PCR Conditions for HFE C282Y: Lack of Effect of 5569G/A Polymorphism with 55 °C Annealing

To the Editor:
The HFE gene mutation designated C282Y (g.5474G→A; GenBank accession no. Z92910) is usually detected by the primers described by Feder et al. (1) in a PCR to amplify genomic DNA followed by restriction enzyme cleavage based on the production of either a SnaB1 (2) or a RsaI (3) cut site. A polymorphism (5569G/A) located five nucleotides within the 3' end of the binding site for the antisense primer has been reported to lead to false-positive results for C282Y homozygosity (4). The false-positive result occurs in analysis of a C282Y heterozygote compound with the 5569G/A polymorphism because of selective amplification of the C282Y-containing allele. A new antisense primer (5'-TACCTCCTCAGGCACCTCCTC-3') that did not bind the 5569G/A site was designed (4).

We developed a RsaI genotype assay (5) with the primers used by Feder et al. (1) and established the PCR conditions by assessing the genotype results in a group of hemochromatosis cases diagnosed by strict phenotypic criteria. The PCR cycling conditions included a lower annealing temperature (55 °C) than reported previously (2). The conditions were as follows: 94 °C for 3 min, and then 35 cycles of 94 °C for 60 s, 55 °C for 45 s, and 72 °C for 1.5 min, and finally 72 °C for 2 min. The 25-μL reaction volume contained 50 ng of DNA and 5 pmol of each primer.

In response to the possibility of false-positive results for C282Y homozygosity, our RsaI genotype assay was validated by two approaches.

In the first approach, we reamplified 112 DNA samples reported previously as C282Y homozygotes by substituting the newly described antisense primer (4) for the Feder et al. (1) primer in our RsaI method. All 112 samples were confirmed as C282Y homozygotes, indicating that results obtained with the RsaI method were unaffected by the 5569G/A polymorphism.

In the second method, to test for the presence of the 5569G/A polymorphism, DNA from 86 samples reported previously as C282Y heterozygotes was reamplified using the Feder et al. (1) primers and PCR conditions described above, except that the annealing temperature was raised to 62 °C. One C282Y heterozygote sample out of the 86 tested apparently changed genotype to homozygous for C282Y. Sequence analysis of the genomic DNA confirmed that this sample was a C282Y/5569A compound heterozygote.

Single nucleotide polymorphisms (SNPs) occurring at primer binding sites are potentially a serious problem, and the overall genomic prevalence is estimated to be between 1 in 300 and 1 in 1000 bases (6). Testing for occult SNPs that may lead to false-positive or -negative results in a new diagnostic PCR assay is a critical but neglected task. The gold standard for detecting interfering SNPs is to sequence the region under the primer from a large number of subjects. An alternative approach is to perform each PCR reaction twice, using two different sets of nonoverlapping PCR primers. This would double the cost of the PCR analysis but provides added confidence in the results. The possibility of occult SNPs interfering with primer binding should be excluded before introducing PCR-based genotype assays into routine diagnostic practice.

We have demonstrated that the presence of the 5569 G/A polymorphism did not produce false-positive results in our RsaI genotype assay when the primers described by Feder et al. (1) were used. Binding of the antisense PCR primer to genomic DNA containing the 5569A polymorphism was not affected when the annealing temperature was 55 °C. However, we have shown the new antisense primer (4) functions well in the RsaI method, and we advocate its use to avoid any problems caused by the 5569G/A polymorphism.

References

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Changes in Academic Productivity: Implications for Clinical Laboratory Research and Development

To the Editor:
A recent report from the Council of Scientific Affairs of the American Medical Association indicated that the introduction of managed healthcare had a substantially negative influence on the academic productivity of clinical faculty in academic health centers (1). Consistent with this finding is the observed change in the number of scientific abstracts presented during the Annual Meeting of the AACC (2). As can be seen, over the last 4 years there has been a substantial 30% decrease in the num-
Fig. 1. Indicators of AACC academic productivity from 1992 through 1999. Comparison of abstract volume (A and B) and abstract acceptance rate (C) for the AACC annual meetings with student participation (D), COMACC-approved clinical chemistry training programs (E), and AACC membership (F).
ber of abstracts received and accepted from all sources, including academia and industry (Fig. 1, A and B). This decline does not correlate to a change in the abstract rejection rate, which has remained relatively constant (∼20%) since 1992 (Fig. 1C).

To more closely examine this finding, we undertook a retrospective study to determine whether the percentage of abstracts presented at the AACC Annual Meeting from academic institutions was disproportionately decreased relative to the total number of abstracts (including those from industry). Abstracts were assigned on the basis of authors’ affiliation. In the case of multiple affiliations, abstracts were assigned on the basis of the first author’s affiliation. Surprisingly, we found that the percentage of abstracts presented from academia increased from 58.4% in 1992 to 63.0% in 1999. This unexpected change may, however, reflect a more recent policy (1997) of the AACC Annual Meeting Organizing Committee to eliminate duplicate and triplicate abstracts on industry-sponsored instrument evaluations in which the only difference between submitted abstracts was the analyte; this policy may initially artifactually increase the percentage of contributions from academic institutions. However, relatively few abstracts were affected by this policy (∼25 per year). Thus, the large observed decrease in abstract volume cannot be attributed solely to implementation of this policy.

Although the percentage of academic abstracts increased from 1992 to 1999, the number decreased ∼12% (from 426 to 379). The number of students presenting their work at the annual student poster contest has also decreased, most dramatically in recent years (Fig. 1D). The reason for the decline in student participation is not known, but it probably is related to a decreased number of funded fellows in clinical chemistry training programs (A.H.B. Wu, personal communication) and a decreased number of programs approved by the Commission on Accreditation in Clinical Chemistry (COMACC; Fig. 1E). Decreased student involvement may also reflect decreased funding for academically related activities, including travel to the national meeting.

In sharp contrast to the changes above, total AACC membership has remained relatively steady over this period (1995–1999; Fig. 1F) (3). Despite this success, it can be anticipated that the decline in junior faculty participation and training programs will negatively impact long-term membership goals and subsequent clinical laboratory research and development. It is well known that decreased clinical revenue streams substantially limit the flexibility of academic health centers, including the funding of graduate medical education programs, i.e., postdoctoral fellowships, residency training, and the research activities thereof (1, 4, 5). In an effort to stabilize existing revenue sources and prevent further anticipated erosion, performance-based compensation for clinical faculty has been introduced (1, 4). This strategy rewards clinical productivity at the expense of other “nonproductive”, i.e., non-revenue-generating activities. The establishment of a service-based compensation system within an academic medical center dissuades academic productivity, especially among junior clinical faculty (1, 4, 5), i.e., faculty likely to publish preliminary research results in abstract form and participate in poster sessions.

Although most laboratorians are aware that the diagnostic services they provide are now viewed as “cost centers”, these data should compel us to evaluate the long-term implications of these findings. The growth of managed care, the increasing influence of health maintenance organizations, and the changes instituted by the Balanced Budget Act of 1997 will exacerbate an already tenuous situation in the provision of healthcare. Academic health centers will be particularly disadvantaged because of their need to balance increased clinical activity with their primary roles of teaching, research, and education.

Are the above data an indication that academic productivity is decreasing? Whether these observations provide early indicators of future constraint on academic commitment to clinical laboratory research and development missions remains to be determined.

We thank Christine Donnell, Marian Valley, Erika Witherspoon, Kimberly Thompson, and other staff members of the AACC National Office for their cooperation. Dr. Tsongalis was Abstracts Coordinator for the 1999 AACC Annual Meeting and Clinical Laboratory Exposition; Dr. Wu is President, Commission on Accreditation in Clinical Chemistry.

References

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Hemolyzed Specimens: A Reason for Rejection or a Clinical Challenge?

To the Editor:

Clinical laboratories must improve the preanalytical phase, a phase highly susceptible to mistakes (1). In some reports, hemolyzed specimens, the most common reason for rejection, account for ~60% of rejected specimens, fivefold more than the second most common cause (2). Cellular contents can falsely increase values for some plasma constituents, such as potassium, lactate dehydrogenase, and aspartate aminotransferase (3). Moreover, hemolysis produces spectrophotometric interference with other laboratory methods.

In vitro hemolysis depends mainly on the way in which the blood samples are drawn and treated, and it may in particular depend on the blood being forced through too fine a needle (4) or through the large-bore needle of a syringe into a tube; it may also be caused by shaking the tube too vigorously and/or centrifuging blood specimens before clotting is complete. In vivo hemolysis, on the other hand, may have at least 50 causes. We evaluated the causes of hemolysis in samples received by our STAT section in the Department of Laboratory Medicine of the University Hospital of Padova, which performs all clinical chemistry tests on specimens collected using lithium heparin as anticoagulant (Becton Dickinson).

Specimens, collected by physicians or nurses from hospitalized patients, were mainly from the departments of internal medicine (28%) and surgery (21%), intensive care units (23%), the emergency department (16%), and the department of organ transplantation (9%). Over a 30-day observation period, we evaluated 27540 blood specimens from 15323 sample requests for clinical chemistry, coagulation, and toxicological tests. According to the study protocol, each time hemolysis was visually identified, even if it was only slight, the laboratory contacted the phlebotomists to find out the procedure utilized for vascular access, the technique used for drawing blood, and to obtain information on the transportation, preservation, and storage of the specimens. If no errors in these procedures were identified and in vivo hemolysis was not suspected clinically, serum haptoglobin was measured immediately and at 24 h with a view to confirming the presence of any acute hemolysis, which was clinically evaluated and then confirmed in a further phase.

We identified 505 hemolyzed specimens (3.3%); of these, 64% were affected by a small degree (<50 mg/L of hemoglobin) of hemolysis, 31% by an intermediate degree, and 5% by a high degree (>300 mg/L of hemoglobin). The concentration of hemoglobin in plasma was measured by a colorimetric assay (Plasma haptoglobin; Sigma-Aldrich). The percentages of hemolyzed specimens were similar in the internal medicine and surgery departments (3.1%), intensive care units (3.5%), the emergency department (3.3%), and in the department of organ transplantation (3.4%).

In most cases it was possible to relate the presence of hemolysis to a specific cause because of the cooperation of phlebotomists and nurses, and only 26 (5.1%) cases remained unresolved (Table 1). Importantly, hemolysis from excessive aspiration force was relatively frequent, mainly in the case of small or superficial veins. Another frequent cause was the presence of a partial obstruction of an arterial catheter, leading to an increase in the aspiration force when a syringe was used to collect the sample. Yet another cause was hemolysis caused by forcing blood from a syringe into a tube, which was confirmed by observing a difference in the degree of hemolysis in the different tubes filled with blood from the same syringe. Collection of samples by syringe was associated with a higher rate of hemolysis as 83.8% of hemolyzed specimens were collected with syringes, vs 70% of the total number of specimens. In vivo hemolysis accounted for 16 of 505 cases (3.2%); 7 of these cases were associated with prolonged extracorporeal circulation during cardiac surgery; 2 with of acute ethanol toxicosis, 3 with transfusional reactions, 1 with necrotic-hemorrhagic pancreatitis, 1 with rhabdomyolysis from drug overdose, and 2 were of unknown etiology. Importantly, in 5 of the 16 cases, the presence of hemolysis was not suspected by clinicians, and the laboratory finding was essential in identifying the presence of a critical situation, thus potentially improving the medical outcome.

We conclude that:

(a) Hemolyzed specimens are a critical preanalytical problem calling for well-designed and implemented laboratory guidelines and recommendations.
(b) The frequency of in vitro interferences can be reduced by introducing and correctly utilizing evacuated tube systems, instead of syringes.
(c) If a specimen is found to be

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hemolyzed, it cannot simply be rejected, but the laboratory should alert the clinician so that any in vivo hemolysis can be ruled out. The laboratory guidelines for in vivo hemolysis should include the measurement and immediate transmission of results of some laboratory tests, at least potassium in emergencies, which can provide the clinician with essential information, thus allowing identification of clinical situations requiring immediate intervention.

(d) A consensus between the medical laboratory and clinicians should be reached to assure the correct use of and improvement in these guidelines.

References

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Effect of Rheumatoid Factor on Cardiac Troponin I Measurement Using Two Commercial Measurement Systems

To the Editor:
The Abbott AxSYM cardiac troponin I (cTnI) immunoassay may generate false-positive results in the presence of specific human anti-animal antibodies (HAAAs) (1). We use the term HAAAs rather than heterophile antibodies, a term used for weak, multispecific antibodies against diverse antigens (2), either human or...
animal. Techniques are available to detect and correct the interference attributable to HAAAs. We have used the Heterophile Blocking Tube (HBT; Scantibodies Inc.) to identify interference from suspected HAAAs. Anecdotal reports and experience in our laboratory have also suggested that the Abbott AxSYM cTnI may also be susceptible to interference from rheumatoid factor (RF), a heterophile antibody. Abbott Laboratories has recently modified its cTnI reagent system to address the issue of HAAA and heterophile antibody interference. The objective of this study was to compare the Beckman Access and Abbott AxSYM (original and modified) cTnI assays in serum samples containing RF, before and after pretreatment with HBT.

We first designed a retrospective study on 23 sera with RF >80 kIU/L. We measured cTnI on both analytical systems with and without prior HBT treatment. In the second part of the study (during the phase-in period of the modified formulation), separated sera of 19 RF-positive samples (>80 kIU/L) were analyzed with Abbott’s original and modified cTnI immunoassay. Samples for both studies were obtained with all patient identifiers removed.

In the first study (Table 1A), the Beckman Access method gave cTnI results of 0 µg/L for 22 of 23 samples, with the remaining sample having a result of 0.08 µg/L. On the Abbott AxSYM, values ranged from 0 to 13.1 µg/L. Pretreatment of specimens with the HBT did not change the results with the Beckman Access method. For the Abbott AxSYM original formulation after pretreatment with the HBT, the results for eight samples remained unchanged, five showed increases in value from 1.2- to 4.2-fold, and seven showed decreases in value from 20% to 94%. Pretreatment with HBT reduced only one of the AxSYM cTnI results to 0 µg/L.

In the second part of the study (Table 1B), the original Abbott formulation yielded results of 0 µg/L for 3 of 19 RF samples, with results for the remainder as high as 10.0 µg/L. In analysis of the same specimens with the modified formulation, 17 of 19 samples had values of 0 µg/L. One sample increased from 0.2 to 0.4 µg/L, and one decreased from 4.3 to 0.5 µg/L.

Two interesting cases arose during these studies. The first was an individual with a cTnI of 74.1 µg/L with the original Abbott formulation in spite of little or no clinical evidence of cardiac ischemia. When the serum sample from that patient was reanalyzed with the enhanced formulation, the result fell to 0.4 µg/L. No additional sample was available for study. The second individual had little or no clinical evidence of cardiac ischemia, but had a cTnI of 25.3 µg/L with the original Abbott formulation and 0.8 µg/L with the modified assay. The results of pretreatment of the sample with the HBT before reanalysis with the original formulation were consistent with the presence of a HAAA.

Our results suggest that the Beckman Access is less affected by RF interference than is the original formulation of the Abbott AxSYM system. The results further suggest that treatment of samples with HBT is not an appropriate step to eliminate RF interference in the original Abbott formulation. The introduction of the modified formulation of the cTnI assay kit by Abbott appears to have largely eliminated this problem. In two cases, the newer version did not reduce the cTnI value to 0 µg/L. This may, however, have reflected non-antibody interferents or cTnI in the sample. Furthermore, anecdotal evidence based on two samples suggests that the new formulation is more robust than the original with respect to interference by HAAAs.

We thank Abbott Diagnostics (Mississauga, Ontario, Canada) and Beckman-Coulter (Mississauga, Ontario, Canada) for providing the cTnI kits.

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Correction for Stott et al.: Simple Multiplex PCR for the Simultaneous Detection of the C282Y and H63D Hemochromatosis (HFE) Gene Mutations

To the Editor:

It has been drawn to our attention that the PCR primer 282rev described in our Technical Brief [M.K. Stott, A.P. Fellowes, J.D. Upton, M.J. Burt, and P.M. George. Simple multiplex PCR for the simultaneous detection of the C282Y and H63D hemochromatosis (HFE) gene mutations. Clin Chem 1999;45:426–8] contains a mismatch, at the penultimate base, to the GenBank sequence (GI: 1890179 at www.ncbi.nlm.nih.gov). This is attributable to an inadvertent error during the design of the assay. The primer sequence described has been used successfully for the analysis of many clinical samples. The primer sequence described introduces an A:A mismatch, which is not particularly destabilizing and does not prevent amplification. Although other groups may prefer to use a perfectly matched “correct” 282rev primer (CCA TCC CCT AAC AAA TTA), this may require reoptimization of the primer concentration and PCR conditions.
Common DPYD Mutation Associated with 5-Fluorouracil Toxicity Detected by PCR-mediated Site-directed Mutagenesis

To the Editor:

The human dihydropyrimidine dehydrogenase gene (DPYD) encodes dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2), the first and rate-limiting enzyme in the three-step pathway of uracil and thymine metabolism. DPD is also the principal pathway of uracil and thymine catabolism. DPYD deficiency leads to increased toxicity of 5-fluorouracil (5-FU) monotherapy or polytherapy (in cancer treatment) and mono- or polychemotherapeutic drugs in cancer treatment (in monotherapy or polytherapy) and the c.1905+1G→A mutation is frequently linked to severe toxicity, molecular screening of cancer patients could be done routinely, coupled with analysis of DPD activity in peripheral blood mononuclear cells, before the start of treatment to avoid the toxic effects of 5-FU. Because economic problems are very important in health-screening strategies, screening tests must be the least expensive. The use of SnaBI in a PSM method produces an 18-fold decrease in the enzyme cost ($0.70 vs $12.87 US per reaction) compared with the previous PCR-restriction method using MaeII (7, 8).

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References


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