Hepatitis B virus infection among first-time blood donors in Italy: prevalence and correlates between serological patterns and occult infection

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Background. A prospective, 1-year study was performed among Italian first-time, volunteer blood donors, who account for 12% of all donations, in order to assess the frequency and serological patterns of hepatitis B virus infection and the presence of occult infection.

Materials and methods. Consecutive donors (n=31,190) from 21 blood transfusion centres, from age classes not subjected to universal HBV vaccination, were tested for HBsAg and anti-HBc by commercial immunoassays. Other HBV serological markers were searched for and qualitative and quantitative assessments of HBV-DNA were made in HBsAg and/or anti-HBc-positive individuals.

Results. Of the 31,190 donors studied, 100 (0.32%) were positive for both HBsAg and anti-HBc, 2 for HBsAg (0.01%) alone, and 2,593 (8.3%) for anti-HBc. Of these last, 86.7% were also positive for anti-HBs (with or without anti-HBe), 2.9% were positive for anti-HBe without anti-HBs and 10.4% had no other HBV markers (anti-HBc alone). A general north-south increasing gradient of HBV prevalence was observed. Circulating HBV-DNA was found in 96.8% of HBsAg-positive subjects as compared to 0.55% (12/2,186) of anti-HBc-positive/HBsAg-negative subjects, with higher frequencies among anti-HBs-negative than among anti-HBs-positive ones (1.68% *vs* 0.37%; p <0.01) and among the 57 cases positive for both anti-HBc and anti-HBe (7%). HBV-DNA levels were significantly higher in HBsAg-positive subjects than in HBsAg-negative ones (median: 456 IU/mL *vs* 38 IU/mL).

Conclusions. The prevalence of HBV infection among Italian first-time blood donors is much lower than in the past. The presence of occult infections in this group was confirmed (frequency: 1 in 2,599), supporting the hypothesis of long-term persistence of HBV infection after clearance of HBsAg. HBsAg and nucleic acid amplification testing for blood screening and vaccination against HBV are crucial in order to further reduce the risk of transfusion-transmitted HBV towards zero.

Keywords: HBV, anti-HBc, blood screening, occult infection, HBV-DNA.

Introduction

Hepatitis B virus (HBV) is easily transmitted by blood products as both cellular and plasma-derived components may be infected. Transfusion-transmitted HBV played a major role in the spread of this infection some decades ago all around the world, and is still a threat in developing countries, where the prevalence of this infection is higher and the donor selection and screening procedures are less tight[1-4].

In Italy, as well as in most industrialized countries, nucleic acid amplification testing (NAT) has been introduced along with serological testing for HBV and for the other major viral agents such as hepatitis C virus and human immunodeficiency virus in order to enhance blood safety[3-9]. As HBV-DNA is a more sensitive marker of ongoing HBV infection than hepatitis B surface antigen (HBsAg), detection of the former is considered to offer the possibility of better prevention of HBV transmission by transfusion, through the identification of blood units collected in the pre-seroconversion phase for HBsAg as well as the detection of occult hepatitis B virus infections (OBI)[8-12]. An OBI is defined as the presence of HBV DNA in the serum and/or in the liver of individuals testing negative for HBsAg by currently available assays. When detectable, the amount of HBV DNA in the serum is usually very low (<200 IU/mL)[13].

Antibodies directed against the HBV core gene products (anti-HBc) are a life-long serological marker

of previous HBV infection, as they are detectable both in acute and chronic infections and after viral clearance; this marker, therefore, indicates a current (recent or chronic) or past HBV infection. Screening for anti-HBc is recommended in several countries in which the prevalence of hepatitis B is low, including Canada, France, Germany and the USA, in order to detect HBV-infected donors[7,10,14-16].

The aim of this study was to evaluate the seroprevalence of HBV infection among first-time blood donors in Italy, to identify the frequency of OBI and to explore the possible correlations between serological patterns and HBV viraemia.

Materials and methods

Study sites

Twenty-one Blood Transfusion Services located in nine of the 20 regions of Italy agreed to participate in this study on a voluntary basis. These centres were selected in order to achieve a representative distribution of the national population of blood donors, as well as the enrolment of at least 15% (n =45,000) of the estimated 300,000 first-time blood donors recruited in Italy over a period of 1 year (April 2004-March 2005).

Screening protocol

Over the period of 1 year, each centre carried out anti-HBc testing, after informed consent, on all first-time blood donors. The same serum sample already obtained for the blood donor's mandatory serological assays was employed; in order to complete the serological picture for reporting purposes and counselling, anti-HBs testing on anti-HBc positive samples was also offered free of charge. Both markers were detected by commercial immunoassays on an AxSYM or ARCHITECT analyser (Abbott Diagnostics, Wiesbaden, Germany). Three aliquots of 1 mL each from samples that resulted positive for HBsAg and/or anti-HBc were stored frozen and shipped to the reference laboratory (Department of Biomedical Sciences for Health, University of Milan). Samples were anonymous and identified only by alphanumeric codes; additionally, each centre reported the total number of first-time donors admitted to the donation in the same period. Donors vaccinated against hepatitis B, according to the current Italian national programme, were excluded from the study.

Serological profile

At the reference laboratory, the serological profile for HBV infection was completed by testing each sample for HBeAg, anti-HBe, anti-HBs (if not tested at the screening sites) and anti-HBc IgM by commercial MEIA assays using an AxSYM analyser. Anti-HBs was considered positive when its level was >3 mIU/mL, corresponding to the functional sensitivity of the assay employed. The anti-HBc avidity[17] was also assayed by an in-house procedure. Briefly, two aliquots from each sample were diluted 1/10, one with the AxSYM working buffer (aliquot B) and the other with guanidine hydrochloride 1M (aliquot G); both aliquots were carefully mixed by vortexing, incubated at 22 °C for 30 minutes and then assayed for anti-HBc by the AxSYM. The avidity index (AI) was calculated as the reciprocal of the ratio of the S/CO value obtained for the G aliquot divided by the S/CO value obtained for the B aliquot. An AI <0.600 was considered as suggestive of a recent infection (within the preceding 6 months)[18]. Samples which were negative for anti-HBc by AxSYM at the 1:10 dilution were retested without dilution by another method (IMx Core, Abbott Diagnostics) in order to confirm the positivity for anti-HBc.

Detection and quantitation of hepatitis B virus DNA

All HBsAg and/or anti-HBc-positive samples were assayed for serum HBV-DNA by a commercial qualitative target amplification method validated for blood donation screening (Cobas Ampliscreen, Roche Molecular Systems, Branchburg, New Jersey, USA). In order to achieve the highest sensitivity allowed by this method (20 IU/mL), testing was performed on each individual sample -without pooling- and increasing the volume for extraction (500 µL). The specimens that resulted positive were further tested by a quantitative method (Cobas Amplicor HBV Monitor, Roche Molecular Systems, Branchburg, NJ, USA) to determine the viral load and amplified by a nested-polymerase chain reaction (PCR) in the S-region in order to determine the HBV genotype by phylogenetic analysis of the directly sequenced PCR products (length 485 bp).

Statistical analysis

The chi square test or chi square for linear trend test was used to compare the different prevalence rates. The Mann-Whitney test was used to compare the median values and 95% confidence intervals (95% CI) were calculated.

Results

A total of 46,147 first-time blood donors were initially enrolled in the 21 Blood Transfusion Services participating in the study. Of these, 27,749 (60.1%) were enrolled in the 13 centres located in northern Italy, 11,019 (23.9%) in the five centres in central Italy and 7,379 (16%) in the three centres in the south of Italy. Of these, 14,957 donors (32.4%) belonged to the age groups targeted by the Italian universal vaccination programme against hepatitis B and were, therefore, excluded from the study. Of the remaining 31,190 donors, 102 (0.33%)

tested positive for HBsAg, and 2,593 (8.3%) tested positive for anti-HBc and negative for HBsAg, for a total seroprevalence of HBV infection of 8.6% (2,695/31,190) (Figure 1).

The rates of anti-HBc positivity varied remarkably among different sites and geographical areas: the overall prevalence was significantly lower (P <0.005) in northern Italy (7.6%) and in the centre of the country (5.2%) than in the south (14.9%) (Table I). More in detail, the prevalence of anti-HBc antibody was unevenly distributed within the same geographical area and also within the same region, ranging from 0.6% to 14.5% in the north, from 3.1% to 6% in central Italy and from 7.4% to 20.8% in the south. A similar pattern was observed for rates of HBsAg positivity, which were 0.3% in the north, 0.2% in the centre and 0.5% in the south of the country.

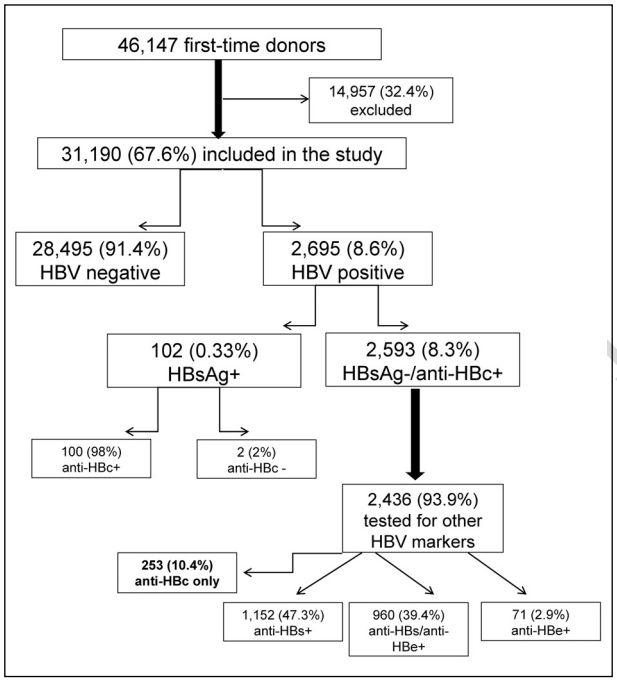


Figure 1 - Cumulative results of HBsAg and anti-HBc testing on first-time blood donors from the 21 Blood Transfusion Services.

Out of the total of 2,695 HBV-positive samples, 2,531 (93.9%) were sent to the reference laboratory and tested for additional HBV markers and for HBV-DNA. The rate of missing samples was almost identical for HBsAg/anti-HBc-positive cases (n=7; 6.9%) and for anti-HBc-positive cases without HBsAg (6.1%).

Of the 95 HBsAg-positive cases, 80.9% were males and 19.1% were females. Their median age was 42 years (range, 26-59 years); 89.1% were Italian and 10.9% were foreigners, the majority of whom coming from North Africa or from Romania. None was positive for HBeAg or anti-HBc IgM; 94.2% were positive for anti-HBe and 7.4% for anti-HBs at low concentrations (range 3-72 mIU/mL). The anti-HBc avidity was measured on 54 subjects and 23 of them (42.6%) had an AI <0.600. Three samples were anti-HBc negative on both diluted aliquots and, when retested undiluted, one was positive and two were negative. Notably, one of the latter (from a 28-year old male donor from northern Italy) showed strong positivity for HBsAg, which was confirmed by neutralization. HBV-DNA was detectable in 92/95 cases (96.8%), with levels ranging from <6 to 53×10^6 IU/mL and a median value of 456 IU/mL. Both HBsAg-positive donors negative for anti-HBc were also HBV-DNA-negative. Phylogenetic analysis, performed on 88 of the 92 HBsAg/HBV-DNA positive samples (95.7%), showed that 76 (86.4%) had genotype D, while the remaining 12 (13.6%) had genotype A. Finally, 3.4% of subjects had raised alanine aminotransferase levels (>1.5 times the upper limit of normal), and one of them was co-infected with the hepatitis C virus.

Among the 2,436 anti-HBc positive samples sent to the reference laboratory, the most frequent pattern was the association of anti-HBc with anti-HBs (47.3%), followed by the same two markers plus anti-HBe (39.4%), by anti-HBc alone (10.4%) and by anti-HBc plus anti-HBe alone (2.9%) (Figure 2). Among anti-HBs positive donors, 5.4% had antibody levels below 10 mIU/mL, 30.7% between 10 and 100 mIU/mL and the remaining 63.9% had antibody levels >100 mIU/mL.

Testing for circulating HBV-DNA was carried out in 2,186 out of 2,436 (89.7%) anti-HBc-positive and HBsAg-negative cases. Initially, 15 samples were found to be HBV-DNA-positive by Ampliscreen; however,

Table I - Study population and cumulative results of HBsAg and anti-HBc testing on first-time blood donors from the 21 Blood Transfusion Services according to geographical area.

Area	Total donors	HBsAg−/anti-HBc+		HBsAg+/anti-HBc+		HBV markers positive	
		N.	% (range)	N.	% (range)	N.	% (range)
North	19,064	1,448	7.6* (0.63-14.5)	64	0.34 (0-1.45)	1,512	7.9 (0.63-15.6)
Centre	6,789	352	5.2# (3.1-6.0)	11	0.16 (0.07-0.45)	363	5.3 (3.3-6.2)
South	5,337	793	14.9*# (7.4-20.8)	27	0.51 (0.4-0.7)	820	15.4 (7.8-21.4)
Total	31,190	2,593	8.3	102	0.33	2,695	8.6

Legend *p <0.005; #p <0.005.

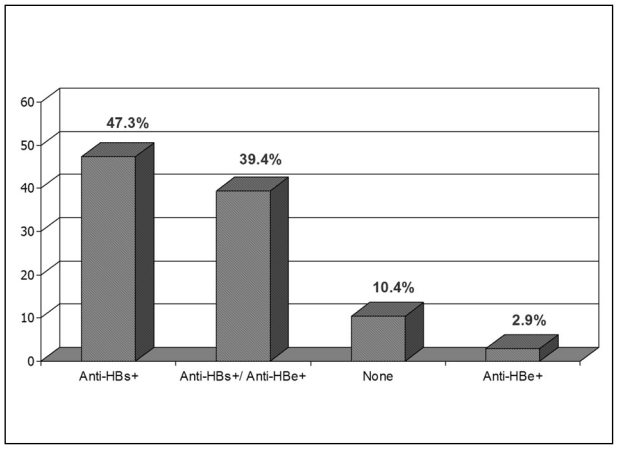


Figure 2 - Serological patterns of HBV markers in 2,436 Italian first-time blood donors negative for HBsAg and positive for anti-HBc.

only 12 of these cases were confirmed by TaqMan and nested-PCR, accounting for a total prevalence of 0.55% (12/2,186). Of these, seven were also anti-HBs-positive (median 63 mIU/mL; range 3.5-114 mIU/mL) and one was anti-HBc-positive alone. The viral load ranged from <6 to 646,854 IU/mL; the median HBV-DNA levels among the HBsAg-negative samples were significantly lower than median levels observed among HBsAg-positive samples (38 IU/mL *vs* 456 IU/mL; P <0.05).

As shown in Table II, both the frequency (1.68% *vs* 0.37%) and the median levels (745 *vs* 16.5 IU/mL) of HBV-DNA were higher among the subset of 298 donors negative for anti-HBs than among the subset of 1,888 donors positive for anti-HBs, but only the positivity rates were significantly different (P <0.01). Finally, in the subgroup of donors positive for both anti-HBc and anti-HBe (n =57), the positivity rate was ten times higher (7.02%) than in the whole group of anti-HBc-positive subjects (p <0.01).

Table II - Results of HBV-DNA testing on 2,186 first-time blood donors positive for anti-HBc and negative for HBsAg, according to the serological profile.

HBV marker positive	N. of donors	HBV DNA +		
		N.	%	Median titre IU/mL
Anti-HBc/ anti-HBe/anti-HBs	802	2	0.25	23.5
Anti-HBc/anti-HBs	1,086	5	0.46	26
Subtotal anti-HBs+	*1,888*	*7*	*0.37**	*16.5#*
Anti-HBc/anti-HBe	57	4	7.02§	1,339
Anti-HBc	241	1	0.41	5
Subtotal anti-HBs−	*298*	*5*	*1.68**	*745#*
Total	2,186	12	0.55§	38

Legend *p <0.01; #p =0.222; §p <0.01.

The phylogenetic analysis performed in 10 out of 12 HBV-DNA-positive samples (83.3%) showed that all sequences clustered with HBV genotype D, *vs* 86.4% among HBsAg-positive cases (P =0.51 by Fisher's exact test). Compared with a consensus sequence of the same genotype, these strains showed some amino acid substitutions in the MHR region of the S gene (Figure 3). The 12 HBV-DNA-positive/HBsAg-negative samples were sent to an external laboratory for testing by a new generation HBsAg assay (Abbott ARCHITECT HBsAg II, Abbott Diagnostics) with increased analytical sensitivity. Four of those (33.3%) then resulted positive for HBsAg, and one was the sample with a high HBV-DNA load (646,854 IU/mL).

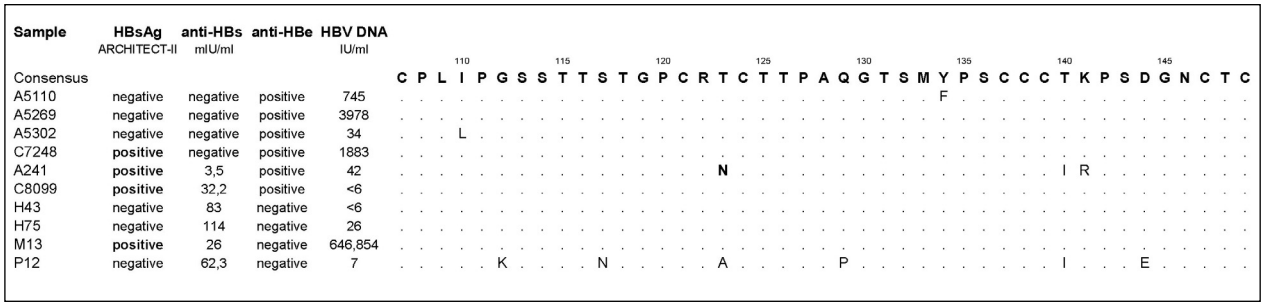
Of the 2,400 samples available for anti-HBc avidity testing, 811 (33.8%) tested negative for anti-HBc on one or both the aliquots diluted 1:10 for the AI determination. Of those, 671 were anti-HBc positive when retested undiluted by IMx, while 140 (17.3%) were negative and were then considered as false positive for anti-HBc at the initial screening. The rate of false positives was significantly higher (p <0.005) among donors reactive only for anti-HBc (49.8%) than among donors positive also for other HBV markers (5.7%). The frequency of false positives among samples reactive for anti-HBc was below 12% in all Centres except two, one in the north of Italy (19.2%) and the other in the south of the country (38.8%) but even adjusting for this factor the prevalence rates of HBV infection in the three areas did not change significantly. According to these results, the specificity of the anti-HBc assays employed at the blood screening sites was 99.48% (95% CI: 99.39-99.57%).

Anti-HBc avidity was measured in 2,260 specimens: a low AI was found in 245 (10.8%), which was significantly (p <0.005) lower than among HBsAg-positive subjects.

The frequency of low AI results was higher (11/54; 19.3%) in the subgroup of specimens reactive only for anti-HBc and anti-HBe and was also significantly different between the specimens positive for anti-HBe and those negative for anti-HBe (16.9% *vs* 5.9%; p <0.01) (Table III).

Discussion

An OBI is defined by the presence and persistence of a circular, episomic form of HBV-DNA (cccDNA) in the nucleus of infected cells in the absence of HBsAg in serum and with circulating HBV-DNA <200 IU/mL[13]. This new biological entity has implications for the screening of blood donations. The possible transmission of HBV by HBsAg-negative blood or blood products has been well documented[1,19], and the need to add testing for anti-HBc to increase blood safety has been envisioned[14-16].



Sample	HBsAg ARCHITECT-II	anti-HBs mIU/ml	anti-HBe	HBV DNA IU/ml	110					115					120					125					130					135					140					145				
Consensus					C	P	L	I	P	G	S	S	T	T	S	T	G	P	C	R	T	C	T	T	P	A	Q	G	T	S	M	Y	P	S	C	C	C	T	K	P	S	D	G	N	C	T	C
A5110	negative	negative	positive	745	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A5269	negative	negative	positive	3978	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A5302	negative	negative	positive	34	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C7248	**positive**	negative	positive	1883	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A241	**positive**	3,5	positive	42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**N**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	R	.	.	.	.	.	.	.
C8099	**positive**	32,2	positive	<6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H43	negative	83	negative	<6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H75	negative	114	negative	26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M13	**positive**	26	negative	646,854	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P12	negative	62,3	negative	7	.	.	.	.	.	K	.	.	.	.	N	.	.	.	.	A	.	.	.	.	P	.	.	.	.	.	.	.	.	.	.	I	.	.	.	E	.	.	.	.	.

Figure 3 - Aligment of amino acid sequences of the S region (MHR, amino acids 107-149) of HBV obtained from 10 first-time donors who were HBsAg negative/anti-HBc positive. The substitution T123N (in bold) is known to be involved in lower HBsAg assay reactivity.

Table III - Anti-HBc avidity index (AI) in samples from 2,260 first-time blood donors positive for anti-HBc and negative for HBsAg, according to the serological profile.

HBV marker positive	N. of donors	AI <0.600	
		n.	%
Anti-HBc/anti-HBe/ anti-HBs	960	161	16.8
Anti-HBc/anti-HBe	57	11	19.3
Subtotal anti-HBe+	*1,017*	*172*	*16.9**
Anti-HBc/anti-HBs	1,102	66	6
Anti-HBc	141	7	5
Subtotal anti-HBe−	*1,243*	*73*	*5.9**
Total	2,260	245	10.8

Legend *p <0.01.

On the other hand, three factors have cast doubts on the feasibility and sustainability of screening blood donations for anti-HBc in addition to HBsAg: (i) the evidence that only a small fraction of anti-HBc-positive donors are infectious (e.g. those with detectable HBV-DNA in the blood)[9,10,15,20-22]; (ii) the relatively high frequency of non-specific results for anti-HBc in the screening of a healthy population[10,16,19,23]; and (iii) the relevant number of blood donations that will be lost because of the high prevalence of anti-HBc in areas at medium- high endemicity for HBV and the relative frequency of non-specific results.

In this study we included more than one fifth of first-time donors enrolled in Italy in one calendar year, a sample size assuring that the selected population is representative of the Italian first-time donor population. The total prevalence of HBV markers in this survey (8.9%) was much lower than prevalences found in studies performed in Italian blood donors in the past[20,21] and consistent with the general decrease in the prevalence of HBV infection in the whole Italian population[22].

The serological frequency of HBV-DNA positivity among HBsAg-negative/anti-HBc-positive donors was indeed low (0.55%) and consistent with the findings of the majority of other published studies[10,15,23,24]. However, a recent survey carried out in the north-west of Italy[25], found a higher (4.9%) positivity rate for HBV-DNA despite the prevalence of anti-HBc in first-time blood donors being somewhat lower than that found in the present study (4.8% *vs* 8.3%). This discrepancy may be due either to a different local epidemiological situation or to the different serological patterns in the two studies, as we noted a much higher prevalence of viraemia among the donors who were also positive for anti-HBe. The differences in the analytical performances of the nucleic acid amplification assays should also be taken in account[26,27], although the assay used in our study has a very high sensitivity and was the same as that used by Manzini *et al*[25].

With regards to the frequency of non-specific anti-HBc results, retesting of more than 800 initially reactive samples at the reference laboratory yielded a positive predictive value of 83.5% and a specificity of 99.48%, which is somewhat better than previously reported results in similar settings[16,28]. Finally, the overall prevalence of HBV markers was nearly 9%, thus the loss of donors possibly discarded because of anti-HBc positivity is still too high to allow anti-HBc detection to be implemented on a routine basis, in our country.

The recent addition of HBV-DNA to HBsAg testing, now established in Italy as well as in most western countries, has further reduced the risk of transfusion-transmitted HBV and has also greatly reduced the relevance of serological testing for preventing the transmission of HBV by blood products[15,24,25,29]. However, although HBV-DNA is the most reliable marker of active viral replication, there is some conflicting evidence concerning the infectivity of blood products contaminated by HBV-DNA. For example, Satake *et al.*[30] failed to document the transmission of HBV infection to the recipients of HBV-DNA-positive blood from donors positive for anti-HBc and anti-HBs, possibly because of viral neutralisation by circulating antibodies. Several

international studies[8,12,15,23,24,31], as well as five studies carried out in Italy[9,25,32-34], have demonstrated that while the samples collected in the infectious window phase usually show very high levels of HBV-DNA, those collected in the post-acute phase of infection often showed very low levels (<100 IU/mL) and can, therefore, be missed when testing is carried out in large pools or by less sensitive assays for HBV-DNA[26,27]. Recently, Stramer *et al.*[35] reported the results of screening for HBsAg, anti-HBc and HBV-DNA in about 6.5 million blood donations in the USA. This study showed that 85% of HBV occult infections were non-reactive when tested in mini-pool NAT, while they became reactive when tested by individual donor polymerase chain reaction. Our experience is consistent with these data, as the majority of HBsAg-negative/HBV-DNA positive blood donors had low levels of viraemia. It is worth noting that the molecular assays currently authorised in Italy for screening blood components for HBV-DNA have an analytic sensitivity of 3.7-10.4 IU/mL and the clinical sensitivity of the two available tests (Roche in pools of six and Novartis in single specimens) is comparable[35,36]. While the infectivity of blood products containing very small amounts of HBV-DNA, especially in the presence of neutralising anti-HBs antibodies, has been questioned[37], very recently Vermeulen *et al.*[38] documented the transmission of HBV infection by a single donation collected from a regular donor in the very early window phase. It should be noted that this donation was negative by individual donor NAT, meaning that "zero risk" for HBV is yet to come[26].

We also tried to evaluate the avidity of anti-HBc in order to identify recently infected individuals. In our experience, 10% of all anti-HBc-positive donors had a low antibody avidity, and both low avidity and HBV-DNA were more frequently found in the subgroups of donors positive for anti-HBc and anti-HBe and negative for anti-HBs, a serological pattern more readily found in the so-called "core window" period occurring some weeks or months after a primary infection. Several studies carried out in Italy, Spain, South Africa and in the USA have determined that the majority of HBsAg-negative, HBV-DNA-positive donors have an occult infection[9,12,36,39], with very few of them being in late convalescent phase or in early seroconversion[11]. In our study, the only possible seroconversion was the case of a young donor with confirmed positivity for HBsAg and negativity for anti-HBc, although we failed to detect the presence of HBV-DNA in the available sample.

The occurrence of the "HBsAg alone" pattern has been recently reviewed[40] and the frequency of its detection may increase with the availability of more sensitive HBsAg assays[35]. On this issue, it is noteworthy that four of the 12 HBV-DNA-positive specimens initially classified as OBI in our study were indeed positive for HBsAg by a novel assay with a greater analytical sensitivity assay (0.02 IU/mL *vs* 0.05-0.08 IU/mL) than the sensitivities of the assays employed at the various study sites[41].

In conclusion, from our data it emerges that over 30% of the first-time donors initially enrolled in the study were vaccinated against hepatitis B, according to the Italian vaccination programme. Due to the long-term immunogenicity and the high effectiveness of hepatitis B vaccination, such donors should be considered protected and at no risk of transmitting HBV infection to the recipients of their donated blood. Of the remaining two-thirds of first-time donors participating in this study, nearly 9% had anti-HBc antibody as a marker of previous or ongoing HBV infection.

It is noteworthy that 0.3% of such anti-HBc positive donors were also positive for HBsAg while 0.55% had a serological profile consistent with a diagnosis of OBI (HBsAg-negative in the presence of low levels of HBV-DNA).

These findings strongly support the use of HBsAg testing together with NAT as the policy of choice for screening donors in order to further reduce and prevent HBV transmission. Current HBsAg assays have a much greater sensitivity than the ones available until a few years ago[41], but nevertheless an improvement is still needed as escape mutations associated with OBI may also lead to decreased reactivity in HBsAg detection[26,42]. The "a" region of MHR is formed of 42 amino acids (107-149) whose specific secondary structure is maintained by cross-linking between eight cysteines, 121, 124, 137, 138, 139, 147 and 149. Substitution of cysteine residues disrupts this structure and considerably reduces recognition by HBsAg assays, sometimes giving false negative results[42,43]. Another factor to consider is impaired production of HBsAg which has been documented in cases of OBI. This phenomenon could have been involved in one donor who resulted negative for HBsAg despite a very high viral load: similar, transient cases were reported in another blood donor[44], who had a ratio of HBV to HBsAg subviral particles of less than 1 to 20 whereas in overt cases the ratio is 1 in more than 1,000, and more recently by Foy *et al.* in a dialysis patient[45], who was vaccinated against hepatitis B, although in this case discrepant results for HBsAg were obtained according to the assays used.

The detection of anti-HBc antibody is currently not recommended in Italy since the relatively high prevalence in the absence of other serological markers of infection (HBsAg and/or HBV-DNA) would currently lead to the refusal of an unacceptable number of donors[46]. Continuous mandatory vaccination of all neonates against hepatitis B will contribute to create

new generations of donors free of HBV, thus further increasing the safety of donated blood.

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Conflict of interest disclosure

Dr. Claudio Galli is currently employed as the Scientific Affairs Manager at Abbott Diagnostics Italy, the other Authors declare no conflicts of interest.

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