)	4 (5.4)
HBV genotype (No. %)	100
A	45 (45.0)
D	49 (49.0)
F	6 (6.0)
HBV core/precore mutations (No. %)	97
Precore (PC)	11 (11.3)
Basal core promoter (BCP)	3 (3.1)
PC + BCP	7 (7.2)
Wild type	76 (78.4)

[a] Cases may have more than one risk factor

[b] Promiscuous sexual activity (more than two sexual partners), and unsafe (i.e., no or seldom condom use during occasional sexual intercourse) homo-bisexual intercourses

[c] Cosmetic treatment with percutaneous exposure (piercing, tattooing, manicure/pedicure, barber shop shaving and acupuncture), living with a HBsAg+ carrier, nosocomial exposure, dental treatment

Of the seven patients who showed mutated sequences in the "a" determinant of the S gene, three patients harbored genotype D and carried three mutations: T126I, S132F, S143L; while four patients harbored genotype A and carried nine mutations: N131T ($n = 2$), F134Y/N ($n = 2$ each) and T143/M/S/F.

The majority of patients (86.4 %) were born and resided in Italy, including all those harboring genotype F. Fourteen patients were immigrants from eastern Europe (Romania [$n = 6$], Ukraine [4], Bulgaria and Poland) and northern Africa (Tunisia and Marocco). No significant difference in genotype distribution was detected according to patient's country of birth. Genotype D occurred more frequently in patients residing in Southern Italy and Islands as opposed to non-D genotypes, which were more frequent in patients from Central Italy ($p = 0.001$) (Table 3).

Interestingly, genotype D was more frequently detected in patients who reported intravenous drug use or other parenteral exposure, while patients reporting high-risk sexual behavior harbored non-D genotypes more often ($p = 0.021$) (Table 3). Overall, more than half of the patients harboring non-D genotypes reported high-risk sexual behavior, (4/6 or 66.7 and 21/45 or 46.7 % of genotype F and A patients, respectively). About 10 % of AHB patients reported homo-bisexual intercourses in the 6 months before the disease onset; this exposure was particularly common among patients infected with non-D genotypes, notably genotype A. However, by comparing only genotype D and genotype A patients, the association between genotype A and high-risk sexual exposure resulted to be only marginally significant ($p = 0.07$).

AHB in previously vaccinated individuals

Two patients enrolled in this study had received a complete and well-documented full course of primary HBV vaccination several years before AHB occurrence, both of whom did not undergo pre- or post-vaccination serologic testing. The first patient, a 44-year-old man suffering from insulin-dependent diabetes mellitus since age 22, received HBV vaccination about 6 years before AHB presentation. At enrolment he was HBsAg-positive, anti-HBs-negative, HBeAg-positive/anti-HBe-negative and harbored genotype F. The second

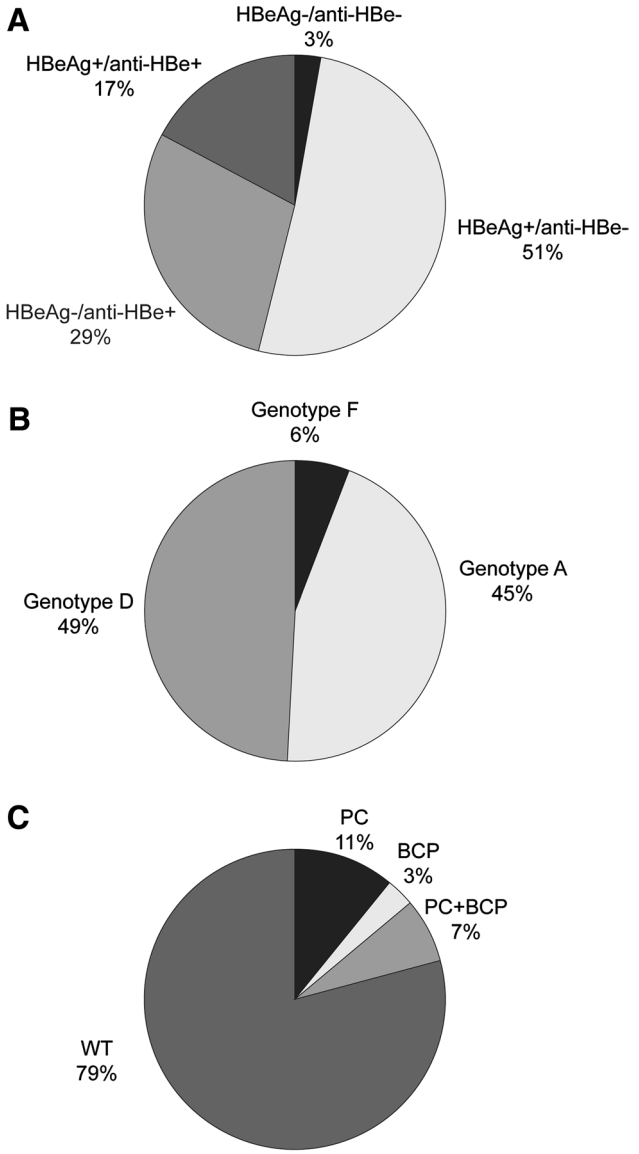

Fig. 1 Distribution of hepatitis B e antigen (HBeAg)/antibodies to hepatitis e antigen (anti-HBe) (**a**), genotypes (**b**) and basal core promoter (BCP)/precore (PC) mutations (**c**) among 103 consecutive patients with acute hepatitis B diagnosed at 15 Italian Infectious Disease Units between July 2005 and January 2007

patient, a 19-year-old man with a history of intravenous drug use, completed primary vaccination about 15 years before AHB presentation. At enrolment, this patient tested HBsAg-positive, anti-HBs-negative, HBeAg-negative/anti-HBe-positive. Besides, he harbored genotype D and showed the T126I mutation in the "a" determinant of the S gene.

Clinical outcome of AHB

Four AHB patients (3.9 %) developed fulminant hepatitis (Table 4). One patient died, two patients underwent transplantation and one other was transferred to a different hospital and lost to follow-up. BCP/PC mutations could be assessed for three of these four patients: one patient, infected with genotype D, showed the simultaneous presence of BCP and PC variants; another one, infected with genotype A, had a BCP mutation; and the last patient, harboring genotype D, had wild-type sequences both in BCP and PC regions. No coinfection with HCV, HDV and HIV was detected in these four patients and none of them reported history of chronic liver disease.

A follow-up of at least 6 months, suitable to establish AHB recovery or chronic evolution, was available for 72 (69.9 %) patients (37 harboring genotype D, 29 genotype A, five genotype F and one with undetermined genotype). Persistence of HBsAg for more than 6 months was observed in two of 72 patients (2.8 %; 95 % confidence interval 0.3–10.0). Both patients were women and aged ≥50 years. One of them was infected with genotype A, while the other with genotype D (Table 3). In both cases BCP/PC wild-type sequences were found. Only one of the two patients was tested for anti-HIV antibodies and was found to be negative. Among the remaining 70 patients who recovered from AHB, 22 (31.4 %) (18 with genotype D and four with genotype non-D), 23 (32.9 %) (six with genotype D and 16 with genotype non-D) and 25 (35.7 %) patients (12 with genotype D and 13 with genotype non-D) cleared HBsAg within the third month, between 3 and 6 months, and at the sixth month after enrolment, respectively. Among these patients, HBsAg clearance occurred significantly earlier ($p = 0.001$) in those harboring genotype D than in those infected with non-D genotypes.

Thirty-one AHB patients were followed up for less than 6 months. Twenty-three of these patients withdrew from the study within the first month after enrolment; they were still HBsAg-positive at the last visit and none of them showed evident decrease (>2 logs) in HBsAg levels during follow-up. Six patients were followed up for a median period of 99.5 days (range 70–115) after enrolment and all of them, albeit still positive at the last visit, showed an evident decrease in HBsAg levels (median decrease 4.5 logs; range 4–6 logs) compared to baseline values. Finally, two patients, who withdrew from the study after 5 months of follow-up, were HBsAg-negative at the last visit.

No significant differences were found at enrolment between subjects followed for at least 6 months and those lost to follow-up regarding epidemiological, clinical, serological and virological characteristics (including HBV genotype distribution) (data not shown).

Discussion

In this study, we have provided an overall comprehensive description of the clinical, epidemiological and virological

Table 2 Distribution of HBV genotypes and precore (PC)/basal core promoter (BCP) region mutations according to HBeAg/anti-HBe status in 103 patients with acute hepatitis B diagnosed at 15 Italian Infectious Disease Units (2005–2007)

HBeAg/anti-Hbe status	PC and/or BCP (No. %)	Wild type (No. %)	Total (No. %)	Genotype D (No. %)	Non-D genotype (No. %)	Total (No. %)
HBeAg+/anti-HBe−	1 (2.0)	49 (98.0)	50 (100.0)	17 (33.3)	34 (66.6)	51 (100.0)
HBeAg−/anti-HBe+	18 (66.6)	9 (33.3)	27 (100.0)	24 (82.8)	5 (17.2)	29 (100.0)
HBeAg+/anti-HBe+	1 (5.9)	16 (94.1)	17 (100.0)	6 (35.3)	11 (64.7)	17 (100.0)
HBeAg−/anti-HB−	1 (50.0)	1 (50.0)	2 (100.0)	2 (100.0)	0 (0.0)	2 (100.0)
HBeAg+	2 (3.0)	65 (97.0)	67 (100.0)	23 (33.8)	45 (66.2)	68 (100.0)
anti-HBe+	19 (43.2)	25 (56.8)	44 (100.0)	30 (65.2)	16 (34.8)	46 (100.0)
HBeAg−	19 (65.5)	10 (34.5)	29 (100.0)	26 (83.9)	5 (16.1)	31 (100.0)
anti-HBe−	2 (3.8)	50 (96.2)	52 (100.0)	19 (35.8)	34 (64.2)	53 (100.0)

anti-HBe antibodies to hepatitis e antigen, *HBeAg* hepatitis B e antigen, *HBV* hepatitis B virus, *BCP* basal core promoter, *PC* precore

Table 3 Demographic, clinical, biochemical and virological characteristics according to HBV genotype in patients with acute hepatitis B diagnosed at 15 Italian Infectious Disease Units (2005–2007)

	Genotype D No. (%)	Genotype non-D No. (%)	*p* value
No. cases	49	51	
Age (years), median (range)	34 (16–80)	37 (21–57)	0.417
Males	28 (57.13)	40 (78.4)	0.023
Geographic area of residence			
North	11 (22.4)	11 (21.6)	0.001
Central	9 (18.4)	26 (51.0)	
Southern and islands	29 (59.2)	14 (27.4)	
Birth country			
Italy	39 (79.6)	47 (92.2)	0.070
Abroad	10 (20.4)	4 (7.8)	
Risk factors[a]			
Intravenous drug use	5 (12.5)	1 (2.6)	0.021
Sexual exposure[b]	14 (35.0)	25 (64.1)	
Other[c]	21 (52.5)	13 (33.3)	
Undetermined	9 (18.4)	12 (23.5)	
Chronic evolution	1 (2.7)	1 (2.9)	1.000
Fulminant hepatitis	2 (4.1)	1 (2.0)	0.614
Deaths	1 (2.0)	0 (0.0)	0.490
Transplantations	0 (0.0)	1 (2.0)	1.000
ALT (IU/ml), median (range)	2354 (851–4974)	2436 (1110–6324)	0.268
AST (IU/ml), median (range)	1857.5 (321–5558)	1795 (361–4230)	0.909
Total bilirubin (mg/dL), median (range)	13.65 (1.1–32)	17.1 (1.6–48.9)	0.014
Precore and basal core promoter variations			
Wild type	27 (58.7)	49 (96.1)	<0.001
Mutant strain	19 (41.3)	2 (3.9)	
HBV DNA (copies/mL), median (range)	66,761 (7–150 × 10^6)	332,485 (31–150 × 10^6)	0.034
HBeAg/anti-HBe status			
HBeAg+/anti-HBe−	17 (33.3)	34 (66.6)	<0.001
HBeAg−/anti-HBe+	24 (82.8)	5 (17.2)	
HBeAg+/anti-HBe+	6 (35.3)	11 (64.7)	
HBeAg−/anti-HBe−	2 (100.0)	0 (0.0)	

[a] Cases may have more than one risk factor

[b] Promiscuous sexual activity (more than two sexual partners during the previous 6 months), and unsafe (i.e., no or seldom condom use during occasional sexual intercourse) homo-bisexual intercourses

[c] Treatment with percutaneous exposure (piercing, tattooing, manicure/pedicure, barber shop shaving and acupuncture), living with a HBsAg+ carrier, nosocomial exposure, dental treatment

Table 4 Clinical and laboratory data of four fulminant hepatitis B cases occurred among a cohort of 103 patients with acute hepatitis B diagnosed at 15 Italian Infectious Disease Units (2005–2007)

Age	Sex	Outcome	HBV Genotype	Precore (PC)/basal core promoter (BCP) variants	Risk factor	HBeAg/anti-HBe status
46	M	Transplantation	Undetermined	Undetermined	Sexual exposure[a]	HBeAg−/anti-HBe+
21	F	Transplantation	A	BCP	Undetermined	HBeAg+/anti-HBe+
30	F	Death	D	Wild type	Undetermined	HBeAg−/anti-HBe+
45	M	Loss to follow-up	D	PC, BCP	Sexual exposure[a]	Undetermined

[a] Promiscuous sexual activity (more than two sexual partners during the previous 6 months), and unsafe (i.e., no or seldom condom use during occasional sexual intercourse) homo-bisexual intercourses

scenario of AHB in Italy as it appeared many years after the occurrence of two events of great epidemiological and public health importance, both dated 1991: the introduction of universal hepatitis B vaccination and the beginning of massive immigration to Italy from countries with high HBV endemicity.

So far only three Italian studies, performed in Southern Italy, have investigated the influence of HBV genotypes on the clinical-epidemiological features of AHB [17–19] and only one of these studies (which focused on patients with severe AHB) has taken into consideration the role of genome mutations deemed able to influence the clinical disease course [19]. Besides, these studies were conducted in one or at most two towns of the same region and enrolled about 100–140 patients during nearly 10 years. Thus, in these studies only the analysis of separate time intervals of recruitment (but with a smaller number of observations in each interval) allowed authors to point out major changes to the clinical-epidemiological and virological profiles of the disease.

A major finding of our study is that more than half (51 %) of the observed AHB cases were due to non-D genotypes, mainly genotype A and F. Genotype A is prevalent in North Europe, the USA, Sub-Saharan and Western Africa [10, 11], while genotype F is common in Central and South America, although it has already been observed in Italy [10, 11, 14, 17, 19, 24]. The prevalence of genotype A (45 %) we found in our study was considerably higher than that reported in the same range time by Coppola et al. (13.7 %) [17] and by Scalia et al. (27 %) [18]. However, these studies were performed in Southern Italy, where non-D genotypes, basing on our findings, are less frequently detected than genotype D.

Thus, looking at our and other available data [17–20, 24], it seems that in Italy the frequency of AHB cases due to genotype A has increased over time, while AHB cases harboring other non-D genotypes have been detected only sporadically. This scenario is consistent with the results obtained in a large sample of CHB patients in North-Eastern Italy during the years 2002–2004; among these patients the prevalence of genotype D and A was 70.6 and 25 %, respectively, while other unusual genotypes (C, E, F, H) were detected in very low percentages, mainly in foreigners [14].

In our study, high-risk sexual exposure was the most frequently reported risk factor for AHB and it was significantly associated with infection by non-D genotypes. An association between infection with non-D genotypes (particularly genotype A) and high-risk sexual exposure was also observed in previous Italian studies [17, 18]. The increasing in prevalence of genotype A over time among AHB patients and its association with high-risk sexual exposure have been well documented by several studies in Japan, where genotype C traditionally predominated [25–27]. The association between genotype A and high-risk sexual exposure has also been reported in studies conducted in some Northern European countries, where genotype A has always been the most prevalent [28, 29]. The above reported change in the molecular epidemiology pattern of AHB over time, well documented in Italy and Japan, has been mainly attributed to the immigration phenomenon [17, 18, 25, 26]. It has been postulated that genotype A was firstly transmitted from infected immigrants to susceptible natives by sexual contact, and then it also spread among the native population, remaining, however, mainly confined to people with at risk sexual behavior [17, 25, 26].

The reasons for the correlation between genotype A and sexual transmission have not yet been fully elucidated. The high viral load that we and others found in genotype A infection [27, 30, 31] increases the HBV concentrations in semen and other body fluids of carriers, thus increasing the risk of transmission by sexual intercourse. Moreover, Japanese studies and our results have shown that genotype A infection tends to persist following AHB and this may further influence its spread [26, 27, 30, 31]. However, also genotype D can be efficiently transmitted through sexual contact: about one-third of our AHB patients harboring genotype D also reported high-risk sexual behavior. Thus, other epidemiological features, in addition to immigration, may have contributed to the current molecular epidemiological pattern of AHB in Italy. Indeed, most probably this pattern is the consequence of the important changes in

HBV epidemiology that occurred throughout the last decades in Italy. These changes are mainly due to the universal vaccination against HBV that has gradually elevated the age of the susceptible population. At the time of the study, all Italians ≤27 years were virtually protected against HBV. The shift in the age of the HBV susceptible population has produced a consequent change in the role of different risk factors, with a significant reduction in the role of intrafamilial contact and intravenous drug use and an increase in the role of unsafe homosexual and heterosexual exposure. This has produced, in turn, an increase in frequency of AHB cases due to non-D genotypes, which preferably spread among people with high-risk sexual behavior. Studies based on phylogenetic analysis of genotype A strains circulating in Italy could certainly contribute to the definition of the source and evolution of this genotype.

Data from the present study showed that, in comparison with AHB patients infected with genotype D, those infected with non-D genotypes were more frequently male and had higher total bilirubin level and HBV viral load at enrolment. Besides, they harbored BCP/PC mutant strains less frequently and were HBeAg-positive more often. All these clinical and virological features have already been reported to be distinctive of AHB by genotype A in other countries [1, 11, 12, 26, 27, 30, 31], but these characteristics have been only incompletely recognized among genotype A (or non-D) AHB patients thus far in Italy [17, 18]. The less frequent detection of PC mutations in non-D genotype, most notably genotype A, has been ascribed to the instability caused by this mutation in the stem-loop structures of the pregenome encapsidation signal [27, 32]. This may explain the association between non-D genotype and HBsAg positivity. However, as we and others have observed, this association seems also to be independent from the BCP/PC status of patients [14].

Two patients, both harboring genotype D, were HBsAg-negative at enrollment. Neither of them showed seroconversion for HBsAg during the follow-up. Low levels of HBsAg in acute hepatitis B have been occasionally reported, usually in patients with low replication levels of HBV [33]. These two cases had indeed a very low viral load (1131 and 7 IU/mL, respectively) on the initial sample that tested negative for HBsAg.

In this study a low rate (2.8 %) of AHB chronic evolution was found. This is in agreement with previous studies showing that in immune-competent adults HBV persistence after AHB is infrequent [1, 17–19, 34]. Several recent studies, mainly from Japan, have suggested that high viral load and chronic evolution of AHB are more frequent with genotype A infections than with infections due to other genotypes [11, 12, 26, 27, 30, 31]. Among patients investigated by Coppola et al., only three patients developed persistent infection and they all harbored genotype D [17]; whereas Scalia et al. [18] found that the mean time to HBsAg clearance was longer when the proportion of AHB cases infected with non-D genotypes increased. We also found that HBsAg clearance was significantly delayed in patients infected with non-D genotypes who recovered from AHB, and this finding is consistent with those of other studies [18, 27, 30, 31]. In our cohort two patients developed persistent infection: one with genotype A, the other with genotype D, with an essentially similar rate of chronic evolution (3.5 vs 2.7 %), but the numbers were too small to enable any meaningful analysis.

Fulminant AHB has been reported to be associated with non-A genotypes, HBeAg-negative status and, above all, the presence of PC e BPC mutations [12, 13]. Although there were only four fulminant AHB cases in our study and BCP/PC mutations could be assessed in three of them, it is intriguing that two patients had BCP/PC mutations. A pre-exiting HCV chronic infection was identified as the only factor associated with a severe or fulminant course of AHB among the cohort of Italian patients investigated by Coppola et al. [19]. No coinfection with other hepatitis viruses was detected in our four patients with fulminant hepatitis and none of them reported history of chronic liver disease.

Two patients developed AHB despite previous vaccination, although G145R mutation, classically associated with vaccine escape, was not detected, suggesting that the possible selective pressure induced by universal immunization has not influenced the emergence and spread of the escape variant.

One of the two vaccine recipients suffered from insulin-dependent diabetes mellitus, a condition already associated with reduced efficacy of HBV vaccination in adults and children [35]. Besides, he was infected with genotype F, which has marked sequence differences in the S and P gene compared to other genotypes. Since current vaccines are based on recombinant HBsAg protein from genotype A and D, they may not result in full protective immunity towards genotype F infection, even in immune competent, fully vaccinated individuals with proper anti-HBs titers [36]. The second patient was infected with genotype D, yet he showed the T126I mutation in the "a" determinant of the S gene. This mutation may have a major impact on HBsAg antigenicity and has been associated, especially in combination with G145R mutation, with diagnosis failure (occult infections), poor disease outcome and possible escape of vaccine-induced neutralizing antibodies [37, 38]. However, since pre- and post-vaccination serological testing was not performed in any of these two patients and both of them were anti-HBs-negative at enrolment, no firm conclusion can be drawn to explain the reasons for their acute infection.

We acknowledge that the sample of AHB patients enrolled in our study was not particularly large. It is

necessary to consider that, as consequence of universal vaccination, the incidence of symptomatic AHB at the time of the study was already very low in Italy and the vast majority of cases were aged over 25 years [6]. Nevertheless, our study population represented 12 % of all the symptomatic cases reported to the Italian surveillance system for acute viral hepatitis in the same time range [6] showing similar age, sex, geographical area of residence and risk factors as those reported by the whole cohort (data not shown). Consequently, our study sample may be considered fairly representative of all the AHB cases reported in Italy in the same period. Another limitation of our study was that about 30 % of patients were lost to follow-up (20 % within the first month after the enrolment). We hypothesize that the low compliance with follow-up among AHB patients was also due to the fact that they were made aware of the favorable disease outcome in most cases. Besides patients, even if still positive for HBV markers, usually feel good after hospital discharge and are probably less inclined to adhere to follow-up visits.

In conclusion, the results of our study show and confirm the increasing spread of non-D genotypes in Italy and the substantial changes in the distribution and relevance of the various risk factors for AHB. High-risk sexual exposure has a major role in HBV acquisition and is significantly associated with non-D genotype infection. HBV vaccination should be recommended and implemented among adults with high-risk sexual behavior.

Further studies on large samples of AHB patients in our geographical area are needed to enable a reliable analysis of the relationship between HBV genotypes and chronic infection, as well as the relationship between BCP/PC mutations and disease severity.

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Conflict of interest All authors, with the exception of Claudio Galli, Alessandro R. Zanetti and Gloria Taliani, do not have potential competing interests. Claudio Galli is currently employed by Abbott Diagnostics Italy as the Scientific Affairs Manager. Abbott Diagnostics Italy has supported this study by providing the reagents for the serological assays and sample transportation from the clinical sites to the reference laboratories. Alessandro R. Zanetti has received a speaker honorarium from Glaxo Smith Kline and a consulting honorarium from Sanofi Pasteur MSD. Gloria Taliani has received speaker's honoraria form Bristol-Myers Squibb, Gilead, Janssen Cilag, Merck and AbbVie.

Ethical approval This study has been performed in accordance with the 1964 Declaration of Helsinki and its later amendments and was approved by the Istituto Superiore di Sanità ethics committee.

Appendix

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