tibodies or other substances in these presumably normal specimens. The Access PRL assay after PEG treatment may be suitable despite the CV of 14% because PEG treatment is frequently used to detect macroprolactin interference (6). It is possible that two samples with low recoveries contained macroprolactin. Our experience with this assay is that samples with PEG recovery values <60% contain high-molecular-weight prolactin immunoreactivity after gel filtration.

The considerable variability seen in the Elecsys PTH and β-CTx and the Access GH assays after PEG treatment probably reflects interference by the PEG in the antibody–antigen reactions of these assays. Similarly, although we do not know the mechanism, it appears that HBT treatment can produce a spuriously high recovery of hormone in some assays. The manufacturers state that HBT contains a “unique blocking agent” limited to use for antigen assays to confirm or disqualify an original result in conjunction with other data (such as symptoms and other testing). Our data suggest a further limitation that its use is assay specific, possibly dependent on the assay configuration. We did not observe overrecovery in the three Elecsys assays (all configured with mouse monoclonal antibodies for both capture and detection). It was, however, apparent in the Access assays (LH, FSH, GH, and to a lesser extent, PRL), which contain solid-phase goat–anti-mouse monoclonal antibody complexes for capture and goat antibodies for detection.

We believe that these high HBT recovery values are likely to be spurious. The heterophile antibody interference in most immunometric assays is generally positive, leading to lower concentrations after blocking treatments, indicating of nonspecific interference in the assay. In contrast, dilution of a HBT-treated aliquot showed poor linearity (170–486%) in all assays, indicative of nonspecific interference in the assay.

In summary, the validity of the technique used to detect heterophile antibody interference is specific to each assay method. Our data suggest that use of HBT tubes may be a suitable simple technique for the Elecsys PTH, β-CTx, and insulin assays but not the Access LH, FSH, PRL, or GH assays. Other blocking agents may be appropriate for these methods. Pretreatment with PEG is suitable for the Elecsys insulin and Access FSH and, possibly, PRL assays. We conclude that the reagents used to test for heterophile antibody interference can themselves cause variable interference in some assay systems; therefore, the validity and expected ranges should be checked for each assay and heterophile antibody detection method.

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References

Hemoglobin H Disease Classification by Isoelectric Focusing: Molecular Verification of 110 Cases from Thailand, Pranee Sutcharitchan,1† Wen Wang,2† Rung Set-tapiboon,1 Supaporn Amornsiriwat,1 Arnold S.C. Tan,2 and Samuel S. Chong3–5† (1 Division of Hematology, Department of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok, Thailand; Departments of 2 Pediatrics, 3 Obstetrics/Gynecology, and 4 Laboratory Medicine, National University of Singapore and Hospital, Singapore, Singapore; 5 Departments of Pediatrics and Gynecology/Obstetrics, and McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD; † F. Sutcharitchan and W. Wang contributed equally to this work and are listed alphabetically; * address correspondence to this author: Department of Pediatrics, National University of Singapore, Level 4 NUH, 5 Lower Kent Ridge Road, Singapore 119074, Singapore; fax 65-6779-7486, e-mail paecs@nus.edu.sg)

Hemoglobin (Hb) H disease is a mild to severe form of α-thalassemia caused by the absence/inactivation of three
of four α-globin genes. As a result, there are insufficient α-globin chains to form HbA (α2β2), the excess β-globin chains forming unstable HbH (β4) (1), which precipitates and attaches to the erythrocyte membrane to cause membrane dysfunction and hemolysis (2). Genetically, there are two types of HbH disease, deletional HbH disease caused by compound heterozygosity for a double α-globin gene deletion on one chromosome and a single α-globin gene on the other (−α/−α), and nondeletional HbH disease caused by compound heterozygosity for a double α-globin gene deletion and a point mutation or small deletion/insertion on a third α-globin gene (αα/−α or αα2/−−) (2). Generally, patients with nondeletional HbH disease present with a more severe phenotype than those with deletional HbH disease (1, 2).

HbH disease is particularly prevalent in Southeast Asia because of the high frequency of α-thalassemia carriers in these areas (1, 2). In northern Thailand, ~1.5% (1 in 65) of babies are expected to be born with HbH disease (3). In addition, nondeleional HbH disease with αConstant Spring (HbHCS) is very common in Thailand, where ~40–50% of patients with HbH disease have nondeletional HbH with αConstant Spring (4).

Since the 1980s, isoelectric focusing (IEF) has been widely used in the identification of hemoglobin variants and in the diagnosis of HbH disease and HbBarts hydrops fetalis because of the good separation of HbBarts, HbH, and HbCS tetramers in IEF gels. The accuracy of IEF in HbH disease classification, however, is unknown. To evaluate the accuracy of IEF in predicting α-globin genotype, we analyzed the genotypes of 110 Thai patients identified as having either deletional HbH disease or nondeletional HbHCS disease by IEF.

Patients were seen at King Chulalongkorn Memorial Hospital, Bangkok, Thailand. Those suspected of having thalassemia on the basis of hematologic markers were further screened by hemoglobin IEF. IEF was performed using the RESOLVE® Hemoglobin Kit (Perkin Elmer) according to manufacturer specifications. Patients were classified as having HbH disease by IEF based on the presence of a HbH band, the farthest migrating on the IEF according to manufacturer specifications. Patients were further screened by hemoglobin IEF. IEF was performed on the basis of hematologic markers were also as the promoter and some upstream sequences of each gene are amplified. Primers α-F, α-BF, and α-R are used for sequencing of gene fragments of both HBA1 and HBA2.

We incubated 3 μL of amplified product with 1 μL of shrimp alkaline phosphatase (1 kU/L) and 0.4 μL of exonuclease I (10 kU/L; United States Biochemical) at 37 °C for 15 min to remove excess PCR primers and unincorporated deoxynucleotide triphosphates. The reaction was stopped by a 15-min incubation at 80 °C.

Purified PCR products were sequenced with primers α-F, α-BF, and α-R (Fig. 1). All three primers will anneal to both the HBA1 and HBA2 PCR products and were designed to work optimally under the same cycle-sequencing condition. Each 10-μL reaction mixture contained 4.4 μL of purified PCR products, 1.6 μL of 1 μM sequencing primer, 2 μL of 5× Q solution (Qiagen), and 2 μL of BigDye® Terminator Ready Reaction Mix v3.0 (Applied Biosystems). An initial 2-min denaturation at 95 °C was followed by 25 sequencing cycles of 98 °C for 30 s, 50 °C for 30 s, and 60 °C for 4 min. Sequencing products were precipitated with ethanol, resuspended in 12 μL of Hi-DiTM formamide, and analyzed by automated capillary electrophoresis on an ABI PRISM® 3100 Genetic Analyzer by a rapid 40-min electrophoresis through POP-4™ polymer across a 36-cm capillary (Applied Biosystems).

Of the 110 Thai patients identified as having HbH disease by IEF, the most common α-globin genotypes were −αα/−−SEA (n = 56; 51%) and αααα/−−SEA (n = 44; 40%). The −−SEA double-gene deletion was present in all but two patients, both of whom carried the −−THAI double-gene deletion.

Of the 67 patients identified as having deletional HbH disease by IEF, 56 were compound heterozygous −αα/−−SEA, 2 were −αα2/−−SEA, 5 were αααα/−−SEA, 1 was αααα/−−SEA, and 1 had an apparent −−SEA/−−SEA genotype, whereas the remaining 2 had an apparent αααα/−−SEA genotype. The IEF misclassification of the six patients with a nondeletional HbH genotype as being deletional HbH was presumably attributable to false-negative detection of the HbCS band. More surprising were the three patients in this group with
apparent $-^{\text{SEA}}/_{\text{SEA}}$ (one patient) and $\alpha\alpha/_{\text{SEA}}$ (two patients) genotypes, which are inconsistent with HbH disease. However, the genotype assignments for these three patients may simply reflect the limitations of the molecular genotyping assays, which screen only for specific mutations. For example, the patient with an apparent $-^{\text{SEA}}/_{\text{SEA}}$ genotype could in fact be compound heterozygous for $-^{\text{SEA}}$ and another deletion that is not one of the seven deletions screened for in the multiplex gap-PCR assay. If this other deletion inactivates only one $\alpha$-globin gene, then the genotype would be entirely consistent with a HbH disease phenotype. In addition, although sequencing of the $\text{HBA1}$ and $\text{HBA2}$ genes on the nondeleted chromosome of both apparent $\alpha\alpha/_{\text{SEA}}$ patients did not reveal any mutation that could account for their HbH disease classification, we cannot exclude the possibility that an undetected mutation exists elsewhere within the $\alpha$-globin gene cluster in these patients. IEF is a very sensitive protein detection method, and the presence of very small amounts of HbH protein could account for their HbH disease classification. However, the red cell distribution width (RDW) in individuals with $\alpha$-thal-1 trait individuals may sometimes be misinterpreted as HbH disease. However, the red cell distribution width (RDW) in individuals with $\alpha$-thal-1 trait is almost always within the reference interval, whereas patients with HbH disease generally have higher than normal RDW values as a result of anisocytosis. Because the two patients with an apparent $\alpha\alpha/_{\text{SEA}}$ genotype had RDW values of 18% and 19%, which are higher than the reference interval (10.9% to 15.7%), they may actually be HbH disease patients carrying an undetected mutation in the nondeleted allele.

Among the 43 patients identified as having HbHCS disease by IEF, 39 were compound heterozygous $\alpha^{\text{CS}}\alpha/-^{\text{SEA}}$, 2 were $\alpha^{\text{Ps}}\alpha/-^{\text{SEA}}$, 1 was $\alpha^{\text{CS}}\alpha/-^{\text{THAI}}$, and 1 was $\alpha^{\text{Ps}}\alpha/-^{\text{THAI}}$. The Pakse (Ps) mutation involves an A$\rightarrow$T transversion in the termination codon (Cd142) of the HBA2 gene, causing its conversion to tyrosine [TAA(ter)$\rightarrow$TAT(tyr)]. Like the Constant Spring (CS) mutation [Cd142, TAA(ter)$\rightarrow$CAA(glu)], the Pakse mutation produces an aberrant $\alpha$-globin peptide that is longer by 31 amino acids. Thus, the migrations of the mutant HbPs and HbCS molecules in an IEF gel are likely to be very similar, if not identical, in which case the IEF assay would be unable to distinguish between them. Unfortunately, there were no significant differences in hematologic profiles between HbHPs ($n = 4$) and HbHCS patients ($n = 45$) to aid in differentiating between them (Table 1).

To detect as many nondeletional $\alpha$-thalassemia mutations as possible, we sequenced the high-GC-content $\alpha$1- and $\alpha$2-globin genes. Consistent with previous studies, Constant Spring was the most common nondeletional mutation (45 of 49 patients; 92%). Interestingly, the Pakse mutation was observed in four patients. Pakse was first reported in 1994 in a single Laotian patient with HbH disease (6) and initially thought to be rare, but there have recently been new reports of the mutation (7, 8). Our findings confirm that after Constant Spring, Pakse might be the second most common nondeletional $\alpha$-thalassemia determinant in Thailand and neighboring regions.

In summary, patients classified by IEF as being nondeletional HbH showed 100% (43 of 43) concordance with molecular genotyping results, although IEF could not distinguish between types of point mutations and double-gene deletions. Molecular genotyping results for patients classified by IEF as having deletional HbH, however, were only 87% (58 of 67) concordant because of misclassification of several HbHCS, HbHPs, apparently homozygous $-^{\text{SEA}}$, and apparently heterozygous $-^{\text{SEA}}$ patients as having deletional HbH. Overall, the majority of these discrepancies are not anticipated to pose a major problem in patient management, given the broad and overlapping hematologic profiles of deletional and nondeletional HbH disease (Table 1). However, the true prevalences of various deletional and nondeletional $\alpha$-thalassemia determinants is masked. Molecular diagnosis is still necessary for accurate $\alpha$-thalassemia genotyping, confirmation of clinical/hematologic diagnoses of $\alpha$-thalassemia, and prenatal counseling and diagnosis/prevention of severe $\alpha$-thalassemia in at-risk pregnancies.

**Table 1. Hematologic profiles of patients with deletional and nondeletional HbH disease, HbPakse, and HbHCS.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>$\alpha/\alpha$ (n = 58)</th>
<th>$\alpha^\text{Ps}\alpha/-$ (n = 49)</th>
<th>$\alpha^\text{CS}\alpha/-$ (n = 4)</th>
<th>$\alpha^\text{SE}\alpha/-$ (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb, g/L</td>
<td>8.58 (1.16)</td>
<td>7.23 (2.26)</td>
<td>8.33 (1.11)</td>
<td>7.14 (2.32)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>5.7–11.1</td>
<td>3.2–14.6</td>
<td>6.8–9.4</td>
<td>3.2–14.6</td>
</tr>
<tr>
<td>MCV, fL</td>
<td>62.8 (5.84)</td>
<td>72.0 (6.83)</td>
<td>73.9 (4.31)</td>
<td>71.8 (7.01)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>48.2–79.3</td>
<td>55.5–90.3</td>
<td>70.1–80.1</td>
<td>55.5–90.3</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>19.8 (1.73)</td>
<td>21.1 (2.44)</td>
<td>20.3 (1.12)</td>
<td>21.2 (2.52)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>17.0–24.6</td>
<td>14.4–29.3</td>
<td>18.8–21.5</td>
<td>14.4–29.3</td>
</tr>
<tr>
<td>Hb H, %</td>
<td>7.9 (5.51)</td>
<td>11.4 (6.80)</td>
<td>13.7 (8.14)</td>
<td>11.2 (6.74)</td>
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<td>Mean (SD)</td>
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<tr>
<td>Range</td>
<td>0.99–23.10</td>
<td>0.74–27.00</td>
<td>9.06–25.86</td>
<td>0.74–27.00</td>
</tr>
</tbody>
</table>

* MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.
Establishing a Reference Interval For Measurement of Flux through the Mitochondrial Fatty Acid Oxidation Pathway in Cultured Skin Fibroblasts, Srirucus B. Narayani, Richard L. Boriack, Bette Messmer, and Michael J. Bennett

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Mitochondrial fatty acid oxidation (FAO) represents a normal metabolic response to increased energy demands during periods of fasting, febrile illness, or muscular exertion. FAO is a complex pathway involving activation of free fatty acids to acyl-CoA species, transport into mitochondria, and cyclic oxidation to break the long-chain fatty acids into acetyl-CoA and a two-carbon chain-shortened acyl-CoA, which is then recycled. Acetyl-CoA is used in the Krebs cycle and in liver as a substrate for ketogenesis (1). Measurement of FAO flux in cultured cells provides valuable information about the integrity of the enzymes and transporters involved in FAO at all stages. Clinical indications for the measurement of FAO flux in skin fibroblasts include fatty acid oxidation enzyme defects, unexplained myopathies, cardiomyopathies, hypoglycemia, liver disease, sudden unexplained infant death, and hypoketotic hypoglycemia. FAO flux is typically measured by monitoring the conversion of radiolabeled fatty acids to carbon dioxide and water. Labeling can be with $^{14}C$ with final analysis of the rate of production of $^{14}CO_2$, or with $^3H$ and analysis of production of $^3H_2O$ (2, 3). Palmitic acid (C16) is used to detect long-chain defects, including deficiencies in very long chain acyl-CoA dehydrogenase, long-chain l-3-hydroxy acyl-CoA dehydrogenase, long-chain 3-keto acyl-CoA thiolase, carnitine/acylcarnitine translocase, carnitine palmityltransferase 1, and carnitine palmityltransferase 2. Myristic acid (C14) is used to detect defects in medium-chain FAO, such as medium-chain acyl-CoA dehydrogenase deficiency. We use tritiated fatty acids labeled in the (9,10) position. The assay will not detect defects in carnitine palmitoyl transferase 1B; short-chain FAO defects, including short-chain acyl-CoA dehydrogenase and short-chain l-3-hydroxy acyl-CoA dehydrogenase; and short-chain-3-keto acyl-CoA thiolase deficiencies.

Historically, patient samples have been compared with batch controls, and a reference interval was not established for this assay. In the present study we have assigned a reference interval for the assay, using FAO flux results from triplicate assays performed on 1000 fibroblast samples. These samples were derived from individuals with clinical signs indicating impaired energy metabolism but with apparently normal FAO compared with data obtained from patients with well-characterized, genetically confirmed FAO defects. Assigning a reference interval is critical for a clinically reportable assay because it simplifies the interpretation of the results and provides a clear definition of disease vs nondisease.

The FAO flux assay consists of measurement of FAO flux in fibroblasts obtained from one T-25 flask for each patient, typically with fewer than eight passages of culture. Fibroblasts are trypsinized and resuspended in 12–36 mL of DMEM (cat. no. 11885-084; Gibco Life Technologies) containing 1 g/L glucose and supplemented with 100 mL/L fetal calf serum (cat. no. 26140-053; Gibco Life Technologies). From this suspension, 1 mL is transferred to 2 wells in each of five 24-well tissue culture plates. Three nondisease control fibroblast and two disease control fibroblast lines are simultaneously seeded in the same plate along with one to six additional patient lines. Cultures are placed in an incubator to allow the fibroblasts to attach and then grow for 48 h at 37 °C, 5% CO₂, 100% humidity (CO₂ Incubator; NUAIRE). The cells are then washed with phosphate-buffered saline (pH 6.9) and incubated with preincubation medium (Krebs Ringer Bicarbonate Medium; cat. no. K-4002; Sigma) containing 0.5 g/L bovine serum albumin (fatty-acid free, cat. no. A-6033; Sigma) in the assay plates or with inhibitor medium [preincubation medium + FAO inhibitors consisting of 0.45 mmol/L malonyl-CoA (cat. no. M-4263; Sigma), 0.02 mmol/L glybenclamide (cat. no. G-0639; Sigma), 0.79 mmol/L 3-mercaptoproprionic acid (cat. no. M-6750; Sigma), and 2.74 mmol/L glucose (cat. no. G-5767; Sigma) in a buffer containing 1.0 g/L bovine