

Cutoff Levels of Immunoglobulin M Antibody against Viral Core Antigen for Differentiation of Acute, Chronic, and Past Hepatitis B Virus Infections

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The titer of antibody against core antigen of hepatitis B virus in the immunoglobulin M class (IgM anti-HBc) was determined by an IgM capture assay of reduced sensitivity (30 arbitrary units). The distribution of titers among 235 acute hepatitis patients who were hepatitis B surface antigen (HBsAg) positive suggested that 600 U forms a lower cutoff value for acute hepatitis B. Clinically apparent cases of acute hepatitis with high IgM anti-HBc and without HBsAg were rare (2.6%). Acute, non-B hepatitis in HBsAg carriers was more frequent (9.4%). In chronic hepatitis B, 39% of 174 biopsy-proven cases had moderate titers of 30 to 600 U, whereas healthy HBsAg carriers were rarely (4/84) positive. In mild or inapparent infections without HBsAg, titers were between 50 and 400 U. Thus, sufficiently accurate and sensitive quantitation of IgM anti-HBc allows for (i) differentiation of acute and nonacute hepatitis B virus infection in acute hepatitis, (ii) partial differentiation between clinically symptomatic and asymptomatic chronic infections, and (iii) identification of recent subclinical infections.

Acute viral hepatitis may be caused by hepatitis A virus, hepatitis B virus (HBV), hepatitis delta virus, and an unknown number of not yet identified non-A, non-B hepatitis viruses. In many regions HBV is the most important cause of acute and chronic viral hepatitis. Diagnosis of HBV infections is usually achieved by assay of surplus HBV surface antigen (HBsAg) in the blood, but this marker does not distinguish between acute, chronic symptomatic, and chronic asymptomatic HBV infections. Moreover, HBsAg may be undetectable in a certain proportion of acute or even chronic infections.

Such infections may be detected by antibody (anti-HBc) against HBV core antigen (HBcAg) (13, 19). Anti-HBc persists for a long time after acute or transient inapparent infections, and it is permanently present in chronic infections. Acute infections may be differentiated from past infections by assay of specific immunoglobulin M (IgM) antibodies, and many studies have shown the presence of IgM anti-HBc in acute hepatitis B (1, 5, 7, 14, 15, 22-24, 28, 33). Its diagnostic value has, however, been drawn into doubt (16) because IgM anti-HBc has also been detected in many chronic carriers of HBsAg (10, 15, 20, 31). Moreover, the very high sensitivity of the widely used IgM capture assay makes IgM anti-HBc sometimes detectable for years after the acute infection (1, 15, 28), if the sensitivity is not purposely reduced.

In view of this situation, it has been noted that only quantitation of IgM anti-HBc could allow distinction of recent, chronic, and past HBV infections (15, 28, 32). There is, however, no general agreement in the literature and among the producers of test kits on whether a clear distinction is possible at all and which titer could serve as the cutoff level. This uncertainty originates partly from the lack of a generally accepted reference sample.

This study describes how the assay can be standardized, and it reports quantitative data on several hundred cases of acute, chronic, and past HBV infections. The data show that

different cutoff levels exist for the following clinical situations: distinction of acute and chronic infections in HBsAg-positive persons; distinction between symptomatic and asymptomatic chronic carriers; detection of HBsAg-negative acute hepatitis B; and detection of transient inapparent HBV infections without HBsAg.

MATERIALS AND METHODS

Reagents for the assay of IgM anti-HBc. Aggregated IgG was prepared from a pool of anti-HBc-negative, recalcified human plasmas (NHP) by gel chromatography through Bio-Gel A5M (Bio-Rad Laboratories, Richmond, Calif.) and by subsequent heating of the IgG main fraction to 65°C for 10 min.

Microplates (Nunc II; Nunc, Roskilde, Denmark) were incubated with a 1:4,000 dilution of anti- μ -chain antiserum (Dakopatts, Hamburg, Federal Republic of Germany) in 0.075 M carbonate buffer (pH 9.6) overnight at 4°C. The solution was aspirated, and 0.1% bovine serum albumin in 0.1 M NaCl-10 mM Tris hydrochloride (pH 7.4)-1 mM EDTA (buffer for all dilutions) was added for 2 h at room temperature. Plates were emptied, washed three times with 0.01 M sodium phosphate (pH 7.4)-0.13 M NaCl-0.5% Tween 20 (buffer for all washings), air dried, sealed in plastic bags, and stored at 4°C until use.

HBcAg was isolated from HBV-infected human liver and partially purified by zonal sedimentation as described previously (14). Recently, HBcAg from a transfected *Escherichia coli* strain (V. Bruss and W. H. Gerlich, unpublished data) was also used. This antigen was purified from bacterial lysate by zonal sedimentation as described before and further purified by gel chromatography through Bio-Gel A15M. Before use, HBcAg was diluted in 1% NHP.

The anti-HBc IgG fraction was isolated from HBsAg-positive plasma which had a high titer of anti-HBc (1:160,000), but no anti-hepatitis B surface antibody, anti-hepatitis B e antibody, or hepatitis B e antigen, using threefold precipitation with 17% (wt/wt) sodium sulfate. This IgG was coupled with peroxidase as described previously

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(14), and 1:1 conjugates were isolated by gel chromatography through Sephacryl S300 (Pharmacia, Uppsala, Sweden). The conjugate was prediluted to 0.1 mg/ml in 10% fetal calf serum and stored at -20°C . Before use, the conjugate was diluted 1:50 in 10% NHP.

The substrate for peroxidase was 4 μg of *o*-phenylenediamine per ml–0.006% hydrogen peroxidase in 0.068 M citric acid–0.2 M phosphate buffer. The stop solution was 2 N H_2SO_4 .

Assay of IgM anti-HBc. Serum or plasma samples were diluted in two steps using dilution buffer to 1:400 and thereafter in 0.5% aggregated IgG–1% NHP to 1:2,000. The NHP contained 1.27 mg of IgM per ml. Highly positive samples were diluted further to 1:20,000 in 0.5% aggregated IgG–1% NHP. Dilutions were incubated in anti- μ -chain-coated microplate wells for 2 h at 37°C . Plates were washed three times, and HBcAg was added for 12 to 18 h at 4°C . The dilution of HBcAg was adjusted to produce an absorbance of 1.2 to 1.6 as the maximal final result. Approximately 10 ng of purified HBcAg was necessary per well. After two washings, the anti-HBc conjugate was added for 2 h at 37°C . After five washings, substrate was added for 30 min and the color of the stopped reaction mixture was measured in a microplate photometer at 490 nm using a reference beam at 630 nm. All volumes were 100 μl , except for the stop solution (200 μl).

Standardization of the assay. Each test run included eight different negative sera and a dilution series of a highly positive reference serum which was typical for sera from patients with acute hepatitis B. The reference sample had, per definition, 3,000 U of IgM anti-HBc. It was prediluted 1:20 in buffer, stored in small portions at -20°C , and thawed only directly before use.

As was shown previously, the results of the assay depend on the ratio of anti-HBc-specific IgM to total IgM (14). Dilution up to many 1,000-fold in buffer did not alter the results as long as the anti- μ -coated surface remained saturated by IgM. Addition of negative IgM to the sample, however, decreased the binding of IgM anti-HBc. To generate a calibration curve, the reference serum was first diluted 1:2,000 as described above, and this sample was further diluted geometrically by 1:4 in 1% NHP to generate values of 750, 187.5, 46.8, and 11.5 U. The effect of the 1:2,000 dilution was neglected as a constant factor which applied to the reference and test samples. The 1:2,000 working dilution of the reference sample was assumed to contain 3,000 U as well. This is possible because the units define only the proportion of IgM anti-HBc to total IgM and not its absolute amount in a sample.

The absorbancies of the reference series were plotted against a logarithmic scale of the units. IgM anti-HBc units were read for each positive test sample from this calibration curve. To avoid saturation phenomena, samples with more than 750 U were repeated at a dilution of 1:20,000 and the result of this assay was multiplied by 10.

The detection limit was assumed to be 2.1-fold the mean value of the negative samples, which was between 6 and 25 U. Recalcified plasma, containing 100 U of IgM anti-HBc, was deposited in small portions as the national reference sample at the German Institute for Sera and Vaccines (Paul-Ehrlich-Institute, Frankfurt am Main, Federal Republic of Germany). The units of this sample are smaller by a factor of 3.0 than the arbitrary units reported in two previous reports (14, 15).

Classification of cases. Within 1 year, 1,253 anti-HBc-positive samples were identified among 21,214 sera. The clinical states of the persons whose samples were tested

were evaluated on the basis of data from questionnaires, which are required for all submissions to our laboratory. Acute hepatitis B was accepted as the diagnosis if the clinical observations and high alanine aminotransferase (ALT) levels (>300 U/liter) were confirmed by a positive HBsAg test, an IgM anti-HBc titer of more than 600 U, or both. If several samples from one person were available, the highest IgM anti-HBc result was taken for evaluation. Acute hepatitis was considered HBsAg positive if at least one available sample was HBsAg positive. Accidentally observed ALT rises were considered to represent acute hepatitis if the peak value exceeded 300 U/ml in the absence of other potential liver diseases.

Chronic hepatitis as a diagnosis was only assumed if confirmed by liver histology. Blood donors with HBsAg were assumed to be healthy carriers if they had normal ALT (<25 U/liter), high anti-HBc, and low or undetectable IgM anti-HBc levels. In approximately 30% of the cases positive for HBsAg, IgM anti-HBc, or both, no clear diagnosis was available, or the serological findings were contradictory. In these cases, a special questionnaire inquired again 6 to 12 months later about the clinical course and peak ALT levels, and in 89% of the cases a diagnosis was possible.

HBsAg and anti-HBc were assayed by solid-phase radioimmunoassay or enzyme immunoassay as described previously (14, 15).

RESULTS

Optimization of the assay. The assay used for this study was reported in 1979 to work well at a dilution of 1:100 and at a detection limit of approximately 3 U of IgM anti-HBc (15). During the following years, the binding capacity of the microplates and the anti- μ -chain antibody for total IgM increased considerably. This resulted in an unwanted increase in sensitivity and in prozones with highly positive samples as also recorded by others (28). Prozones were observed even at dilutions of 1:2,000. To avoid uncomfortably high working dilutions, negative human IgM in the form of 1:100-diluted NHP was used as the diluent for 1:2,000-diluted samples. Under these conditions, the detection limit was 5 to 25 U, and the absorbance of the enzymatic assay product was roughly proportional to the amount of IgM anti-HBc to at least 750 U. Samples with more than 750 U were tested again at 1:20,000 in 1% NHP.

The addition of excess negative IgM also standardizes the amount of total IgM in the sample, which may vary considerably. Under these conditions, the absorbance of the assay was proportional to the endpoint dilution titer. The endpoint titer was, however, not determined for practical reasons in most of the sera. Reading of arbitrary units from a calibration curve was accurate within $\pm 20\%$ and much less laborious than endpoint titration. To avoid inconsistent results caused by a variable detection limit, all values below 30 U were considered negative.

The nature of the IgM capture assay requires precautions against nonspecific results caused by rheumatoid factor. HBcAg from human infected liver always contains some IgG anti-HBc, and this binds to rheumatoid factor as has been noted by others (4). The conjugate of IgM anti-HBc and peroxidase also contains some rheumatoid-factor-binding components despite purification (4). An easy way to block out rheumatoid factor is addition of heat-aggregated negative IgG to the diluent of the samples. An amount of 0.5% aggregated IgG has been found sufficient at a 1:100 sample dilution (14), and this amount was maintained for the 1:2,000 dilution. An additional advantage of this method is that even

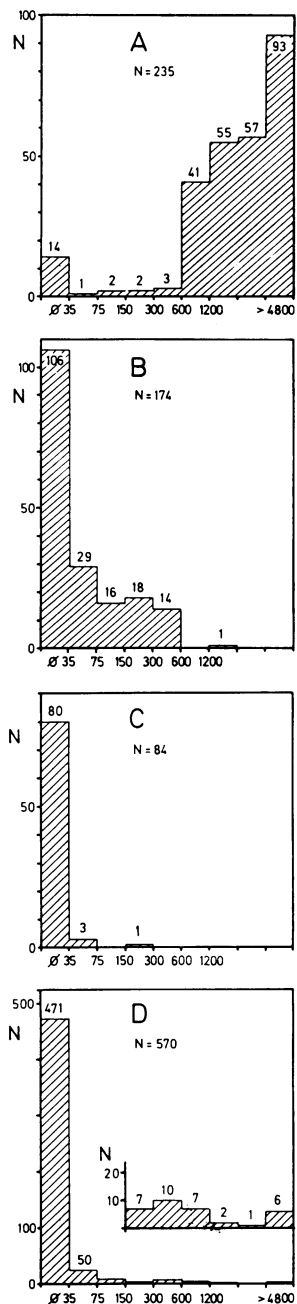


FIG. 1. Histograms of IgM anti-HBc titers in HBsAg-positive acute hepatitis (A), HBsAg-positive chronic hepatitis (B), asymptomatic HBsAg carriers (C), and anti-HBc-positive persons without HBsAg (D). The clinical state of group D is specified in the text.

extremely high titers of IgG anti-HBc in the sample are prevented from nonspecific binding to the microplate by a 1,000-fold excess of negative IgG which may otherwise occur (3, 4).

The assay worked well with HBcAg from both liver and HBV particles which had been isolated from serum. As has been noted by others (8, 18, 29), the superior source of HBcAg is, however, *E. coli* which has been transfected with an HBcAg-expressing plasmid. A total of 245 samples were tested in parallel with identical results, using recombinant HBcAg or HBcAg from liver.

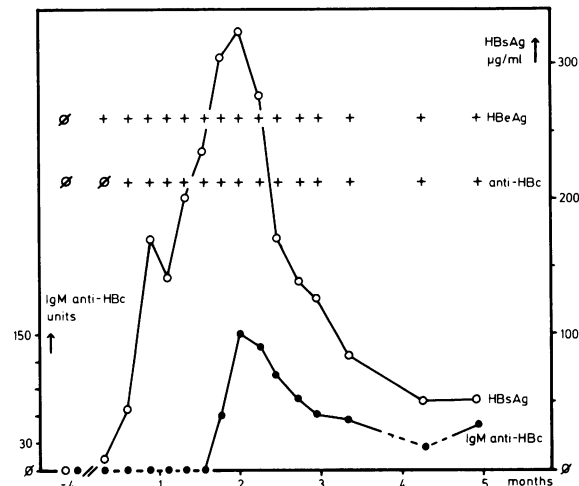


FIG. 2. Time course of IgM anti-HBc (●), HBsAg (○), and other serological HBV markers during asymptomatic HBV infection in an immunodeficient patient with a renal transplant. Note the late appearance and low titer of IgM anti-HBc despite massive infection.

Acute HBsAg-positive hepatitis. Together with the clinical picture of acute hepatitis, HBsAg would usually be taken as a sign of acute HBV infection. This well-founded rule is wrong in cases in which non-B hepatitis may occur in chronic HBsAg carriers. In fact, 14 of 235 evaluated HBsAg-positive cases (5.6%) had no detectable IgM anti-HBc. In all 14 cases the clinical diagnosis was confirmed at a second inquiry. In some cases a high risk of acquiring non-A, non-B hepatitis was known. Hepatitis delta antibody was found only in one patient of Italian origin.

The titers of IgM anti-HBc were very high in the great majority (Fig. 1A), and they often exceeded the upper limit (7,500 U) of the quantitation range. Only eight patients (3.7%) had low titers between 30 and 600 U. The sharp discontinuity of the histogram at 600 U (Fig. 1A) suggests that this value may serve as a lower cutoff value for HBsAg-positive acute hepatitis B. If this is accepted, it appears unlikely that the eight cases with values below 600 U suffered from acute HBV infection. Thus, only 213 of the 235 patients (90.6%) had acute type B hepatitis.

It was occasionally noted that the IgM anti-HBc titer was low (<600 U) or absent in the first available serum sample. In a second serum sample, which was available several days or weeks later, titers were very high but HBsAg was already negative. Such cases were diagnosed as HBsAg-positive acute hepatitis B. If only the first sample had been available, the diagnosis would have been impossible. If only the second sample had been obtained, a classification as HBsAg-negative hepatitis B would have resulted.

The postulated cutoff value of 600 U does not apply to recent HBV infections in immunodeficient patients who never fully develop acute hepatitis. Figure 2 gives an example of a hemodialyzed patient who had a newly acquired HBV infection but no clinical hepatitis. Because of the reduced sensitivity of the assay for IgM anti-HBc, it became detectable only after total anti-HBc was positive, and it never exceeded 150 U. A similar course was observed in some, but not all, infants after perinatal infection.

Persistent HBV infections. Samples were available from 174 HBsAg-positive patients with histologically confirmed chronic hepatitis. In many cases, no history of acute hepatitis B was known. In most other cases, the acute infection

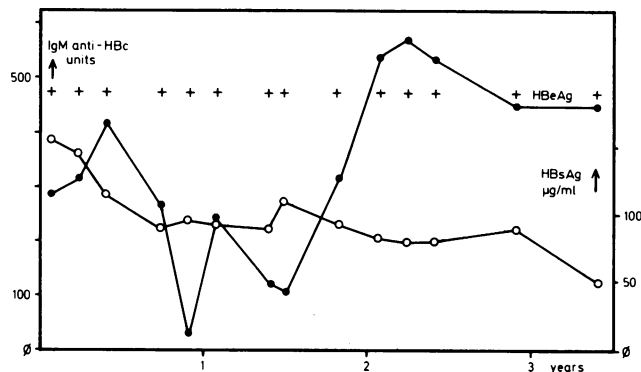


FIG. 3. Time course of IgM anti-HBc (●), HBsAg (○), and hepatitis B e antigen (HBeAg) in a patient suffering from chronic, aggressive hepatitis. Anti-HBc and HBV DNA were also positive throughout. Note the variable titer of IgM anti-HBc.

was several years ago. A considerable proportion (39.1%) of the patients had IgM anti-HBc in moderate titers between 30 and 600 U (Fig. 1B). Only one patient had a very high titer typical of acute hepatitis B. One might assume that all these titers were remnants of the acute phase (E. G. Lindenschmidt, Zentralbl. Bakteriologie, Mikrobiologie, Hygiene, I. Abteilung, Originalarbeiten, in press), since it is known that IgM anti-HBc decreases very slowly in developing chronic hepatitis B (15, 28). However, in most of the patients who could be followed up, there was no steady decrease of the titer but instead an irregular titer pattern (Fig. 3). In several cases the titer was close to the detection limit, and it became transiently positive after negative periods.

A correlation between the titer and the severity of chronic hepatitis could not be deduced from our data. In favor of such a correlation, however, is the fact that asymptomatic HBsAg carriers rarely (4/84) had detectable IgM anti-HBc (Fig. 1C). Since biopsies were not available, mild liver disease could not be excluded in these four positive persons.

HBsAg-negative, anti-HBc-positive samples. By evaluation of all HBV marker-positive samples, 570 persons were identified who had anti-HBc (total antibody) as a marker of previous or ongoing HBV infection. The absence of HBsAg suggested that the majority of these persons had no active infection. Persons known to have had clinical hepatitis B or transient HBsAg were not included in this group. The great majority of these persons were clinically healthy but belonged to high-risk groups. A significant proportion (17.4%) also had IgM anti-HBc, but titers were mostly below 150 U (Fig. 1D).

The 32 cases (5.8%) with higher titers formed two peaks in the histogram for the titers. In 24 cases the clinical state was known to us. The eight persons with titers higher than 1,200 U (Fig. 1D) all had fully developed hepatitis with ALT values of more than 300 U. In two of these eight cases, weak, transient HBsAg findings of another laboratory became known to us after inquiry. Compared with the 215 HBsAg-positive cases with high IgM anti-HBc, the complete absence of HBsAg in six cases (2.6%) is a relatively rare finding.

The serological picture is probably very different in asymptomatic or mildly symptomatic cases of transient HBV infection. In 10 of 16 persons who had moderate titers (150 to 1,200 U), exposure or a very high risk of infection was known, and only this fact led to the identification of a recent HBV infection. The infection was usually accompa-

nied by moderate ALT elevations (<300). Anti-hepatitis B surface antibody was mostly present even in the first positive serum sample. The histogram of Fig. 1D suggests that titers between 150 and 1,200 U in the absence of HBsAg are typical for mild HBV infections. These titers usually last for several weeks, but in some cases IgM anti-HBc remains elevated (>150 U) for more than 1 year without clinical symptoms.

The large number of low-positive (<150 U) results (67/570) suggests that silent HBV infection occurred recently in many of the persons examined. This reflects the origin of the samples, which came mainly from high-risk groups. Owing to the highly variable persistence of IgM anti-HBc, no conclusion is possible when HBV infection has occurred.

DISCUSSION

Acute hepatitis. This study confirms the usefulness of IgM anti-HBc in the diagnosis of acute hepatitis, which has been suggested by many previous studies. Two types of error would arise if this diagnosis relied exclusively on HBsAg. One error would be the assumption of non-A, non-B hepatitis in HBsAg-negative cases of acute hepatitis. Such a false conclusion would raise unjustified concerns, because non-A, non-B hepatitis has a much worse prognosis than does HBsAg-negative hepatitis B. However, this form of hepatitis B was less frequent (2.6%) in our study than has been reported by others (22). This may be due to earlier sample taking, improved detection of HBsAg, exclusion of subclinical cases from the group with acute hepatitis B, or all of these factors. Frequent absence of HBsAg has been reported in fulminant hepatitis B (17, 31), but such cases were not reported to us.

A second error would be to overlook non-B hepatitis in a chronic HBsAg carrier. In our study with cases from northern Germany, this situation was present in 10% of the HBsAg-positive cases. This is comparable to data from other countries (25, 26). Here the clinical prognosis is worse than in uncomplicated hepatitis B, and close clinical supervision, including liver histology, may be indicated. Hepatitis delta virus was found only once in this group, which is much less than was found in Italy (9, 21). The relatively high rate of non-A, non-B hepatitis in our group is not surprising, if one takes the similar route of infection into account.

Chronic HBV infection. Evaluation of several hundred HBsAg-positive patients in our study confirmed previous assumptions that a clear cutoff level between acute and chronic HBV infections exists (6, 9, 15, 26, 29, 33). Owing to the much larger group of chronic patients, we now adjust this value to a higher level (600 U) than in our previous study (15). We suggest, however, as do others (12), that results below this cutoff value not be ignored but that accurate quantitation be carried out. Moderate IgM anti-HBc titers (between 30 and 600 U) are a strong sign of liver disease in a chronic HBsAg carrier as shown by this and other studies (3, 30-32, 34). Only if the assay is used in its original, overly sensitive form are healthy HBsAg carriers often positive (1, 10, 11, 15, 20, 28, 31). On the other hand, a negative IgM anti-HBc finding does not exclude chronic liver disease. Moreover, IgM anti-HBc is certainly not a marker of viremia (27; unpublished data).

Subclinical HBV infection. Another severe disadvantage of neglecting moderate titers would be the incomplete detection of mildly symptomatic or asymptomatic transient HBV infection. Samples drawn very early or very late during fully developed hepatitis B might also be missed, and the seem-

TABLE 1. Serological findings and diagnoses

IgM anti-HBc titer (U)	HBsAg (RIA) ^a	Suggested diagnosis
>600	+	Acute hepatitis B
30-600	+	Chronic hepatitis B, early acute phase, immune deficiency
<30	+	Healthy HBsAg carrier, chronic hepatitis B, incubation period, immune deficiency
>1,200	-	Acute hepatitis B, rare course
150-1,200	-	Mild transient infection, early convalescence
<150	-	Past infection, silent seroconversion

^a RIA, Radioimmunoassay.

ingly negative result would distort the true situation. This would be especially regrettable because HBsAg is often negative in these situations. Detection of silent HBV infection is of epidemiological importance, and it is greatly enhanced by testing IgM anti-HBc at sensitivity levels below 600 U.

Significance of various titers. In favor of a fixed, high cutoff level at 600 U is its clear correlation with fully developed, acute hepatitis B. As soon as lower values are also considered, certain ambiguities have to be accepted. Table 1 summarizes the possible meanings of various serological findings. Many of the seeming ambiguities can, however, be resolved if the clinical state is known or if a second serum sample is obtained soon after.

Standardization of the assay. The scheme of Table 1 is only applicable for IgM capture assays. This type of assay determines the proportion of specific IgM anti-HBc to total IgM. Ideally, it is independent of anti-HBc in other immunoglobulin classes. In its current form it is free of nonspecific side reactions, and it is of high but not excessive sensitivity. All other assays determine the ratio of IgM anti-HBc to total anti-HBc (2, 5, 7, 30). They may be of advantage during the early acute phase but would be of low sensitivity during late stages or chronic infections. All producers of test kits for IgM anti-HBc and most scientific studies have adopted the IgM capture principle. Use of recombinant HBcAg (8, 18, 29) will further improve the assay.

A generally recognized quantitative reference sample for IgM anti-HBc applicable to all techniques would be highly desirable. Ways to standardize the assay were described previously (14, 33) and applied successfully in this study. A reference sample was deposited at a German control institution, and its validity was confirmed in a cooperative trial involving 29 diagnostic laboratories (R. Thomssen, unpublished data). An international trial and stability studies would be required to establish an international standard.

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