

Quantitative analysis of HBsAg, IgM anti-HBc and anti-HBc avidity in acute and chronic hepatitis B

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Abstract

Background and objectives: We evaluated hepatitis B virus (HBV) serological markers by novel, quantitative immunoassays in order to study their behaviours and possible role in the various phases of HBV infection.

Study design: The quantitative determination of HBsAg and anti-HBc/IgM by chemiluminescent immunoassays (Abbott Architect) and the calculation of anti-HBc avidity index have been carried out on repository specimens from patients with acute or chronic hepatitis B.

Results: In acute hepatitis the levels of HBsAg were generally >10,000 UI/mL and decreased sharply in the recovery phase. In 35 anti-HBe-positive chronic hepatitis cases HBsAg levels were generally lower than 10,000 UI/mL (mean: 2655), whereas in five HBeAg-positive chronic hepatitis patients the mean value was 78,756 UI/mL and 90% of specimens exceeded 10,000 UI/mL. The lowest values (mean: 1029 IU/mL) were found in the seven patients with minimal hepatic damage. IgM anti-HBc antibodies were positive in all acute cases and in 68/207 samples (32.85%) from patients with chronic hepatitis, with significantly lower levels (average sample/cutoff (S/CO) ratio: 2.95 in chronic cases versus 25.96 in acute cases; $p < 0.005$).

A S/CO value of 10 for anti-HBc IgM had a 100% negative predictive value and a 99.13% positive predictive value for acute hepatitis B. The study of anti-HBc avidity by an experimental procedure showed that an avidity index (AI) threshold of 0.7 had a good efficacy to discriminate the cases of chronic hepatitis, among whom only 2 specimens out of 193 (1.04%) had an AI < 0.7.

Conclusion: The quantitative determination of HBsAg, anti-HBc/IgM and anti-HBc avidity provides additional information and may be useful in the differential diagnosis of acute and chronic HBV infections and in the follow-up of chronically infected patients.

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1. Introduction

The burden of infection by hepatitis B virus (HBV), a major cause of chronic hepatic damage and of hepatocellular carcinoma worldwide (Fattovich, 2003; Lai et al., 2003) has decreased substantially in Italy since the late 1980s, mainly due to the strong reduction of intrafamilial spread, and further after the introduction of mass vaccination of newborns and adolescents, that started in 1991 (Zanetti et al., 2005). Even if the long-term perspectives are encouraging, in Italy

there are still more than 1.5 million chronic HBV carriers, and among acute hepatitis cases reported to the surveillance systems, HBV is still the second cause after hepatitis A, with an increase in recent years.

The majority of acute as well as of chronic infections are asymptomatic, and simple and inexpensive methods that may be useful differentiating acute from chronic HBV infection are needed. To do that, the measurement of the avidity index of anti-HBc antibodies, that are detectable in all phases of HBV infection, may represent an interesting tool (Thomas, 1997), but no assay is currently available. Another diagnostic dilemma is the differentiation between inactive carriers and chronic hepatitis cases, since both forms are asymp-

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tomatic for long periods of time. Quantitative methods for evaluating viral replication and disease activity need to be employed on this purpose, and such methods must also have a detection range wide enough to be employed also in the follow-up of chronic cases, either untreated or during antiviral treatment. Serum HBeAg and HBV-DNA are currently the most important markers for both purposes; unfortunately, in Italy as well as in other Mediterranean countries, over 90% of chronically HBV-infected patients are negative for HBeAg, due to the absolute prevalence of the “e-minus” pre-core mutant strains (Bonino and Brunetto, 2003; Lai et al., 2003). As for HBV-DNA, current assays are technically complex and expensive and, although their value has been clearly established in controlled trials, they cannot be employed for routine monitoring outside of specialized clinical settings, and their cost allows for testing only at intervals of several months.

The surface antigen of HBV (HBsAg) is used typically as a qualitative serological marker for diagnosing an ongoing HBV infection. Recently, a quantitative, fully automated chemiluminescent microparticle immunoassay (Abbott Architect HBsAg QT) for the detection of HBsAg at a wide range of concentrations became available (Deguchi et al., 2004). Another serological marker that gained importance as an indicator of the host's active immune response, and also for monitoring disease activity, is represented by the IgM antibodies towards the HBc antigen (anti-HBc/IgM) (Brunetto et al., 1993; Colloredo et al., 1996, 1999; Marinos et al., 1994).

We aimed to determine quantitatively both HBsAg and IgM anti-HBc, as well as to study the anti-HBc avidity, in a selected population of acute and chronic hepatitis B in order to evaluate their patterns and prospect their possible use in the serological diagnosis and monitoring of hepatitis B, and especially on the purpose of differentiating acute from chronic infection.

2. Methods

2.1. Patients

Cases of acute and chronic hepatitis B infection have been retrospectively selected from the hospital database of the Spedali Civili of Brescia (Italy) in the years 1999–2004 according to the following criteria:

- (a) Acute hepatitis B: patients with no previous history of hepatitis B virus infection, admitted with signs or symptoms suggestive of acute hepatitis (nausea, jaundice, fever, abdominal pain, enlarged liver) and with the following laboratory data: positive for HBsAg and IgM anti-HBc, negative for anti-HCV, anti-HAV/IgM and anti-delta, alanine aminotransferase (ALT) values exceeding eight times the maximum normal value of 40 U/L.

- (b) Chronic hepatitis B: HBsAg chronic carriers with biopsy-proven chronic liver disease at different stages and with a follow-up of at least 36 months.

2.2. Routine testing

All samples included in the study had been previously assayed for hepatitis B serological markers (HBsAg, HBeAg, anti-HBe, anti-HBc/IgM) by commercial methods (MEIA, Abbott Diagnostics or EIA, Sorin Biomedica) during the hospitalization period for acute cases and at follow-up visits for chronic cases. Many samples had also been assayed for ALT values by standard biochemical assays with an upper normal value at 40 U/L and for HBV-DNA by quantitative assays (Digene Hybrid Capture II or Roche Amplicor); for the purpose of this study, HBV-DNA results are expressed in genome copies/mL.

2.3. Quantitative serological assays

Frozen serum specimens from patients fulfilling the above mentioned criteria for whom at least 500 μ L were available were thawed at 0–4 °C, centrifuged to separate particulate material and assayed as follows:

- (a) HBsAg: quantitative determination by the Architect chemiluminescent assay (Abbott GmbH, Wiesbaden Delkenheim, Germany). This assay, previously described (Deguchi et al., 2004), is calibrated against the WHO standard and allows the quantitation of HBsAg from 0.05 to 250 international units (IU)/mL: for the purpose of this study, and based on previous experiences (Chen et al., 2004; Deguchi et al., 2004), samples were diluted 1:20 and 1:500 with the Architect HBsAg diluent in order to expand the upper limit of the dynamic range. The analytical correlation between the results obtained in a preliminary study with the two dilutions was good ($r=0.937$; data not shown).
- (b) IgM anti-HBc: the chemiluminescent immunoassay on the Abbott Architect was employed. Briefly, human serum was incubated with paramagnetic particles coated with anti-human IgM antibodies: after washing and addition of acridine-conjugated recombinant HBcAg, the reaction was revealed by addition of pre-trigger and trigger solutions. The positivity is defined by a sample/cutoff (S/CO) ratio ≥ 1.0 , corresponding to 50 PEI Units, while samples with a S/CO between 0.500 and 0.999 are classified as “gray zone” and contain low levels of anti-HBc-IgM.
- (c) Anti-HBc antibody avidity: we adopted the same experimental procedure employed for assessing the avidity of anti-HIV antibodies (Suligoi et al., 2002). For each sample, two aliquots of 0.1 mL each were subjected to a pre-test 1:10 dilution, respectively with the AxSYM working buffer (B) and with 1 M guanidine chloridrate (G). Both aliquots were vortexed and incubated at room temperature for 30 min and then assayed for anti-HBc by

the automated AxSYM Core assay (Abbott GmbH) without any modification of the test procedure. Since this is a competitive assay in which reactive samples are lower than the cutoff ($S/CO < 1$), the avidity index has been calculated by dividing the reciprocal (1 divided by S/CO) of the S/CO value obtained on the G aliquot by the reciprocal of the S/CO value of the B aliquot.

2.4. Statistical analysis

Sensitivity, specificity and predictive values were calculated by 2×2 contingency tables. Differences between percentages were evaluated either by chi-square test with Yates' correction or by Fisher's exact test; differences between means were evaluated by Student's *t*-test.

3. Results

3.1. Acute hepatitis

Thirty-six patients (26 males, 10 females, mean age: 40.4 ± 15.5 years, range: 20–89 years) fulfilled the inclusion criteria. All patients included in our study showed a spontaneous recovery from acute hepatitis B after 6–8 weeks from admission to the hospital.

A total of 42 samples were available for the study: 21 (from 20 patients) were collected during the first 3 weeks after admission and 21 (from 16 patients) at later stages, during the convalescent phase but before complete recovery. All acute phase sera but 2 (19/21, 90.5%) were positive for HBeAg and 12/14 (85.7%) were positive for HBV-DNA (median value: 38 million copies/mL; range: 2.4 millions–4.4 billions); conversely, 16/21 convalescent sera (76.1%) were negative for HBeAg and positive for anti-HBe and all but 1 was negative for HBV-DNA. The Digene assay was employed in all acute and convalescent cases.

Substantial differences in HBsAg levels were recorded in the two groups: on acute phase samples the mean value was 25,767 IU/mL (range: 5–90,575); and 71% of values exceeded 10,000 IU/mL, whereas in the recovery phase the mean was 1351 IU/mL (range: 0.05–8495) (Fig. 1A). Anti-HBc IgM antibodies were positive in 41/42 samples (Table 1) and levels (S/CO) did not differ significantly between the two subgroups (acute phase: mean 25.96 ± 4.74 , 25–75th percentile: 24.14–29.86; convalescence: mean 25.96 ± 4.84 ,

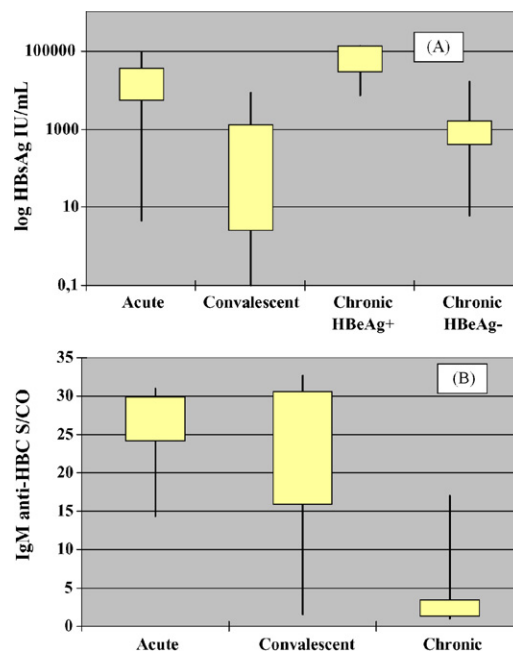


Fig. 1. Levels of HBsAg (A) and IgM anti-HBc (B) in acute, convalescent and chronic hepatitis B cases. The boxes represent the 25–75th percentile values, the two lines represent the range between the minimum and maximum value observed. HBsAg values in chronic cases are shown separately for HBeAg-positive and HBeAg-negative subjects. For anti-HBc IgM only positive results ($S/CO \geq 1.0$) have been considered.

25–75th percentile: 15.90–30.60) (Fig. 1B). The IgG avidity index (AI) was significantly different between the two groups: mean values were 0.65 ± 0.19 in acute phase and 0.91 ± 0.14 in convalescence ($p < 0.01$). An arbitrary cutoff for AI set at 0.70 appeared the best option in order to discriminate the two phases of acute hepatitis B, since 12/21 samples in the first group (57.14%) and only 1 in the second group (4.76%) had an AI < 0.70 ($p = 0.008$; Table 1).

3.2. Chronic hepatitis

We selected 40 patients (28 males, 12 females, mean age: 54.9 ± 13.8 years, range 20–81 years). Thirty-two of them had a chronic active hepatitis (CAH, one evolved in cirrhosis over the follow-up period), seven had a mild form of hepatitis with minimal histological changes and one had liver cirrhosis. All patients except five CAH cases were HBeAg-negative and anti-HBe-positive (82.5%). The mean follow-up period was 50.5 ± 10.7 months (range: 36–79 months); over this period,

Table 1

Study population (number of patients and number of specimens) and summary results for anti-HBc IgM and anti-HBc avidity index (AI), based on a threshold of 0.7 AI

Category	No. of patients ^a	No. of samples	No. of samples IgM anti-HBc-positive (%)	No. of samples with an AI < 0.70 (%)
Acute hepatitis B florid phase	20	21	21 (100)	12 (57.14)
Acute hepatitis B convalescence	16	21	20 (95.2)	1 (4.76)
Chronic hepatitis B	40	207	68 (32.85)	2 ^a (1.04)

^a Data available on 193 samples.

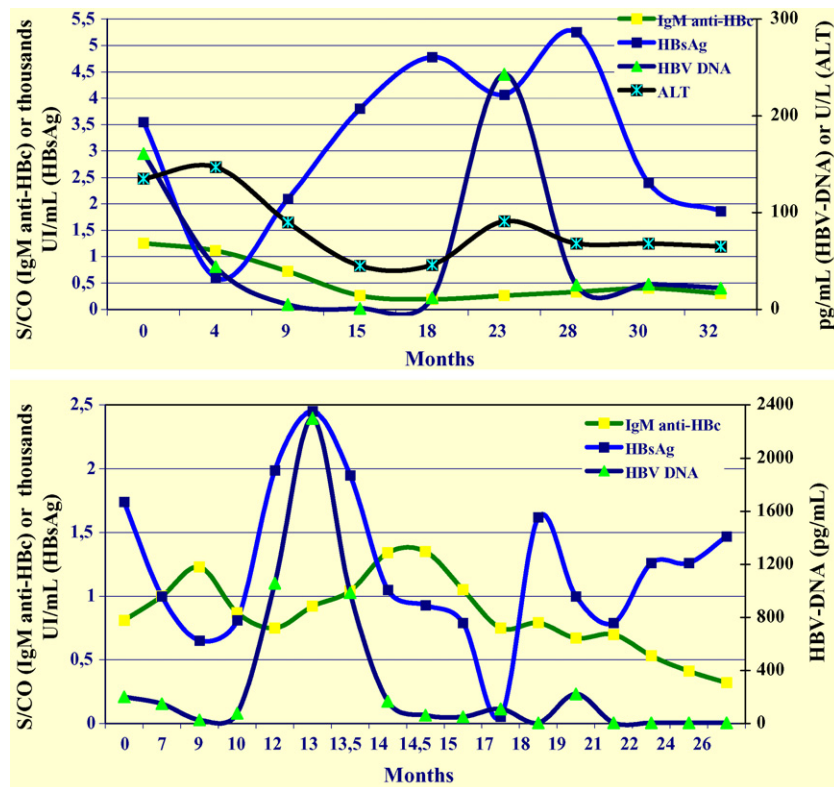


Fig. 2. Profiles of HBsAg (UI/mL), IgM anti-HBc (S/CO) and HBV-DNA (pg/mL) in the follow-up of two patients with HBeAg-negative chronic hepatitis B. For the first patient ALT values (U/L) are also included.

the positivity rates for HBV-DNA (Digene in all cases except seven tested by Amplicor) and ALT values >40 U/L were 77.18% and 81.61%, respectively. A total of 207 specimens (average: 5 per patient; range: 3–17) were still available for retrospective serological testing.

HBsAg levels were markedly higher in HBeAg-positive patients than in HBeAg-negative patients, and significant differences were found both between means: $(78,756 \pm 49,448 \text{ IU/mL}$ versus $2192 \pm 2401 \text{ IU/mL}$; $p < 0.001$) and between rates of samples exceeding 10,000 IU/mL (90% versus 3.12%; $p < 0.001$). Finally, among the 35 anti-HBe-positive cases, the mean levels were significantly lower ($p < 0.01$) in the seven patients with mild disease ($1029 \pm 916 \text{ IU/mL}$) than in the 28 patients with CAH or cirrhosis ($2655 \pm 2626 \text{ IU/mL}$).

Anti-HBc IgM antibodies were positive in 68/207 samples (32.85%) and in the gray zone (GZ) in 47 more samples (22.71%); thus, 55.5% of specimens from chronic hepatitis patients had detectable levels of IgM antibodies directed against the capsid antigen. The frequency of IgM positivity was significantly lower ($p = 0.03$) in the seven patients with mild hepatitis (15.15%) than among patients with chronic active hepatitis or cirrhosis (36.48%). No relationship between IgM positivity and HBsAg levels, neither with ALT values, resulted from our data. The mean values and range of anti-HBc/IgM and HBsAg in acute, convalescent and chronic

cases are shown in Fig. 1. A threshold of 10 S/CO seems best suited for differentiating acute from chronic hepatitis, since it yielded a sensitivity and negative predictive value of 100%, a specificity of 99% and a positive predictive value of 99.13%.

In chronic hepatitis the IgG avidity index (AI) was higher than 0.70 in 191 out of the 193 specimens assayed (98.96%), the only exceptions being 2 specimens from 2 different patients. One of them, an HBeAg-positive CAH, had an AI of 0.68 on the first available sample and values of 0.90 and 1.05 on samples obtained after 34 and 40 months. Interestingly, in the second case, an anti-HBe-positive CAH, a low IgG AI (0.61) was recorded 3 weeks after the temporary reappearance of HBeAg accompanied by a positive IgM result (S/CO: 2.25), whereas the other four samples obtained before or after that one over a period of 19 months had an AI above 0.70 (mean: 1.10). In our experience this arbitrary threshold had a specificity of 99%, a positive predictive value of 85.7% and a negative predictive value of 95%.

For two patients (both males, anti-HBe-positive CAH) we processed enough samples to construct follow-up charts for HBsAg and IgM anti-HBc in comparison with HBV-DNA and (for one case) ALT values. The results are reported in Fig. 2. Though HBsAg levels fluctuated less than IgM levels, in the first patient the highest values were recorded after a replicative flare and in the second one the two peaks of HBsAg were coincident or anticipated a reactivation of HBV

infection, evidenced by peaks of HBV-DNA and IgM anti-HBc.

4. Discussion

The serological diagnosis of hepatitis B virus infection has relied for more than three decades mainly on a combination of qualitative assay results, whose different patterns were considered representative of acute and chronic disease or of a chronic “healthy” state. In chronic hepatitis B quantitative testing of serological markers may represent an attractive alternative to the evaluation of ALT and HBV-DNA. In fact, while such determinations are both quantitative, and thus appear more suited for following up a chronic disease, the former lacks specificity, being just an index of hepatic cytonecrosis (Brunetto et al., 1993; Lai et al., 2003) and the latter is still fairly imprecise, difficult to standardize and, for the time being, too expensive and technically demanding to be adopted on a routine basis in general hospital settings.

For many years anti-HBc IgM have been considered a specific marker of acute hepatitis B. Indeed, this was substantially true with the old enzyme immunoassays, that were standardized at a threshold value corresponding to 600–700 Paul-Ehrlich (PEI) units, and were then able to give a positive result only in the presence of the high IgM levels that are usually present in acute HBV infections (Gerlich et al., 1986; Brunetto et al., 1993; Marinos et al., 1994). However, it has been demonstrated by several authors that lower levels of IgM anti-HBc are often detectable also in chronic hepatitis B (Brunetto et al., 1993; Colloredo et al., 1996; Gerlich et al., 1986; Marinos et al., 1994) by more sensitive methods, that may attain an analytical sensitivity as low as 7 PEI units (Brunetto et al., 1993). This level seems adequate to differentiate chronic, inactive carriers (Brunetto et al., 1993; Cacciola et al., 2005) from asymptomatic patients suffering from chronic hepatitis B (Brunetto et al., 1993; Colloredo et al., 1996; Gerlich et al., 1986; Marinos et al., 1994), an important clue in countries where the negativity for HBeAg is a common trait for both groups (Bonino and Brunetto, 2003; Fattovich, 2003). The quantitation of IgM values allows also to establish their relationship with the HBV replicative flares and with disease activity (Colloredo et al., 1996, 1999). Our data, obtained with a new assay with a wide dynamic range on well-characterized specimens from patients with acute and chronic hepatitis B, confirmed those early findings, with a relevant percentage of positive cases among chronic hepatitis cases, but with significantly lower levels than in acute hepatitis, and higher positivity rates in patients with chronic active hepatitis than in mild chronic hepatitis with minimal histological changes.

The possible role of the quantitative determination of HBsAg has been initially prospected by Froesner et al. (1982), who studied the concentration of the surface antigen over time in patients with acute and chronic hepatitis B and concluded that in acute hepatitis B cases a reduction of

at least 50% of values was attained on average in 16.6 days in patients who recovered spontaneously. The diagnostic assays available at that time did not allow routine quantitative testing for HBsAg, and a decade passed before other authors (Zoulim et al., 1992), employing an automated assay, suggested the possible role of this marker also in the monitoring of chronic HBV infection, and in particular its predictive value on the outcome of interferon therapy (Burczynska et al., 1994). Two weak points of those studies were the limited dynamic range of the assays, with the ensuing need of performing multiple dilutions, and the need to employ an external standard, also at serial dilutions, since all HBsAg assays were qualitative. The dynamics of HBsAg levels in acute infections and their relationship with HBV-DNA and other HBV markers have been recently reviewed (Chulanov et al., 2003; Erhardt et al., 2000). While Laurell's electrophoresis has been demonstrated to be accurate and clinically useful for the quantitation of HBsAg (Gerlich et al., 2004), the recent availability of a quantitative, fully automated assay has started a new wave of studies: a correlation between HBsAg and HBV-DNA was found by Chen et al. (2004) in asymptomatic carriers stratified by HBV-DNA levels and by Kohmoto et al. (2005) in lamivudine-treated patients. In contrast, a retrospective study (Kuhns et al., 2004) failed to find a correlation between HBsAg and HBV-DNA in untreated blood donors; a recent observation (Werle-Lapostolle et al., 2004) linking HBsAg load to the amount of covalently closed circular HBV-DNA (ccc-DNA), whose dynamics are different from serum HBV-DNA, may help to explain the contrasting finding among studies carried out only on serum specimens.

Interestingly, in two different studies (Wolters et al., 2000; Kohmoto et al., 2005) a decrease of HBsAg concentration, up to the negativization in some cases, was observed only in long-term sustained responders to lamivudine, while an increase was observed in breakthrough cases after the selection lamivudine-resistant YMDD mutants. In our study we focused mostly on the levels of HBsAg in acute and chronic hepatitis, without investigating the response to treatment. Our findings support the hypothesis that HBsAg levels reflect the replicative state and also disease activity, since the mean values were higher both in acute hepatitis and in chronic, HBeAg-positive hepatitis and were lower both during the convalescent phase of acute patients who showed a spontaneous recovery and among HBeAg-negative chronic hepatitis cases. In the latter group, cases with a mild disease had significantly lower levels than patients suffering from chronic aggressive hepatitis. This is of great importance in our geographical area, where the absolute majority of chronic hepatitis B cases are infected by HBe-negative strains, while it could be less relevant in other countries where most patients with active disease are positive for HBeAg.

Another diagnostic tool that is not currently employed in viral hepatitis testing is the evaluation of the avidity index of specific antibodies. Anti-HBc antibodies seem best suited for this purpose, since they are the most durable markers of HBV infection, either current or past. We assayed the

anti-HBc avidity by an experimental procedure requiring only a 1:10 predilution of the specimens in a chaotropic agent and in neutral buffer before anti-HBc testing, thus minimizing pre-analytical variations (Suligoi et al., 2002). In the only systematic experience published so far (Thomas, 1997) an increase in the avidity of this specific antibody, similarly to what happens in other viral infections, has been observed in patients with an acute, resolving infection, while low avidity antibodies persisted in HBeAg-positive patients who progressed towards chronicity. The possible occurrence of an increase of anti-HBc avidity in acute cases before sero-conversion from HBeAg to anti-HBe is consistent with our data obtained on convalescent specimens, mostly HBeAg-negative, who had an average AI close to 1 (0.91 ± 0.14) and only one result below the proposed threshold of 0.70. This is of peculiar relevance, since one of the unsolved diagnostic problems concerning HBV is the distinction between an acute infection which proceeds to chronicity and an acute flare of an already chronic HBV infection. Both cases may be IgM anti-HBc-positive and remain HBsAg-positive, and our data suggest that the avidity index of anti-HBc avidity assay provides an adequate solution, since it was most powerful for excluding acute hepatitis B.

In summary, our results suggest that the quantitative determination of HBsAg and IgM anti-HBc may represent a useful, simple and fairly economic tool that may be added in the serological algorithm for HBV infection. For anti-HBc IgM, a threshold of 10 S/CO seems best suited for differentiating acute from chronic hepatitis, while for HBsAg no clear difference appeared, since the values recorded showed a wide range in both acute and chronic infections, but HBsAg levels reflected quite well the high replicative phases, being much higher in HBeAg-positive subjects, as already reported in two experiences carried out in the Far East (Chen et al., 2004; Deguchi et al., 2004).

Further studies are needed in order to establish the spontaneous variations of HBsAg in chronic HBV carriers and thus to prospect which level of decrease may be significant for a long-term remission or, conversely, what increase may represent a marker of disease reactivation.

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