The use of LR values to check the best fit of cut-off values in G6PD deficient cases

S.M. Castro a,⁎, R. Weber a, U. Matte b, G.J. Reclos c, K.A. Pass d, T. Tanyalcin e, R. Giugliani f

a School of Pharmacy, UFRGS, Av. Ipiranga, 2752, Porto Alegre, RS, Brazil
b Center for Gene Therapy, HCP A, UFRGS, Rua Ramiro Barcelos, 2350, Porto Alegre, RS, Brazil
c R&D DIAGNOSTICS Ltd, 33 Alevizatou street, 15669 Papagos, Greece
d New York State Department of Health, Wadsworth Center, Albany, NY, USA
e Tanyalcin Medical Laboratory, Newborn Screening and Metabolism Unit, 1359 Sokak No:4/3 Alsancak 35220 Izmir, Turkey
f Medical Genetics Service, HCP A, UFRGS, Rua Ramiro Barcelos, 2350, Porto Alegre, RS, Brazil

Received 16 October 2006; received in revised form 23 December 2006; accepted 5 January 2007
Available online 19 January 2007

Introduction

Glucose 6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) deficiency is one of the most common enzymopathies throughout the world with an incidence as high as 20% in some populations [1]. Although most affected individuals are asymptomatic, there is a risk of neonatal jaundice and acute haemolytic anaemia, triggered by infection, the ingestion of certain pharmaceuticals and, in weaned individuals, fava beans [2]. Inheritance of G6PD shows a characteristic X-linked pattern. Since X-inactivation may be nonrandom the RBCs of heterozygotes may exhibit normal, intermediate or grossly deficient G6PD activity.

Although several variants of G6PD have been described in Southern Brazil, there is a homogeneous predominance of G6PD A− variant in the Brazilian population [3]. The red blood cells of individuals with this mutation show a residual enzymatic activity between 5% and 15% as compared with normal individuals.

Because of this apparent discrepancy between phenotype and genotype, diagnosis of the G6PD genotype in female subjects and individuals with some variants is particularly problematic. There is a need for a simple, sensitive and reliable test for G6PD deficiency for clinical laboratories to predict more accurately the risk for a patient. Only when we can predict more accurately the natural course for a patient can we make a valid risk–benefit and cost–benefit assessment for therapeutic options [4]. The objective of this work was to evaluate the accuracy of a quantitative G6PD kit employing haemoglobin normalization, considering DNA analysis as a gold standard.

Materials and methods

Subjects recruitment

Blood samples were collected from patients under investigation for G6PD deficiency from Porto Alegre, in the southern part of Brazil. The study was conducted between July 2004 and October 2005. The samples were taken in EDTA tubes, transported at 4 °C and stored at this temperature for no more than 3 days before enzyme assay and DNA extraction for molecular analysis.

Detection of enzyme activity

The Interscientific Neolisa G6PD (Interscientific Corporation, 2700 North 29th Avenue – Suite 220, Hollywood, FL-USA Cat. Nr 3570-050) was used for the quantitative measurement of G6PD activity as previously described [5,6]. The controls used were supplied by Interscientific, in three levels of G6PD activity (normal = 15.8 U/g Hb, intermediate = 4.7 U/g Hb and deficient = 1.3 U/g Hb).

Determination of average G6PD activity in the study population

The cut-off values for G6PD deficiency and the normal reference values were previously obtained from a normal group of individuals representing the population living in the Porto
Alegre area, employing the Haemoglobin Normalization procedure recommended by the manufacturer. We check the whole population, get an average, remove the lower 60% and then take another average of the healthy population [6]. Samples with an activity between 20% and 60% of the mean (12.83 U/g Hb) were classified as having intermediate activity. Values lower than 20% were classified as deficient samples. The cut-off points for the population were set below 2.6 U/g Hb and between 2.7 and 7.7 U/g Hb for deficient and partially deficient respectively.

**Mutation detection**

Genomic DNA was extracted from peripheral blood leukocytes. Three mutations were studied: the A− (202 G→A and 376 A→G) and the Mediterranean (563 C→T) G6PD variants. These three mutations account for 98% of G6PD deficiency in the Brazilian study population [3]. The coding region of the G6PD gene encompassing Mediterranean and A− mutations was analyzed by polymerase chain reaction (PCR) using primer sets to amplify exons 6, 4 and 5 [7,8].

**Statistical analysis**

Statistical Packages for Social Sciences (SPSS v.12.0) software was used to evaluate the results. Continuous variables were expressed as mean±SD. To evaluate diagnostic performance sensitivity, specificity and likelihood ratios (LR) to obtain posttest probability using Bayes’ Theorem were calculated. The LR can be calculated for each level of the diagnostic test result and the different LRs indicate by how much a given diagnostic test result will raise or lower the pretest probability of the target disorder [9]. The steps are briefly summarize: estimate pretest probability (population prevalence); convert pretest probability to pretest odds; multiply pretest odds by LR to obtain posttest odds; and convert posttest odds to posttest probability.

**Results**

A total of 35 specimens from the 73 tested were found to be abnormal by the DNA assay, of which 20 were male hemizygous for the G202A; A376G, 4 were female homozygous and 10 females were identified as carriers for the same double mutations. One subject showed the Mediterranean variant (C563T). In our study, two deficient individuals were negative for the mutations screened. We can observe that all subjects with G6DP activity ≥12 U/g Hb are negative for these mutations.

According to the mutation analysis, subjects were reclassified as homozygous or hemizygous, heterozygous or normal (Fig. 1).

ROC curve analysis was performed to define the sensitivity and specificity in the enzyme assay for different levels of G6PD, using molecular analysis as a gold standard. Calculated sensitivity and specificity for cut-off values established for the normal population were 2.9 U/g Hb (11.4% and 100%), 8 U/g Hb (77.1% and 94.7%) and 11.5 U/g Hb (97.1% and 76.3%); the area under the curve was 0.972.

In order to calculate sensitivity and specificity, one has to classify test results in only two categories (normal and abnormal). For quantitative traits, however, a test may have different “patterns” for values far low (or above) cut-off but the critical points are those intermediate values. For this reason, we used likelihood ratio (LR) to calculate posttest probability in three different levels of enzyme activity (Table 1).

It is estimated that combined deficiency of G6PD is approximately 8% in a population sample from Rio Grande do Sul [10]. So, from a pretest probability of 8%, after enzyme assay the odd ratio of a given person to be G6PD deficient (having enzyme levels lower than 8 U/g Hb) becomes 55.9%. On the other hand, for enzyme levels higher than 11.5 U/g Hb, this probability goes down to 0.37% (Table 1).

Two subjects with G6PD activity below 8.0 U/g Hb had negative DNA test for the three mutations used, but molecular analysis does not rule out the possibility of other mutations.

**Discussion**

About 400 million of individuals are estimated to be affected by G6PD deficiency worldwide [2] and a large proportion of these individuals will necessarily be heterozygous. The most commonly used methods to diagnose G6PD deficiency are able to detect totally deficient cases, but fail to detect partially deficient cases [5]. Only part of the affected heterozygotes will be detected by simple screening methods or even by enzymatic assays, in particular those patients with mutations resulting in severe loss of enzymatic activity like the Mediterranean one.
The correlation between biochemically and molecularly characterized variants is not always straightforward. As more than 140 variants have been described [11], a complete analysis of all mutations is costly and time consuming. Therefore, frequency-oriented studies are more effective, as can be seen by the 94.4% of deficient patients genotyped in this study analyzing G6PD A– and G6PD Mediterranean variants. This panel of mutations should allow for the detection of more than 90% of complete G6PD deficiency cases in Brazil population and allow detection of female carriers who may be partially deficient due to selected X chromosome inactivation.

Diagnostic accuracy refers to the quality of the information provided by the laboratory test. Diagnosis of the G6PD genotype using an enzymatic assay is particularly problematic. First the G6PD enzymatic activity measured by any kit can be easily affected by a great number of factors, most notably transportation, temperature and time elapsed between blood collection and sample analysis [12]. Second, G6PD deficiency is caused by a very large number of mutations resulting in different levels of enzyme deficiency [11].

As previously reported by Lin et al. [13], the cut-off value for the enzymatic assay is difficult to set. Using the recommended cut-off values from our enzymatic assay the sensitivity was 77.1%, specificity 94.7%. Still some partially deficient females could be missed. It is important to stress that, as not all known mutations have been tested in this study, the two deficient individuals may be misclassified by mutation analysis, therefore interfering with the sensitivity and specificity test.

Using only sensitivity and specificity must discard important information or recalculate sensitivity and specificity for every cut-off point. The LR approach is simpler and more efficient. LR posses some properties which makes it a very powerful diagnostic strategy. It is more stable than sensitivity or specificity when prevalence changes. As a result, the LR expresses the probability that a given level of a diagnostic test result would be expected in a patient with (as opposed to one without) the target disorder [9]. Our study showed that from a pretest probability of 8% of deficiency, the probability posttest of a given person to be G6PD deficient (G6PD activity < 8 U/g Hb) becomes 55.9%. On the other hand, for enzyme levels higher than 11.5 U/g Hb, this probability goes down to 0.37%.

Molecular data can identify the variant of the mutation but cannot indicate the ratio of G6PD+/G6PD-RBC populations which may result in residual enzymatic activities ranging from 30% to 60% [13]. Thus, the information obtained from an enzymatic method run alone is enough to identify the cases that are at greater risk, but it cannot give definitive answers about the underlying genotype. In this respect, the complementary use of a molecular technique for samples showing an intermediate residual enzymatic activity would greatly enhance the efficacy of the test, providing the clinician with more conclusive data.

Checking the cut-off points derived from a simple statistical analysis of the values obtained from the population tested, it could be concluded that Intercientífica kit is particularly suitable for the reliable evaluation of the enzymatic activity in whole blood samples, for large scale screening in field studies and in the public health setting. It is a reliable method for detecting G6PD deficiency, giving a satisfactory evaluation of the guide for severity of G6PD deficiency in heterozygous females and even in individuals with mutations causing less severe enzyme deficiency.

Acknowledgments

This work was supported by the Brazilian commercial company Empresa Intercientífica, São Paulo, Brazil and the Brazilian Federal Agency Fundo de Incentivo a Pesquisa e Eventos (FIPE-HCPA), Porto Alegre-RS, Brazil.

References