A statistical model based on serological parameters for predicting occult HBV infection: implications for organ/ blood donations

Sabrina Coen1, Claudio Angeletti2, Pierluca Piselli2, Michele Tronchin3, Donatella Vincenti1, Maria Rosaria Capobianchi1, Claudio Galli3, Stefano Menzo1,4
1Laboratory of Virology, National Institute of Infectious Diseases “L. Spallanzani”, Roma, Italy; 2Department of Epidemiology, National Institute of Infectious Diseases “L. Spallanzani”, Roma, Italy; 3Abbott Diagnostics Division, Roma, Italy; 4Department of Biomedical Sciences, Università Politecnica delle Marche, Ancona, Italy

INTRODUCTION

Occult hepatitis B is defined as the presence of persistent HBV infection in the face of serological clearance of HBV surface antigen (HBsAg). This phenomenon has been associated with the persistence of a limited number of infected cells which survive the immune system by bearing either scarcely active cccDNA or inactive HBV DNA integrated in the cell chromosomes (Lai et al., 2003). In a minority of these patients, viral production is sufficient for detection as circulating HBVDNA in peripheral blood (Al-lain and Cox, 2011). The clinical significance of occult HBV infection is still unclear (Raimondo et al., 2008). Despite the attempts of many research groups to correlate this condition to an enhanced risk for liver cirrhosis or liver cancer, consistent data are still lacking, and the results appear biased by confounding factors, such as the duration of the previously active infection, coinfections, immunosuppression, and HBsAg mutations. Occult hepatitis B infection can sometimes reactivate, as has been well-documented in immunosuppressed patients (Lubel and Angus, 2010), and it can also be transmitted to other individuals by means
of blood transfusion or organ donation (Inaba et al., 2006; Riediger et al., 2007; Satake et al., 2007; Allain et al., 2013). In the great majority of immunocompetent individuals, the presence of residual replicating HBV is accompanied by the persistence of anti-HBc antibodies that are therefore the most reliable low-cost and easily accessible single marker of potential occult HBV infection. Anti-HBc testing is currently included in the guidelines for the screening of blood, tissue and organ donors in many countries with low HBV prevalence, but while anti-HBc positive blood donors are usually excluded, they are considered suitable for organ and tissue donation. In Italy, anti-HBc testing is discretionary for blood donations (Velati et al., 2011), and anti-HBc positive, HBsAg-negative individuals are considered valid donors of any organ (not tissue), excluding the liver (which can only be assigned for emergency interventions or to previously HBV-positive recipients). Unfortunately, this approach leads to a few de novo HBV infections, especially in kidney recipients, for whom prophylaxis is currently not suggested (Dhillon et al., 2009). A possible solution to this problem is to perform HBV nucleic acid (NAT) screening systematically to directly identify circulating HBV-DNA positive donors, which should advise prophylaxis in the recipient. However, some evidence currently exists that also HBV DNA-negative anti-HBc-positive donors have transmitted the infection (Inaba et al., 2006; Vermeulen et al., 2014). Another possibility would be to gather and use additional information from the combination of an array of serologic markers. This could represent a valuable and economic tool (including blood donations) in settings with limited resources, where NAT is not performed and HBV infection is common and anti-HBc is performed (Dhawan et al., 2008), but could also add complementary information for organ donations in settings where NAT is performed. Among potentially useful markers, a central role could be played by anti-HBc antibody levels as a means for stratifying donors for the risk of carrying circulating HBV DNA. Anti-HBc antibody titer might be a surrogate marker of the probability of finding circulating HBV DNA, as has been demonstrated in the case of HCV antibodies for HCV RNA (Bossi and Galli, 2004; Gale et al., 2011). Extension of this concept may also provide clues to the probability of carrying functional HBV virions, even at levels undetectable by current NAT techniques. A more elaborate evaluation of serological markers may also help to define with greater precision the risk of endogenous HBV reactivation in patients who will be subjected to a immunosuppressive regimen, such as transplant recipients, oncologic patients or those with autoimmune disease. Quantitative evaluation of anti-HBc antibodies, together with that of other traditional serologic markers of HBV infection, might reveal yet unknown diagnostic or prognostic properties. The aim of this study was to analyze the correlation of anti-HBc antibody titer with other HBV serological and molecular markers and other patient characteristics to investigate the potential usefulness of this approach for the screening of blood/tissue/organ donors and for other clinical applications.

MATERIALS AND METHODS

Patients and selection criteria
All anti-HBc positive samples consecutively collected and tested at the Virology laboratory of INMI “L. Spallanzani” Hospital from February to June 2010, also subjected to HIV (or with known HIV status) and HCV testing, were included in the study. In order to minimize the inclusion of samples from patients undergoing anti-HBV treatment, or pharmacologically immunosuppressed subjects, the following exclusion criteria were adopted:
1) previous HBV testing or any hematological or biochemical testing already present in the lab archives, back to 2001;
2) a concomitant request for HBV DNA or HBV drug resistance.

These criteria also avoided multiple sampling from the same subject. This of course cannot be considered a “general anti-HBc positive population” comparable to donors, but, predictably, it should include acute infections, inflammatory and metabolic disorders of all sorts, and among these, active forms of hepatitis, either chronic HBV or HCV infections. However, the study of occult HBV infection in these subjects should not suffer from unacceptable biases for the pur-
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In an additional part of the research, samples from HIV-infected patients were compared to HIV-negative subjects to understand how HBV serology is conditioned by the virus-immune system interplay. In this group HBV-DNA was not evaluated because it was assumed that an unknown but significant (>80%) proportion of these patients were subjected to antiretroviral (and hence almost inevitably also anti-HBV) treatment.

Serologic and molecular testing
All serologic testing was performed by the CMIA technique on the Architect platform (Abbott Diagnostics, Wiesbaden, Germany). The signal to cutoff (S/CO) indexes for anti-HBc and anti-HBe (competitive assay) were considered for quantitative analysis. The system provides standardized quantitative results for HBsAg and anti-HBsAg, expressed as International Units and milli-International Units (IU/ml and mIU/ml), respectively; values exceeding 0.04 IU/ml and 10 mIU/ml were considered positive for HBsAg and anti-HBsAg respectively. Molecular testing was performed by a commercial real-time amplification technique (Abbott Molecular, Wiesbaden, Germany) on an m2000 integrated system (Ismail et al., 2011). The technique provides quantitative results above 10 IU/ml (1 IU=3.41 HBV genomic equivalents), but allows qualitative detection of HBV DNA also in samples with lower concentrations. In addition, an in-house real-time amplification method for HBV-DNA was developed and used on the same samples. This assay is based on the amplification of a 112 base pair sequence in a conserved region of the core gene (primers: sense-AGGCAG-GTCCCCCTAGAAG, antisense-TGAGTCCAAG-GAATACATAC, as synthesized), detected by a fluorescent hydrolysis probe (AGAUCTCCCTC-GCCTCGCAGAC). The assay sensitivity (LOD) was evaluated on a series of dilutions of the Abbott standard samples (probit analysis 95% confidence interval) at 18 IU/ml.

Statistical analysis
When not specifically stated, differences in the averages of quantitative parameters were calculated by the Mann-Whitney/Wilcoxon test, differences in frequencies by the chi square test or Fisher exact test where required. A logistic regression model was used to evaluate the role of selected laboratory and clinical parameters (Anti-HBc, anti-HBs, anti-HBe, anti-HBc IgM) as predictors of HBV DNA presence in the peripheral blood.

A penalized maximum likelihood approach proposed by Firth (1993) was used in fitting the logistic model. This approach is a general method to reduce the bias in maximum likelihood estimates that converges to zero as the sample size increases. The phenomenon of “separation”, that is the presence of one or more covariates perfectly predicting the dichotomous outcome, can be considered an extreme case of small sample bias. In fact, data sparseness causes empty cells in the 2x2 table formed by the outcome and the covariate, with parameter estimates becoming infinite. The penalized maximum likelihood approach yields finite estimates of Odds Ratios (OR) despite the ‘separation’ problem (Heinze and Schemper, 2002; Heinze 2006).

All tests were two tailed and p-values less than 0.05 were considered statistically significant. For all statistical analyses we used STATA 10.1 statistical software (Stata Corp, College Station, TX, USA), in particular the firthlogit package was used for fitting the penalized maximum likelihood logistic regression model.

RESULTS

Quantitative serology of HBV markers and impact of HIV infection
A total of 723 anti-HBc-positive subjects (461 HIV-seronegative and 262 HIV-seropositive), were selected for the study. To understand the dynamics of anti-HBc serology and its relationship with other serological markers of HBV infection, the anti-HBc S/CO value was associated with the qualitative values of HBsAg, anti-HBs and anti-HBe. For this analysis, serological results from 461 consecutive (February-June 2010) HIV-negative subjects were included, comprising 282 HBsAg-negative and 179 HBsAg-positive subjects. HIV-positive patients (n=262 from the same year) were also studied as a comparative group to gain insight in the effects of HIV infection on HBV sero-
logical markers. HBsAg-positive subjects were significantly younger than HBsAg-negative subjects (43 vs 51 years, p<0.001), in agreement with the known trend towards spontaneous HBsAg clearance. In order to investigate the effect of ongoing expression of HBV genes in the liver (HBsAg production) on anti-HBc antibody response, HBsAg-negative and positive subjects were compared for the S/CO index. HIV-positive patients were analyzed separately. Figure 1 shows the distribution of anti-HBc S/CO values in four distinct populations based on HBsAg and HIV status. HBsAg-positive subjects displayed a different distribution of anti-HBc values, resulting in significantly higher anti-HBc S/CO values both in HIV-negative and HIV-positive patients (Figure 2A). HBsAg/HIV-positive patients showed generally lower anti-HBc S/CO values than their HIV-negative counterparts (9.2 vs 10.9, p<0.0001). Surprisingly, when HBsAg-positive subjects were further stratified for the presence of HBeAg (Figure 2B), anti-HBc S/CO values resulted higher in HBeAg-negative subjects, as if the extremely high concentration of HBeAg could partially mask anti-HBc antibodies, similarly to anti-HBe or anti-HBs. In HBsAg and HIV-negative subjects, anti-HBc S/CO values were associated with the presence of the other serological markers of HBV infection. Anti-HBs positive subjects displayed only slightly higher S/CO values (8.4 vs 7.4, not significant), while anti-HBe-positive subjects had significantly higher anti-HBc S/CO values compared to anti-HBe-negative ones (9.5 vs 6.7, p<0.0001). This was true also for anti–HBc IgM-positive subjects (9.5 vs 7.9, p=0.05), albeit
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anti-HBc IgM-positive subjects accounted for only 10 out of 282. The close relationship between anti-HBc and anti-HBe was also demonstrated by a direct correlation between the respective S/CO values in double positive subjects (R²=0.22, p<0.001). Despite the generally lower anti-HBe S/CO values, the same pattern was also true for HIV-positive patients. In addition to the lower production of anti-HBc antibodies, HIV-positive HBsAg-negative patients also displayed a significantly lower frequency of anti-HBs positivity (62% vs 74%, p=0.0036) and anti-HBe positivity (31% vs 43%, p=0.0144) and significantly lower anti-HBs titers compared to HIV-negative subjects (respectively 493 vs. 653 mIU/ml, p=0.032 and 0.451 vs. 0.304 S/CO values, p=0.005).

HBV DNA in the peripheral blood of anti-HBc positive, HBsAg-negative subjects

In order to establish a relationship between occult HBV infection (in peripheral blood) and serological response, HBV DNA was evaluated in the 282 HIV/HBsAg-negative subjects, among the mentioned anti-HBc-positive population. HIV positive-subjects were excluded from this analysis because it was assumed that an unknown but significant (>80%) proportion of these patients were subjected to antiretroviral (and hence almost inevitably also anti-HBV) treatment. 12/282 patients showed a positive result for peripheral HBV DNA (4.2%) by the Abbott assay. Viral load ranged from 994 IU/mL to “detected” (i.e. less than 10 IU/mL), with a median value of 27 IU/mL. An alternative in-house HBV DNA assay for a different genomic segment was used to overcome potential problems due to viral genetic variability. This test successfully amplified 9 samples but failed to yield a positive result in 2 of the 4 samples with HBV DNA detected below 10 IU/ml by the Abbott assay, revealing a slightly lower sensitivity. The one successfully amplified was quantified as 9 IU/mL. No additional sample resulted positive compared to the Abbott assay. Table 1 shows the virological parameters in these subjects. In 2 out of 12 HBVDNA-positive subjects, anti-HBe was the only serological marker present, while 6 were anti-HBsAg-positive, 7 were anti-HBe-positives and 2 were anti-HBc IgM-positive. In the patients studied, no HBV DNA was found in patients with anti-HBc S/CO values below 7.

HBV DNA was strongly associated with anti-HBc IgM: only 12 out of the 282 subjects were positive for anti-HBc IgM, and HBV DNA was detectable in 2 of them, 16.7%, compared to 3.7% in anti-HBc IgM-negative subjects (p=0.01). Anti-HBc S/CO index was quantitatively evaluated, and resulted higher in HBV DNA-positive than in HBV DNA-negative subjects (respectively: 9.9, range 7.65-11.3, vs 7.9, range 1.16-13.3; p=0.045) and all HBV DNA-positive subjects showed medium to high titers (>7.5 S/CO) of anti-HBc, as shown in Figure 3. Although low anti-HBc S/CO values (<7)

<table>
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<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>HBV DNA IU/ml</th>
<th>Anti-HBc S/CO</th>
<th>Anti-HBs mIU/ml*</th>
<th>Anti-HBe</th>
<th>Anti-HBc IgM</th>
<th>Score§</th>
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*Positive above 10 mIU/ml. §Probability of HBV DNA positivity calculated by the logistic regression model.
can reasonably predict HBV DNA negativity in immunocompetent subjects, these values are found only in a minority of anti-HBc-positive, HBsAg-negative patients, and other HBV serological parameters were explored as possible complementary predictors. HBV DNA proved twice as frequent in anti-HBe-positive subjects than in negative subjects (6% vs. 3%), although this difference was not statistically significant. In addition, HBV DNA-positive subjects displayed a lower average of the S/CO index for anti-HBe antibodies (0.13 vs 0.31; not significant), meaning higher titers by this assay (competitive, hence lower values). Interestingly, none of HBV DNA-positive subjects were positive for anti-HCV antibodies and the frequency of anti-HCV positivity was significantly different between HBV DNA-positive and negative subjects (0/78 vs 12/204, 5.9% p=0.040, 2 tailed Fisher exact test). In addition, in HBsAg-negative/anti-HBc-positive subjects (all HIV negative), the frequency of both anti-HBs and anti-HBe positivity was significantly lower in HCV positive than in negative subjects (64% vs 78% and 25% vs 49% respectively; p=0.03 by chi square). By multivariate analysis all the serological parameters analyzed (anti-HBc, anti-HBs, anti-HBc/IgM and anti-HCV) showed a p-value less than 0.2 in their association with the presence of HBV DNA, except anti-HBe, that was excluded from further analysis (Table 2). The quantitative evaluation of anti-HBs did not add significance compared to the qualitative evaluation (not shown) and was not included in the analysis. A multivariate logistic model was therefore used to evaluate whether these combined parameters could achieve a better predictive value for the presence of HBV DNA than anti-HBc alone. Anti-HCV was maintained in the model as potentially useful for allocating anti-HBc+, HBsAg-, HCV+ organs to HCV+, HBV- recipients. The discrimination ability of this model was assessed using the area under the receiver operating characteristics (ROC) curve, whereas its calibration was evaluated by comparing predicted and observed probabilities of a positive outcome stratifying the studied population according to the values of predicted probabilities. Figure 4 shows the ROC curve for the estimated model, including 4 covariates (pan-
el A): anti-HBc S/CO (quantitative), anti-HBc IgM, anti-HBsAg and anti-HCV (all qualitative). Panel B shows the calibration curve of observed versus predicted risk. By this model, HBV DNA-positive subjects displayed an average score (indicative of the probability of being HBV DNA positive) of 0.15, compared to 0.04 of negative subjects (p<0.0001, Wilcoxon test), and a fair predictive value for HBV DNA positivity (AUC=-0.85) was observed. Based on the prevalence of HBV DNA-positive tests observed in the study population and on the score derived from the logistic model, we computed the negative predictive value (NPV) and the proportion of patients potentially excluded from a donation as a function of the score cut-off variation (Figure 4 C).

DISCUSSION

HBV infection can be transmitted by blood, tissue or organ donation by HBsAg-negative subjects, carriers of occult HBV infection (Hennig et al., 2002; Kleinman et al., 2003; Inaba et al., 2006; Manzini et al., 2007; González et al., 2010; Zheng et al., 2011; Vermeulen et al., 2014). The risk of transmission, except by liver donation where the potential presence of intrahepatic cccDNA greatly enhances the risk, is presumably posed by complete HBV virions in the peripheral blood of these subjects, and is therefore possibly correlated to the amount of peripheral HBV DNA. This study investigated the association between HBV serological parameters and the presence of circulating HBV DNA in anti-HBc-positive, HBsAg-negative individuals, to identify predictors of its presence. To achieve this goal, the semiquantitative titers of anti-HBc (Han et al., 2011) and anti-HBe, conventionally considered only qualitative markers, were evaluated along with anti-HBs titers and antiHBc IgM positivity in order to build a logistic prediction model. This proof-of-concept approach is of course limited by the fact that it is calibrated on the Abbott CMIA diagnostic platform S/CO values, as current diagnostic systems do not provide quantitative results based on International Units for anti-HBc and anti-HBe. In addition, to understand the meaning of generally neglected (in terms of risk assessment) HBV serological markers, we took advantage of the study population to focus on the general dynamics and on the interdependence of their titers in the context of the natural history of HBV infection, including the impact of HIV infection. The results have clearly shown that a higher risk of carrying HBV DNA is associated with higher anti-HBc S/CO values, the presence of anti-HBe, anti-HBc IgM and the absence of anti-HBsAg antibodies. The value of qualitative anti-HBc testing has already been established (Hoofnagle et al., 1978; Allain et al., 1999), while the higher frequency of HBV DNA in HBsAg-negative donors with high anti-HBc...
antibody titers was already observed in donors from Venezuela (Gutierrez et al., 2004). Here we confirm the observation, provide a statistical analysis of the phenomenon, and by showing, like others (Taira et al., 2013), that anti-HBc by itself is not a sufficiently reliable predictor of risk, we extend the analysis to anti-HBs, anti-HBe and anti-HBc IgM.

In a simple interpretation, anti-HBc and anti-HBe may relate to the presence of HBV DNA as the ongoing expression of all viral proteins (which in turn is necessary for the production of viral particles), albeit in minimal amounts, that clearly serves as an antigenic stimulus for antibody production. Thus, ongoing replication of HBV apparently stimulates the production of anti-HBc antibodies. This was evident when comparing the high anti-HBc and anti-HBe (in HBeAg-negative patients) titers of HBsAg carriers with the lower ones of HBsAg-negative subjects, but the study also showed that among HBsAg-negative subjects, HBV DNA carriers had significantly higher anti-HBc titers than HBV DNA-negative subjects. The fact that anti-HBV antibodies are relatively diminished in HIV patients compared to the HIV-negative HBV-infected population, further suggests that antibody production is continuously modulated by T helper cells and hence by an ongoing antigenic stimulus. As previously observed, anti-HBc IgM are independently associated with the probability of finding HBV DNA in HBsAg-negative subjects. Possibly, in recent infection (or reactivation), HBV DNA may persist longer than detectable HBsAg in the peripheral blood of some patients and anti-HBc IgM should be regarded as a reliable alarm bell for circulating HBV DNA in HBsAg-negative patients.

By contrast with these positively associated markers, the presence of HBV DNA was shown to be preferentially associated with the absence of anti-HBs antibodies, in agreement with previous work (Han et al., 2011; Romanò et al., 2013). In the context of relatively low concentrations of both antigens and antibodies, this might be explained by the fact that HBsAg may mask anti-HBs antibodies, similarly to what is observed in active hepatitis B patients until seroconversion. In addition, as in most occult HBV infections the HBsAg sequence is heavily mutated, it may escape detection due to reduced antibody binding “false” HBsAg negatives (Svicher et al., 2012; Lieshout-Krikke et al., 2014), or its secretion may be impaired (Biswas et al., 2013), leading to failure to detect the presumably low amounts of free circulating antigen.

An intriguing unexpected result from these subjects is the strong negative association between HCV positivity and HBV DNA and serological parameters in HBsAg-negative HBV-infected subjects. Although with a limited importance for blood or tissue donation, this notion may be useful for allocating HCV-positive, anti-HBc-positive organs to HCV-positive HBV-negative patients and for evaluating the risk of endogenous OBI reactivation in HCV-positive immunosuppressed patients. The results on this aspect of HCV-HBV coinfection fill a knowledge gap concerning the effect of HCV on the outcome of HBV infection, indicating that specific immunological investigations are required for a better comprehension of the interplay between the responses against the two viruses. Generally speaking, because of some common infection pathways, the HCV-positive population has a higher risk of being infected by HBV than the HCV-negative population (Hatzakis et al., 2011). However, the results from this study indicate that once infected with HBV, HCV-positive subjects clear HBV infection more radically than HCV-negative individuals, suggesting a more efficient clearance of infected hepatocytes. Previous data also support this hypothesis: among untreated HIV/HBsAg-positive patients, HBV DNA detection was significantly less frequent in HCV-positive than in HCV negative subjects (Morsica et al., 2009). In addition, acute HBV infection was shown to clear (in terms of HBe antigen and HBV DNA) more rapidly in HCV-infected than in HCV-negative patients (Sagnelli et al., 2012).

The relationships between serological and molecular parameters in HBsAg-negative HBV infections allowed the development of a predictive model for the presence circulating HBV DNA in occult infections, based on simple serologic parameters available in all laboratories within an hour from sampling. With very limited expense in terms of time and resources, as only anti-HBsAg, antiHBeAg, anti-HBc (total Ig and IgM) are needed, this approach could
quantify the potential infectivity of a donor and provide a numerical threshold for safe donations. For example (as inferable from chart in Fig. 4C), a cut-off score of 0.03 (3% probability of being HBV DNA positive calculated by the logistic model) would exclude 50% of the anti-HBc-positive HBsAg-negative study population from donation, including all 12 HBV DNA subjects. The remaining 50% of these potential donors, in many countries excluded from blood donation independently from NAT testing, might be considered acceptable donors (Dhawan et al., 2008). Clearly, such a predictive model may represent a valuable tool for countries with limited resources, trying to improve blood safety without supporting the costs of NAT. A possible limit to this application would be the extremely high prevalence of anti-HBc in some countries (such as China), where the predictive model would discard an unacceptable number of donors. In the context of solid organ transplantation, where HBV NAT testing is not routinely performed for non-liver organ donations (as in Italy and many other European countries), such a model could provide an indication for a time-limited prophylaxis with hyperimmune immunoglobulins and antivirals in recipients of organs from donors with higher scores. The modulation of the cut-off towards more or less stringent values may prove functional for evaluating blood/organ donors for specific needs. In fact, its predictive value may reach beyond the identification of carriers of detectable circulating DNA, possibly providing some clues also on the transmission potential of anti-HBc positive, NAT-negative donations. This may prove useful, as these donations have also occasionally transmitted the infection (Inaba et al., 2006; Taira et al., 2013; Vermeulen et al., 2014). Liver donation may also be another interesting area of application, but will require the investigation of biotopic specimens to establish a liver-specific model. Further studies based on large numbers of donations are required to identify and validate the predictive value of the model on infection transmission, both for blood and for organ/tissue donations (Avelino-Silvo et al., 2010), where NAT testing is not generally required at present. Finally, a logistic risk assessment model, including multiple HBV serological markers in addition to HBV DNA, could be conveniently implemented for assessing the risk of HBV reactivation in anti-HBc, HBsAg-negative patients who will be subjected to immunosuppression as transplant recipients or for oncologic/autoimmune diseases. Currently, the periodic evaluation of HBsAg and HBV DNA is used for the follow-up of these patients and for antiviral treatment, but a more sophisticated risk assessment could allow a rational approach, with follow-up intervals and prophylactic interventions tailored to risk.

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