Hepatitis B Surface Antigen: Decreased Need for Confirmation of Reactive Results

To the Editor:

Increasingly large numbers of specimens are referred to laboratories with orders to perform an assay for hepatitis B surface antigen (HBsAg). In our laboratories, reagents from Abbott, either RIA or enzyme immunoassay (EIA), are used for this purpose.

The product insert for the RIA assay states: “It is desirable to perform a specificity analysis before informing a donor/patient that he is an HBsAg carrier with an FDA-licensed neutralization confirmatory test (e.g., the Abbott AUSRIA II-125 confirmatory neutralization test). A repeatedly reactive specimen, confirmed by neutralization with human Anti-HBs must be considered positive for HBsAg”.

Similar advice is given in the EIA insert.

Given this advice, it has been our practice to follow all repeatedly reactive or borderline results with a neutralization assay. This practice leads to delayed reporting and additional expense. We have analyzed data obtained over a period of ~2 years to study the utility of our current protocol.

There were 14,733 records with a repeatedly positive or borderline positive RIA result for HBsAg. These records showed confirmation, with the neutralization assay, for 14,714 of initially positive HBsAg assays. The distribution of S/N ratios (cpm of sample/mean cpm of negative controls) for the 19 specimens that were not confirmed is given in Table 1. All but 2 of the 19 specimens that failed to confirm with the neutralization assay had S/N ratios <6.0. The specimen that gave a S/N ratio of 85.6 was from a 30-year-old HIV-seropositive male. This patient’s record also showed a positive hepatitis B core antibody and a negative hepatitis B surface antibody. His alanine aminotransferase was 1008 U/L, and his aspartate aminotransferase was 475 U/L. Two months later, his alanine aminotransferase and aspartate aminotransferase values were within the reference intervals.

A review of laboratory records for the specimen with an S/N ratio of 170.6 showed a failure by the technologist to adhere strictly to the test protocol. It seems reasonable to conclude that failure to confirm the reactive result for these two specimens, despite the very high ratios, represents a laboratory error.

There were 4514 records with a positive or borderline positive EIA result for HBsAg. Confirmation of these results with the neutralization assay was obtained for 4467 specimens. The distribution of absorbances for the 47 specimens (41 patients) that failed to confirm is also listed in Table 1. No additional information is available concerning the two patients with EIA absorbances >1.0 and non-confirmed results.

We believe that the information obtained by this review of records supports a change in testing protocol. For the RIA assay, a reflex to the neutralization assay for positive or borderline results is not necessary when the S/N ratio is >6.0. For the EIA assay, the reflex is not necessary for specimens with an absorbance >1.0. The analytical specificity of the RIA assay, using this amended protocol, is 99.87%, and that of the EIA is 99%. The number of confirmations required would be reduced 97.7% for the RIA assay and by 94.7% for the EIA assay. A decrease in complexity, improved turnaround time, and decreased expense are expected outcomes of this change in protocol.

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Erratum

To the Editor:

In our letter (Clin Chem 1999;45:1576–8), we erroneously stated that the patient’s AxSYM cTnI values were normal when in fact we should have stated that the values were below the cutoff value for acute myocardial infarction (AMI; ≤2.0 μg/L). We also stated that the study performed by McLaurin et al. (Clin Chem 1997;43:976–82) made use of M11.7 and M7 monoclonal antibodies (MAbs) to detect skeletal isofoms of cardiac troponin T (cTnT) in patients with renal disease. McLaurin et al. used the JS-2 MAb.

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Table 1. Distribution of samples that were positive in the RIA and EIA assays but failed to confirm in the neutralization assay.

<table>
<thead>
<tr>
<th>S/N ratio</th>
<th>Number of samples</th>
<th>RIA</th>
<th>Absorbance</th>
<th>Number of samples</th>
<th>EIA</th>
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<td></td>
<td>Cutoff–0.059</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>2.1–3.0</td>
<td>8</td>
<td>0.060–0.100</td>
<td>13</td>
<td></td>
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</tr>
<tr>
<td>3.1–4.0</td>
<td>4</td>
<td>0.101–0.222</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.1–6.0</td>
<td>1</td>
<td>0.223–1.00</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;6.0</td>
<td>2*</td>
<td>&gt;1.00</td>
<td>2*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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*The S/N ratios were 85.6 and 170.6.
*The absorbances were 1.047 and 2.023.
Evaluation of a Minipool Reverse Transcription-PCR Screening Method for the Detection of Hepatitis C Virus Infection in Hemodialysis Patients

To the Editor:

The prevalence of anti-hepatitis C virus (HCV) antibody among patients on hemodialysis (HD) is consistently higher than in healthy populations, suggesting that dialysis patients may be at a higher risk of acquiring HCV infection (1).

At present, the diagnosis of HCV infection relies mostly on immunoserological screening assays (ELISA) because they combine a direct evaluation of immune anti-HCV response, simplicity in both handling and performance, and a substantially lower cost (2). However, anti-HCV positivity may indicate past infection, current infection, or even nonspecific reactivity (3). HCV RNA determination through qualitative reverse transcription-PCR (RT-PCR) screening assays allows direct HCV detection before any serological alteration, including generation of antibodies or an increase in aminotransferase (4). In fact, several recent studies have demonstrated that de novo cases of HCV infection occur in HD units in the absence of other parenteral exposure (5). This finding underlines the spread of HCV among HD patients and the need for HCV RNA screening assays in the diagnosis of acute HCV infection in seronegative HD patients (6). Until recently, both qualitative and quantitative RT-PCR techniques had no practical application in diagnostic laboratories. Recently, several commercial diagnostic kits have been developed, offering high reproducibility and reliability (7). However, applying HCV genetic testing by means of an approved HCV RNA detection assay to each HD patient often is not suitable because it is a relatively expensive assay and should be performed frequently if acute infection is to be detected. Recently, transfusion services have applied nucleic acid amplification technology by means of the “minipool” methodology (8). In this screening strategy, samples are pooled together and tested at once. Hence, we investigated whether pooling candidate HCV samples before testing them individually, by means of a minipool RT-PCR methodology, may decrease the cost without markedly decreasing the sensitivity of the assay.

We examined 200 HD patients (age, 46.5 ± 18 years; 96 males and 94 females) three consecutive times over a 1.5-year follow-up period for the presence of anti-HCV antibodies and HCV RNA. The procedures followed were approved by our institution’s responsible committee.

HCV RNA qualitative determination was performed in three well-isolated areas (sample preparation, amplification, and detection) to avoid contamination. Specimen preparation was performed with an optimized isopropanol-based method, and detection of HCV RNA was performed by a nonisotopic method adapted to the microwell format, as instructed by the manufacturer (HCV Amplicor™, La Roche). Both positive and negative controls were treated as unknown samples and were included in the initial step of sample preparation. For the minipool RT-PCR qualitative determination, we produced 20 master pools of 10 samples each. If a minipool was reported as HCV RNA negative, then no further HCV RNA evaluation was performed. On the other hand, if a minipool was reported as HCV RNA positive, it was divided in two smaller primary pools of five samples each. Subsequently, if a five-sample minipool was reported as positive, each of the five samples was tested separately for the presence of HCV RNA. In the minipool methodology, the RNA extraction step was performed separately for each sample. Samples were pooled together before the cDNA amplification step. Before the minipool testing, positive controls were diluted 10-fold and tested separately to confirm the sensitivity of the assay. The remaining procedure was performed according to the manufacturer’s instructions supplied by the provided protocol.

Serum samples were tested three consecutive times during the study period for the presence of anti-HCV antibodies by the ORTHO ELISA III (ELISA III HCV; Ortho Clinical Systems) and the MONOLISA anti-HCV New Ag (Monolisa HCV; Sanofi Diagnostics Pasteur) assays. The ORTHO ELISA III detects antibodies directed to core, nonstructural 3 (NS3), NS4, and NS5 antigens; the MONOLISA anti-HCV plus detects antibodies directed to core, NS3, and NS4 antigens. Both assays were performed according to the manufacturers’ instructions. Low- and high titer-positive controls were included in each assay, and these were always positive. Detection of anti-HCV antibodies (c33, N55, C22p, and c100p) by means of the RIBA third-generation assay was also performed three consecutive times according to the manufacturer’s instructions (Chiron). Reverse transcription, PCR amplification, and detection and colorimetric quantification (based on a method adapted to the microwell format) were performed simultaneously, with the incorporation of an internal standardized target sequence control according to the manufacturer’s instructions (HCV-Monitor™ Test; La Roche). Results were expressed as viral copies/mL of human serum.

Results obtained with the minipool RT-PCR methodology were HCV positive for 16 of 200 HD patients (8%). All 16 were found anti-HCV positive by the ORTHO ELISA III, MONOLISA anti-HCV plus, and RIBA assays. Quantitative RT-PCR analysis showed that 3 of 16 PCR-positive samples had viral loads of >500,000 copies/mL and that each patient’s viral load remained relatively stable in three consecutive measurements.

Some of the difficulties in formulating strategies to control the transmission of HCV infection include the high prevalence of HCV in HD patients, the limitations of immunoserological tests in identifying these patients, and the uncertainties regarding the modes of transmission within dialysis units. At this point, detection of acute HCV infection in seronegative HD patients by the
highly sensitive RT-PCR screening assay may represent a critical step in preventing patient-to-patient HCV transmission. Pooling candidate HCV samples may have certain limitations in detecting low concentrations of HCV RNA. This inadequacy may be further strengthened by the nonrandom distribution of HCV quasispecies in plasma and peripheral blood mononuclear cell subsets (9). A role for additional refinement of a HCV diagnosis using tests to determine genotype and subtype as well as quasispecies should be explored. We cannot confirm the sensitivity of this methodology in samples with low HCV RNA; the cutoff for the quantitative HCV RNA determination assay for this study was <2000 viral copies/mL. However, quantitative assays are not intended for use in HCV diagnosis. These tests, in conjunction with the clinical presentation and other laboratory markers, may provide an aid in assessing viral response to antiviral treatment as measured by changes in serum or plasma HCV RNA concentrations. In essence, our findings suggest that pooling candidate HCV samples before testing them individually decreases the time and cost of qualitative RT-PCR analysis up to 50% without reducing sensitivity. In seronegative HD patients, this screening approach may be helpful in identifying currently HCV-infected patients, thus allowing a higher flexibility in implementing policies to prevent patient-to-patient HCV transmission.

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References

To the Editor:

Increased Carbohydrate-deficient Transferrin Concentration and Abnormal Protein Glycosylation of Unknown Etiology in a Patient with Achondroplasia

We report the observation of abnormal protein glycosylation in a patient with achondroplasia in whom known causes of impaired glycosylation could not be found. Automated isoelectric focusing (IEF) was performed in our laboratory for the investigation of glycosylation disturbances of transferrin (1, 2). All procedures were carried out in accordance with the Helsinki Declaration of 1975 as revised in 1996. We used surplus serum samples as controls, and surprisingly, in one of those, IEF revealed a moderate increase in disialotransferrin and a slight increase in asialotransferrin (Fig. 1, lane 1) compared with control serum (Fig. 1, lane 2). A similar IEF pattern has been described, e.g., in alcohol abuse (3) or in carbohydrate-deficient glycoprotein (CDG) syndrome (4). The patient’s isotransferrin pattern had been stable in different blood samples for more than 3 years. The hypoglycosylation of transferrin was confirmed by an increased carbohydrate-deficient transferrin (CDT) concentration of 44 units/L (upper reference limit, 20 units/L) determined by the CDTect assay (Pharmacia & Upjohn).

Clinically, the patient was a 41-year-old man in good general health. His past history included well-controlled arterial hypertension (treated with low-dose β-blockers) and two generalized seizures at the ages of 35 and 37 years. His height was 142 cm, his weight was 57 kg, and his head circumference was 62 cm (75th centile for achondroplasia). He had shown the stigmata of achondroplasia from the neonatal period, and his motor milestones were slightly delayed, which is usual in subjects with achondroplasia. Mental development had been normal. Detailed neurological examination was normal, including the absence of any tremor. Typical skeletal features of achondroplasia were present on radiologi-
Heterozygosity for a g1138a transition leading to a Gly380Arg substitution in the gene coding for fibroblast growth factor receptor 3 confirmed the clinical and radiological diagnosis. This is the most common mutation causing this disorder (5). The patient had worked as an optician for the same small company for 20 years, and continues to do so.

The most obvious suspicion in an adult with increased CDT values is alcohol abuse (3, 6). This had been thoroughly investigated. The clinical findings, history, interview of the patient, and the history given by his relatives gave no evidence of alcohol abuse. Conventional biochemical markers of alcohol abuse (7) determined in this patient were within the reference interval: γ-glutamyltransferase, mean corpuscular volume, aspartate aminotransferase, alanine aminotransferase, and aspartate aminotransferase/alanine aminotransferase ratio. Additionally, we tested for apolipoprotein Al, an additional marker of alcohol abuse (8), which was within the reference interval as well. After carbamazepine therapy for seizures, γ-glutamyltransferase, HDL-cholesterol, and apolipoprotein Al were moderately increased, a known consequence of anticonvulsant medication (9). In addition to negative biochemical markers and history, working for the same small company for 20 years in a job requiring good fine motor skills seems a strong argument against the hypothesis of alcohol abuse. There is a theoretical possibility that low-dose treatment with a β-blocker can cause disturbed glycosylation. We found one study investigating CDT values in treated hypertensive men >50 years with at least one of the following additional problems: tobacco smoking, diabetes mellitus, and/or hypercholesterolemia (10). Increased CDT values were reported to be overrepresented in patients receiving calcium antagonists but not in those receiving β-blockers or diuretics. Meerkerk et al. (11) could not confirm this observation in their study and reported unaffected specificity for CDT concentrations when investigating the possible influence of several common diseases and common medications individually. In the group with false-positive CDT tests, they found more and heavier smokers as well as statistically higher mean corpuscular volumes. Our patient did not smoke.

The patient’s isotransferrin band pattern was also similar to CDG syndrome type I, albeit mild. Various skeletal abnormalities, including dysplasia (12), have been described in patients with CDG syndromes, all of which were different from the typical findings in achondroplasia. The patient’s professional and private activities are incompatible with known CDG syndromes. Furthermore, the biochemical markers that may suggest CDG syndromes, such as albumin, thyroid-stimulating hormone, factor XII, and proteins C and S had been within the appropriate reference values on more than one occasion. Similarly, other biochemical markers known to be pathological in patients with various CDG syndromes were within reference values. Phosphomannomutase and phosphomannose isomerase activities, measured in leukocytes and fibroblasts according to the method described by Van Schaftingen and Jaeken (13), were within reference values. Other well-documented causes of transferrin hypoglycosylation or increased CDT concentrations, such as biliary cirrhosis or chronic active hepatitis (3), fructosemia (14), galactosemia (15), and reduced ferritin concentrations (16) could be ruled out clinically or by routine laboratory analyses. Genetic transferrin D variants known to cause increased CDT values (3) were excluded by IEF after neuraminidase treatment of serum samples. In addition to abnormal transferrin glycosylation, the altered IEF pattern of \( \alpha_1 \)-antitrypsin (data not shown) hinted at a generalized disturbance of N-glycosylation of proteins. We also studied the O-glycosylation of apolipoproteins E and C III by IEF according to Hackler et al. (17) and Noll et al. (18), which did not reveal abnormalities.

To elucidate whether there is a causal link between achondroplasia and disturbed N-glycosylation of proteins, the investigation of additional patients and studies of the glycosylation pathways in cultured fibroblasts are necessary. The CDT test currently is the most specific biochemical marker of alcohol abuse, with a specificity of >90% (11). However, laboratories involved in the interpretation of increased CDT values or screening for CDG patients should be aware of the existence of...
glycosylation disturbances that may not be caused by alcohol abuse or other known reasons. When alcohol abuse is considered, conventional biochemical markers must also be determined as well as a detailed clinical investigation. This is particularly important when the wrong interpretation of increased CDT values could have an impact on the social lives of individuals. Additional investigations of false-positive CDT test results may in the future lead to new insights in the biology of protein glycosylation.

We are grateful for the mutation analysis performed in the Department of Metabolic and Molecular Genetics, University Children’s Hospital, Zurich, Switzerland, Dr. Superti-Furga. We also acknowledge the Department of Clinical Chemistry and Pathobiocchemistry, University Hospital Marburg, Germany, for the determination of CDT concentrations, and thank Dr. Robert Surtees for reading the manuscript and helpful discussions.

References


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Effects of Carboxyhemoglobin on Hemoglobinometers

To the Editor:

During a recent comparison of two portable hemoglobinometers designed for near-patient testing, we noted disparate readings on a quality-control material derived from human hemoglobin (Level 1, Multi-4® CO-Oximeter Controls; Instrumentation Laboratory). The insert sheet listed an acceptable range of total hemoglobin readings of 159–185 g/L (15.9–18.5 g/dL) for the OSM3 co-oximeter (Radiometer A/S). Our results on the OSM3 were at the midpoint of this range, as were results with one of the hemoglobinometers, the Hb-Quick® hemoglobinometer from Avox Systems [mean (SD), 175 (0.5) g/L; n = 5]. By contrast, readings on a HemoCue hemoglobinometer (HemoCue AB) were 16 g/L higher than the mid-range value and averaged 188 ± 1.5 g/L. Both hemoglobinometers had recently been calibrated by factory-trained employees of the respective companies.

Because Level 1 controls contained ~60% carboxyhemoglobin, we made additional measurements on fresh whole blood from a nonsmoking donor and on other controls with low carboxyhemoglobin (Level 2, Multi-4 CO-Oximeter Controls). The same blood samples and controls were then equilibrated with carbon monoxide by rotating a syringe containing a small volume of blood or control and a much larger volume of carbon monoxide gas. With CO equilibration, the carboxyhemoglobin fraction (percentage of HbCO), as measured by the OSM3, increased from 2.9% to 94.9% in Level 2 controls, from 0.2% to 96.8% in whole blood, and from 57.3% to 93.1% in Level 1 controls, respectively.

The Hb-Quick and HemoCue both initially gave readings on Level 2 controls that agreed with the insert sheet (Table 1). With increased HbCO, the HemoCue’s readings on Level 2 controls increased spuriously by 17 g/L, whereas readings on the Hb-Quick were not affected (P >0.05). Equilibrating whole blood...
with CO increased the Hb-Quick’s average reading by a small although statistically significant 2.6 g/L, but the HemoCue’s reading increased by 12.4 g/L. The effect of CO on the HemoCue was not consistent: equilibrating Level 2 control with CO increased readings on the HemoCue, but equilibrating Level 1 control with CO reduced the HemoCue’s average reading by 3.0 g/L. With CO equilibration, the Hb-Quick’s readings on Level 1 control rose slightly, by 2.2 g/L.

The two hemoglobinometers operate on different principles. To measure the total hemoglobin concentration spectrophotometrically in a typical clinical blood sample, these instruments must solve two problems: the intense light scattering caused by red blood cells, and the fact that the four principal species of hemoglobin (oxy-, deoxy-, carboxy-, and methemoglobin) each have distinct but overlapping absorbance spectra. The Hb-Quick hemoglobinometer (1) measures directly in unaltered whole blood by a proprietary optical design and mathematical algorithms to compensate for light scattering, and it uses a sufficient number of wavelengths to include all four principal species of hemoglobin in its measurements. By contrast, the HemoCue system (2,3) uses disposable cuvettes preloaded with dry reagents that first hemolyze the sample to eliminate light scattering and then convert the various hemoglobin types into a single species (azide-methemoglobin) so that the total hemoglobin concentration can be measured by the optical absorbance at a single wavelength.

In view of the principle of operation used by the Hb-Quick, it is understandable that this hemoglobinometer was relatively unaffected by carboxyhemoglobin because it includes that species in its measurement of total hemoglobin. The most likely explanation for the HemoCue’s aberrant readings is that carboxyhemoglobin is relatively resistant to conversion into azide-methemoglobin. In fact, carboxyhemoglobin is a well-known source of error in the cyanmethemoglobin method and others that depend on chemical conversion (4–6). In further support of this explanation, when HemoCue cuvettes are filled with blood or hemoglobin-based controls, the sample usually turns brown within 1–2 min as the azide-methemoglobin is formed; however, we noticed that samples with high carboxyhemoglobin concentrations remained bright red, indicating that some or all of the carboxyhemoglobin was not converted into azide-methemoglobin.

As Table 1 shows, the effects of carboxyhemoglobin on the HemoCue were inconsistent and seemed to depend on the initial percentage of HbCO. Therefore, the HemoCue should be used with caution if quality-control solutions, proficiency-testing materials, or clinical blood samples contain significant fractions of carboxyhemoglobin. By contrast, the Hb-Quick gives accurate readings in the presence of high concentrations of carboxyhemoglobin both in whole blood and in hemoglobin-based controls.

We thank Olle Hagström for calibrating the HemoCue and providing a generous supply of cuvettes.

References

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A representative of HemoCue AB responds:

To the Editor:

It is well known that increased concentrations of carboxyhemoglobin (COHb) will lead to overestimation of hemoglobin concentrations as measured photometrically at a single wavelength.
wavelength \((1)\). In the study above, at COHb concentrations of 60–90%, the HemoCue instrument read <3 g/L (0.3 g/dL) outside the acceptable range.

The relevance of these findings to clinical settings is tenuous. The normal COHb concentration in blood is 0.4–0.8%. In urban environments, nonsmokers have COHb concentrations typically in the 1–2% range. In smokers, COHb is typically 4–5% and as high as 9%. Values >10% are abnormal. At 20%, dizziness, nausea, and syncope develop, and at 50%, seizures and coma develop; 60% is associated with death \((2)\).

The conversion of COHb to azide-methemoglobin in the HemoCue system or to cyanmethemoglobin in the International Reference Method takes longer than conversion of other hemoglobin derivatives. Because the absorbance of COHb at 570 nm is \(\sim 14.3 \, \text{L} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}\), a total non-conversion of COHb produces higher values for the hemoglobin concentration. The upper limit of this error can be calculated. At 5% and 10% COHb, the maximum errors are 1.5% and 3%, respectively.

The authors of the above letter conclude that the effect of COHb is inconsistent on the HemoCue system. Only one fresh whole blood from a single nonsmoking donor and two control materials were assayed.

When control material is assayed, matrix effects have to be considered. Careful study of additional samples is needed before this conclusion can be supported.

References

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Correction
In the article by M.N. Nanjee and E.A. Brinton, entitled “Very Small Apolipoprotein A-I-containing Particles from Human Plasma: Isolation and Quantification by High-Performance Size-Exclusion Chromatography” (Clin Chem 2000;46:207–23), on page 214 (left column, fourth line from bottom) and page 219 (left column, last line), the reference to Fig. 4 should in fact be to data shown in Fig. 5. The authors regret any confusion this may have caused.