Improved Quantitative Method for G6PD Deficiency Detection

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Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common hereditary enzyme defect that affects over 400 million people worldwide. Deficiency in G6PD can cause hemolytic anemia, which can be fatal if it is not treated properly. Mass qualitative G6PD-deficiency screening methods have been available for some time; however, in this study we developed the first high-throughput quantitative method for

measuring G6PD activity. A patient's blood can be analyzed directly from the original collection tubes on a Hitachi P modular serum work area (SWA), which dramatically reduces the analysis time and the cost of the assay. The results presented here may enable the development of other improved, automated assays of whole-blood samples. *J. Clin. Lab. Anal.* 21:107–113, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme that plays a key role in metabolism of glucose. Specifically, G6PD converts glucose 6-phosphate into 6-phosphogluconate in the hexose monophosphate pathway (Fig. 1). During this step it catalyzes the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form, NADPH, which protects red blood cells from oxidative stress (1–5). Therefore, G6PD deficiency increases the vulnerability of erythrocytes to oxidative stress.

G6PD deficiency is the most common human enzyme deficiency and is most prevalent in the Mediterranean, Middle East, Southeast Asia, and West Africa (3–5). Many countries have established nationwide screenings for newborns (8–10). There are more than 400 identified variants of G6PD. The gene encoding G6PD is located on the distal long arm of the X chromosome. Therefore, G6PD deficiency mainly affects males, since in males a single defective G6PD gene is sufficient to cause the deficiency. The phenotype of a G6PD deficiency in affected individuals can range from mild (asymptomatic to neonatal jaundice) to severe (hemolytic anemia (6)). Many individuals with this condition can lead normal lives but are prone to hemolytic anemia triggered by exposure to oxidants, such as antimalarial drugs. The major symptoms of hemolytic anemia include dark urine, back pain, and fatigue. If a hemolytic episode is not accurately diagnosed and properly treated, it can

result in death. Thus, it is important to identify G6PD-deficient individuals before prescribing antimalarial drugs.

Accurate detection of individuals with this abnormality is especially important in the military. When military personnel are deployed to malaria-endemic areas, they are routinely treated with antimalarial medication while they are in theater and for 2 weeks after they return. Soldiers have been known to develop hemolytic anemia while receiving Primaquine prophylaxis for malaria, and subsequently found to have G6PD deficiency (7). This could be avoided by prescreening all military personnel for G6PD deficiency. All known antimalarial drugs are contraindicated for G6PD-deficient individuals; however, in cases of acute uncomplicated malaria, most antimalarial drugs can be administered with careful supervision.

Standardized tests for G6PD deficiency have existed since 1967 (11). Currently, the most popular method that can handle a large volume of samples is the fluorescent spot test, in which the generation of

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Fig. 1. Hexose monophosphate pathway.

NADPH is detected visually under ultraviolet light (12–14). This method is only qualitative but is generally adequate for detecting hemizygous males; however, it sometimes misses certain partially deficient females (15). The best way to reduce false-negative results is to perform a quantitative analysis. The best-known method for quantitative analysis is an enzyme kinetic method that measures the activity of G6PD (4). All existing quantitative methods are more labor-intensive and time-consuming than the qualitative methods.

Although new quantitative methods have recently been developed (16–18), it is not economically practical to use these protocols to test all military personnel for G6PD deficiency. Since medical capabilities to treat soldiers abroad may be limited, it is important to have a quantitative rather than a qualitative analysis. Given the lack of an instrument designed for automated high-throughput quantitative G6PD activity analysis, we modified our existing semiautomated method to develop a simple, automated mass quantitative screening method. In this article we describe the first automated high-throughput quantitative G6PD deficiency detection method using patients' blood directly from heparinized tubes. On average, in this study we were able to analyze approximately 1,000 samples in 8 hr.

MATERIALS AND METHODS

Sample Collection

The patients' blood was collected into a plastic tube with a Hemagard closure by a standard venipuncture technique with EDTA heparin. The blood specimens were shipped to the laboratory on ice via overnight express mail. In whole blood, G6PD is stable for 1 week when stored at 2–8°C. Freezing of blood is not recommended.

Reagents

The G6PDH kit (catalog no. 345B) and red cell lysing reagent (catalog no. R1129) were purchased from Trinity Biotech (Co. Wicklow, Ireland). The G6PDH kit consists of G6PDH reagent and G6PDH substrate

solution. The G6PDH reagent was reconstituted with an equal volume of type 1 deionized water before use. The diluted reagent was mixed by inversion (not shaking). The hemoglobin reagent (catalog no. 1108912), control (catalog no. 08H5801) and calibrator (catalog no. 08H5701) were purchased from Abbott Diagnostics (Abbott Park, IL). G6PDH-normal (catalog no. G6888) and G6PDH-deficient (catalog no. G5888) samples were purchased from Sigma (St. Louis, MO) and used for quality control.

Software and Equipment

G6PD activity and hemoglobin concentration were measured on a MODULAR[®] ANALYTICS P800 system (Roche Diagnostics, Indianapolis, IN).

A correlation analysis was performed with the use of the EP Evaluator[™] (release 6, David G. Rhoads Associates, Inc., PA).

Calibration and Quality Control

Calibration of the G6PD procedure was accomplished with the use of a zero calibrator, 0.9% NaCl, and a predetermined "K" factor. The K factor was determined at the time of system installation according to the following equation:

$$K = (C_c - C_b) / (A_c - A_b)$$

where C_c is the concentration of the standard, C_b is the concentration of the blank, A_c is the absorbance of the standard, and A_b is the absorbance of the blank.

G6PDH-normal (catalog no. G6888), G6PDH-intermediate (catalog no. G5029), and G6PDH-deficient (catalog no. G5888) samples with known concentrations were used as the standards, and a 0.9% NaCl solution was used as the blank. The absorbance of each standard and the blank was measured at 340 nm following the same protocol described in the G6PD Activity Determination section below. The K-values calculated from these three standards with the blank should be very close due to the linearity property. Once the K-factor is determined, the concentration of an unknown sample can be calculated. The value of K does not need to be

recalculated, if not indicated by the quality control analysis.

A hemoglobin calibrator from Abbot was used to calibrate the machine before each run of the hemoglobin assays or when the reagent lot was changed. A two-point calibration was used in the hemoglobin procedure to establish the linear range of the machine.

We performed quality control tests for both G6PD activity and hemoglobin concentration assays daily before each run, following the same procedures used for the test specimens. For the G6PD assay, G6PD-deficient and -normal samples from Sigma were used, and for the hemoglobin assay the control sample from Abbott was used.

G6PD Activity Determination

We prepared the G6PDH assay solution by mixing two parts of G6PDH substrate solution with one part of the reconstituted G6PDH reagent. The assay solution was allowed to reach instrument storage temperature (5–10°C) prior to use. The blood in the heparinized tubes was shaken gently on a sample rocker for 5 min to ensure homogeneity and then put on the MODULAR. The MODULAR was programmed to mix 25 µL of blood with 250 µL of red cell lysing reagent for 3 min at 5–10°C. Then 6 µL of the above mixture were added into 180 µL of G6PDH assay solution. The final mixture was mixed for 7 min at 5–10°C before the absorbance was measured at 340 nm. Deionized water was used to rinse the machine.

Hemoglobin Concentration Determination

To determine the hemoglobin concentration, we followed the same procedure used in the Core Laboratory at Brooke Army Medical Center (Abbott Cell-Dyn® 4000; Abbott Diagnostics, IL), with the exception that we used a Modular P. We measured the hemoglobin concentration by mixing 2 µL of the same blood samples from the G6PD study with 250 µL of the hemoglobin reagent for 10 min at 5–10°C. The concentration of the hemoglobin was measured at 546 nm. Deionized water was used as the rinsing solution.

Correlation Studies

We assessed the correlation between the semiautomated and automated methods for G6PD deficiency by testing 425 samples with both methods. The same 425 samples were also used for the correlation analysis between hemoglobin concentration results obtained from MODULAR and Cell-Dyn 4000. The correlation was calculated using the Deming regression on the EP evaluator with a 95% confidence level.

RESULTS AND DISCUSSION

The G6PD method was based on the rate of NADPH formation from NADP in the presence of G6PD (Fig. 2). The rate of formation of NADPH was proportional to the G6PD activity in the sample, and was measured spectrophotometrically as an increase in absorbance at 340 nm. NADP and the substrate (glucose-6-phosphate (G6P)) for the G6PD were from G6PDH reagent and substrate solution, respectively.

To accurately measure the activity of G6PD, the subsequent step (Fig. 3) has to be inhibited to prevent the production of the second molar equivalent of NADPH produced by the presence of erythrocyte 6-phosphogluconate dehydrogenase (6-PGDH) would have interfered with the absorbance readings. The maleimid in the D6PDK1 reagent inhibits the 6-PGDH activity, and subsequently prevents the formation of the NADPH.

The hemoglobin concentration was determined by the formation of hemoglobin-imidazole complex based on the change of absorbance at 540 nm. The hemoglobin reagent from Abbot contained both lysing agent and imidazole for the reaction.

The initial G6PD results were reported in absorbance unit (U) per each deciliter (dL) of blood. The hemoglobin concentrations were converted automatically on the instrument as gram (g)/dL of blood. We then calculated the final G6PD activity results on the analyzer by normalizing the initial G6PD results with the hemoglobin concentration into U/g of hemoglobin (HGB). We used the concentration of hemoglobin to normalize the G6PD activity so that the numbers would be more comparable for samples with abnormal

Fig. 2. Conversion of G6P to 6-PG.

Fig. 3. Production of the second molar equivalent of NADPH.

Fig. 4. Reference interval histogram.

TABLE 1. Results distribution

G6PD Concentration (U/g HGB)	Count	Percentage (%)
<7.0	32	5.0
7.0–8.8	3	0.5
8.9–10.7	4	0.6
10.8–12.7	40	6.3
12.8–14.7	268	42.1
14.8–16.7	228	35.8
16.8–18.6	37	5.8
18.7–20.5	17	2.7
>20.5	8	1.3

hemoglobin concentration. We analyzed over 600 random samples to establish our reference range. According to our results, 95% of the samples (Fig. 4, Table 1) fall above the cutoff point of 7 U/g HGB established by the World Health Organization (WHO). Therefore, we decided to follow the same reference range published by WHO to identify individuals with a deficiency. Any individual with a G6PD reading less than 7 U/g HGB would be considered deficient in G6PD. In addition, the cutoff of 7 U/g HGB is well above the detection limit of 0.4 U/g HGB and within the linear range of the assay.

When the G6PD-deficiency test was first introduced in our laboratory, we followed a semiautomated method in which the blood was aliquotted and the cells were lysed manually. The hemolysate was put on the MODULAR P to mix with the assay reagent, followed by photometric measurement at 340 nm. MODULAR P was selected because of its high-throughput capability and extendibility. However, the turnaround time was delayed significantly due to the manual preparation of hemolysate. In addition, it became both time-consuming

and mistake-prone to aliquot all of the samples and to add lysing reagent manually when we started to receive 700–900 samples daily. Each new tube containing the hemolysate had to be labeled and checked against the original tube to ensure the correct identity. Just the specimen-processing step alone required a few hours to finish. Since G6PD is not stable in hemolysate, it had to be prepared fresh before each run. Thus, hemolysate preparation became the bottleneck for the whole process, which limited our throughput to approximate 400 samples a day. To reduce the turnaround time, we modified the procedure so that the hemolysate could be prepared on the MODULAR P. By switching from manual preparation of hemolysate to machine preparation, we also reduced the potential human errors that were introduced in these steps. In addition, we had better control of the time from the hemolysate formation to the absorbance readings.

The MODULAR P was designed for serum, urine, and hemolysate analyses; therefore, we were concerned that the same protocol would not work properly on the whole-blood sample without significant modifications. When we incorporated the new step on the SWA, we observed that foaming occurred at the end of the washing cycle and clogged the machine. The foaming problem suggested that one or more of the reagents used to prepare the hemolysate were incompatible with the washing solvent (aqueous NaOH, <5%) in the machine. Therefore, we mixed aqueous NaOH with some whole blood and lysing reagent separately to determine the culprit. The fact that foaming was observed only when aqueous NaOH was mixed with the lysing reagent suggested that it was generated when the syringe and tubing used to deliver the lysing reagent were washed with the aqueous NaOH. We suspect that the methyl 4-hydroxybenzoate (Sigma-Aldrich,

Fig. 5. Correlation between results from Cell-DYN and MODULAR P.

TABLE 2. Deming regression analyses

	Unnormalized G6PD activity calculated (95% confidence level)	Hemoglobin concentration calculated (95% confidence level)	Normalized G6PD activity calculated (95% confidence level)
Slope	0.979 (0.964–0.994)	1.082 (1.053–1.111)	1.008 (0.993–1.022)
Intercept	–3.3 (–6.2)–(–0.4)	–1.514 (–1.901)–(–1.126)	0.012 (–0.195–0.219)
Standard error estimated	13.3	0.693	0.941
R ²	0.949	0.840	0.958

St. Louis, MO) in the lysing reagent was reacting with the aqueous base solution. In the old method the lysing reagent was not used directly on the machine, and the concentration of the lysing reagent was low enough in the preprepared hemolysate to avoid this problem. Since there was nothing wrong with the assay procedure itself, we decided it would be easier to switch the washing solvent than to change the lysing reagent. Fortunately, water proved to be an adequate washing solvent, and no carryover was detected in the samples.

To validate the new method for G6PD-deficiency determination, we randomly chose 425 samples to test using both the semiautomated method (in which the hemolysate was prepared manually) and the fully automated method (in which a whole-blood sample was used). When we performed the Deming regression analysis between the two methods on the initial G6PD activity, without normalizing it with the hemoglobin concentration, we observed a good linear correlation (Fig. 5, Table 2). However, there appeared to be a slight downward bias for the new method, as indicated by a negative y-intercept. We then used the same 425 samples to validate the hemoglobin assay on the MODULAR P. Again, there was a good linear correlation but a negative

y-intercept (Fig. 6, Table 2). Before we started to correct for the downward bias, we decided to analyze the correlation between the two methods on the normalized G6PD concentration that would be reported to the physician. After the initial G6PD activity was normalized with the hemoglobin concentration, the Deming regression analysis (Fig. 7, Table 2) showed that the two methods were statistically equivalent (95% confidence intervals (CIs) for the slope and y-intercept = 1.0 and 0.0, respectively). Thus, no correction was needed to adjust the final results measured from the new method. These regression analyses suggested that both unnormalized G6PD readings and hemoglobin concentrations from the automated method tended to have lower values than the values determined by the old methods. However, the final G6PD concentration from the new method, which is what we would report to the physicians and patients, is statistically equivalent to the final results obtained from the old method.

The results of the correlation studies confirmed that we could perform both tests on the MODULAR P. Since we were able to perform both tests by taking the whole-blood sample directly from the original collection tubes, we were able to run both G6PD activity and

Fig. 6. Correlation of hemoglobin concentration between the Cell-DYN and MODULAR P methods.

Fig. 7. Correlation of final G6PD concentration between the Cell-DYN and MODULAR P methods.

hemoglobin concentration assays together as one panel, which dramatically reduced the turnaround time. In addition, by automating the whole process we lowered the volumes of the specimens and reagents needed to perform the test, which in turn lowered the cost of the assay. Furthermore, one can easily revert the MODULAR P to run serum or urine samples by changing the washing solvent back to aqueous NaOH solution.

CONCLUSIONS

We have reported the first automated high-throughput quantitative G6PD activity test that is rapid and

cost-effective. By automating the whole process we were able to increase our workload and at the same time reduce the operating time and the potential for human error. Few modifications of the procedure and conditions had to be made. However, the correlation showed strong agreement between the semiautomated and new automated methods. Patients' blood was used directly from the heparinized tubes without any manual preparation prior to the screening. All steps were done automatically on a Modular P. On average, we were able to analyze 1,000 samples in an 8-hr period. We believe this method can be easily adapted by other laboratories that are interested in screening for G6PD

deficiency. Most importantly, we can now run tests by taking blood directly from the original collection tubes, so potentially we will be able to automate other tests using whole blood in a similar manner.

REFERENCES

1. Roos R, van Zwieten R, Wijnen JT, et al. Molecular basis and enzymatic properties of glucose 6-phosphate dehydrogenase volendam, leading to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. *Blood* 1999;94:2955–2962.
2. Vulliamy T, Mason P, Luzzatto L. The molecular basis of glucose-6-phosphate dehydrogenase deficiency. *Trends Genet.* 1992;8: 138–143.
3. Beutler E. Glucose-6-phosphate dehydrogenase deficiency and other red cell enzyme abnormalities. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, editors. *Williams hematology*. New York: McGraw-Hill; 1995. p 527–545.
4. Beutler E. G6PD deficiency. *Blood* 1994;84:3613–3636.
5. Jalloh A, Tantular IS, Pusarawati S, et al. Rapid epidemiologic assessment of glucose-6-phosphate dehydrogenase deficiency in malaria-endemic areas in Southeast Asia using a novel diagnostic kit. *Trop Med Int Health* 2004;9:615–623.
6. Lin Z, Fontaine JM, Freer D, Naylor E. Alternative DNA-based newborn screening for glucose-6-phosphate dehydrogenase deficiency. *Mol Genet Metab* 2005;86:212–219.
7. Carr ME Jr, Fandre MN, Oduwa FO. Glucose-6-phosphate dehydrogenase deficiency in two returning operation Iraqi Freedom soldiers who developed hemolytic anemia while receiving Primaquine prophylaxis for malaria. *Military Medicine*. 2005;170: 273–276.
8. Lim HH, Daniel LM, Lee J, Tan MC. Predicting significant hyperbilirubinaemia and early discharge for glucose-6-phosphate dehydrogenase deficient newborns. *Ann Acad Med* 2003;32: 257–261.
9. Joseph R. Mass newborn screening in Singapore—position and projections. *Ann Acad Med Singapore* 2003;32:318–323.
10. Missiou-Tsagaraki S. Screening for glucose-6-phosphate dehydrogenase deficiency as a preventive measure: prevalence among 1,286,000 Greek newborn infants. *J Pediatr* 1991;119: 293–299.
11. World Health Organization. Standardization of techniques for the study of G6PD. *WHO Tech Rep Ser* 1967;366:1–53.
12. Frank JE. Diagnosis and management of G6PD deficiency. *Am Fam Physician* 2005;72:1277–1282.
13. Tan IK, Whitehead TP. Automated fluorometric determination of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) activities in red blood cells. *Clin Chem* 1969;15:467–478.
14. Dow PA, Petteway MB, Alperin JB. Simplified method for G6PD screening using blood collected on filter paper. *Am J Clin Pathol* 1974;61:333–336.
15. Ainoon O, Alawiyah A, Yu YH, et al. Semiquantitative screening test for G6PD deficiency detects severe deficiency but misses a substantial proportion of partially-deficient females. *Southeast Asian J Trop Med Public Health* 2003;34:405–414.
16. Gumuslu S, Yucel G, Sarikcioglu B, Serteser M. Application of a new chemiluminescence method for the determination of glucose-6-phosphate dehydrogenase activity in healthy and enzyme deficient individuals. *J Pharmacol Toxicol Methods* 2005;51: 169–174.
17. Tantular IS, Kawamoto F. An improved simple screening method for detection of glucose-6-phosphate dehydrogenase deficiency. *Trop Med Int Health* 2003;8:569–574.
18. Ye BC, Liu H, Lei Z. Rapid detection of common Chinese glucose-6-phosphate dehydrogenase (G6PD) mutations by microarray-based assay. *Am J Hematol* 2004;76:405–412.