

Original article

Comparative analysis of diagnostic methods for glucose-6-phosphate dehydrogenase (G6PD) deficiency: fluorescent spot test versus quantitative methods

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Abstract:

Introduction: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a prevalent enzymopathy with significant public health implications. This study compares the diagnostic performance of the fluorescent spot test (FST), a qualitative method, with two quantitative assays—namely, the World Health Organization (WHO)-recommended method and the Glock and McLean (G&Mc) method—to assess their reliability and applicability. **Methods:** A total of 221 blood samples collected between 2009 and 2021 were analyzed. Hemolysates were prepared under controlled conditions to preserve enzyme activity. Quantitative assays measured nicotinamide adenine dinucleotide phosphate (NADPH) production using spectrophotometry, while the FST provided visual confirmation under ultraviolet light. Median G6PD activities were calculated, and the sensitivity and specificity of the FST were evaluated against activity thresholds defined by the quantitative methods. **Results:** The adjusted male median G6PD activity was 12.6 IU/gHb for the WHO method and 8.8 IU/gHb for the G&Mc method. The FST exhibited high sensitivity and specificity across different thresholds, with near-perfect performance at 30% activity and above. **Conclusion:** The FST is a reliable tool for field-based screening of G6PD deficiency, demonstrating robust sensitivity and specificity. Quantitative methods offer enhanced precision for detailed evaluations, supporting their complementary roles in diagnostics.

Keywords : ● G6PD deficiency ● Fluorescent spot test ● Quantitative analysis ● Enzyme activity
● Diagnostics

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นิพนธ์ต้นฉบับ

การวิเคราะห์เปรียบเทียบวิธีการวินิจฉัยภาวะพร่องเอนไซม์ กลูโคส-6-ฟอสเฟต ดีไฮโดรจีเนส (glucose-6-phosphate dehydrogenase, G6PD) : fluorescent spot test กับวิธีเชิงปริมาณ

กชปิณฺฐ จันทรสิงห์ วิยการณ อินทรจุฑกุล กัลยา เตชะวณิชย์ กลีบสไบ สรรพกิจ และ เจษฎา บัวบุญนำ
สาขาวิชาโลหิตวิทยาและอองโคโลยี ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

บทคัดย่อ

บทนำ ภาวะพร่องเอนไซม์กลูโคส-6-ฟอสเฟต ดีไฮโดรจีเนส (glucose-6-phosphate dehydrogenase, G6PD) เป็นภาวะที่เกิดจากความพร่องของเอนไซม์ในเม็ดเลือดแดง ซึ่งส่งผลกระทบต่อทางสาธารณสุขอย่างมาก การศึกษาเปรียบเทียบประสิทธิภาพการวินิจฉัยของวิธี fluorescent spot test (FST) ซึ่งเป็นวิธีเชิงคุณภาพ กับวิธีวิเคราะห์เชิงปริมาณสองวิธี ได้แก่ วิธีที่องค์การอนามัยโลก (WHO) แนะนำ และวิธีของ Glock และ McLean (G&Mc) เพื่อประเมินความน่าเชื่อถือและความเหมาะสมในการส่งตรวจ **วิธีการศึกษา** ตัวอย่างเลือดจำนวน 221 ตัวอย่าง ที่เก็บระหว่างปี พ.ศ. 2552 ถึง 2564 ได้รับการวิเคราะห์ โดยเตรียมสารฮีโมไลเซตภายใต้สภาวะควบคุม เพื่อคงไว้ซึ่งการทำงานของเอนไซม์ วิธีการวิเคราะห์เชิงปริมาณใช้การวัดการฟลูออโรสโคปีของโคตินาไมด์ อะดีนีน ไดนิวคลีโอไทด์ ฟอสเฟต ด้วยสเปกโตรโฟโตเมทรี ในขณะที่วิธี FST ใช้การตรวจสอบด้วยการดูภายใต้แสงอัลตราไวโอเล็ต ค่าพิกัดการทำงานของ G6PD เฉลี่ย และประเมินความไวและความจำเพาะของ FST เทียบกับเกณฑ์ที่กำหนดโดยวิธีเชิงปริมาณ **ผลการศึกษา** ค่าการทำงานของ G6PD เฉลี่ยปรับตามเพศอายุอยู่ที่ 12.6 IU/gHb สำหรับวิธี WHO และ 8.8 IU/gHb สำหรับวิธี G&Mc วิธี FST แสดงความไวและความจำเพาะสูงในทุกเกณฑ์ โดยมีประสิทธิภาพเกือบสมบูรณ์ที่เกณฑ์ ร้อยละ 30 ขึ้นไป **สรุป** วิธี FST เป็นเครื่องมือที่น่าเชื่อถือสำหรับการคัดกรองภาวะพร่อง G6PD ในภาคสนาม โดยแสดงความไวและความจำเพาะที่แม่นยำ ขณะที่วิธีการเชิงปริมาณมีความแม่นยำสูงขึ้นสำหรับการประเมินในรายละเอียด จึงมีบทบาทเสริมกันในกระบวนการวินิจฉัย

คำสำคัญ : ● ภาวะพร่องเอนไซม์จีซิกพีดี ● การทดสอบการเรืองแสง ● การวิเคราะห์เชิงปริมาณ ● การทำงานของเอนไซม์ ● การวินิจฉัย

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต. 2568;35:281-8.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a genetic disorder that affects red blood cell metabolism, leading to hemolysis under oxidative stress.¹ It is prevalent in regions with a high incidence of malaria, where G6PD variants may confer a selective advantage.² Accurate diagnosis of G6PD deficiency is essential for preventing adverse drug reactions and managing hemolytic episodes. Current diagnostic approaches include qualitative methods, such as the fluorescent spot test (FST), and quantitative assays, such as the World Health Organization (WHO)-recommended method and the Glock and McLean (G&Mc) method.³ The FST, developed by Beutler, et al.⁴ is widely used for rapid screening of G6PD deficiency due to its simplicity, high sensitivity and specificity. While effective in field settings, the FST provides only qualitative results and lacks precise measurements of enzyme activity. Quantitative spectrophotometric methods, as recommended by the WHO and the G&Mc protocol, remain the gold standard for accurately assessing G6PD activity in IU/gHb. However, these methods are resource-intensive and challenging to implement in low-resource settings.⁵ As such, the FST and quantitative assays serve complementary roles, balancing practicality and precision depending on diagnostic needs.

This study aims to compare the diagnostic performance of the FST with quantitative methods for determining G6PD activity. By evaluating sensitivity, specificity and predictive values at various thresholds, this study seeks to inform the appropriate use of these diagnostic tools in diverse clinical and field settings.

Material and methods

Blood samples were collected from individuals referred for G6PD testing at our laboratory between March 2009 and December 2021. Both quantitative (WHO and G&Mc) and qualitative (FST) G6PD tests were performed. Samples were collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes and

stored at 2 to 8°C. Specimens were processed within one to three days of collection to ensure the stability and reliability of the results. Ethical approval for this study was obtained from the Institutional Review Board of the Faculty of Medicine, Siriraj Hospital, Mahidol University (COA number SI 195/2022).

Hemolysate Preparation

Hemolysate was prepared from whole EDTA blood. Red blood cells were purified by filtration through microcrystalline cellulose and washed twice with cold saline. The washed cells were resuspended in saline to create a 50% suspension. One part of this suspension was mixed with nine parts of a lysing solution containing 0.27 M EDTA (pH 7.2) and 0.7 mM 2-mercaptoethanol to prepare the hemolysate. The optimal hemoglobin concentration for the enzyme assay ranged between 1.0 and 1.5 g/dL. Complete lysis was achieved through freeze-thaw cycles, involving rapid freezing in liquid nitrogen followed by thawing at room temperature. The hemolysate enzyme remained stable for up to 8 hours at 2 to 8°C, but assays were recommended to be performed immediately to minimize variability. To ensure quality, samples were visually inspected for clarity, and any showing signs of contamination or inadequate hemolysis were excluded from further analysis.

Quantitative G6PD Test (WHO Method)

Red cell G6PD activity was measured in duplicate using the WHO kinetic method.⁶ A 20 µL aliquot of hemolysate was mixed with 100 µL of 1 M Tris-HCl buffer (containing 5 mM EDTA, pH 8.0), 100 µL of 0.1 M magnesium chloride, 100 µL of 2 mM nicotinamide adenine dinucleotide phosphate (NADP), and 580 µL of distilled water. The mixture was incubated at 37°C for 10 minutes, followed by the addition of 100 µL of 6 mM glucose-6-phosphate (G6P) to initiate the reaction. A reaction blank was prepared by replacing G6P with distilled water. Reduced NADP (NADPH) production was measured at 340 nm over 10 minutes at 37°C using a spectrophotometer (Evolution 600, Thermo, UK). Enzyme activity was calculated based on the change in

absorbance (ΔA) of NADPH and expressed in international units per gram of hemoglobin (IU/gHb) at 37°C. Quality control samples with known G6PD activity levels were included in each run to ensure the accuracy and precision of the assay.

Quantitative G6PD Test (G&Mc Method)

G6PD activity was measured using a two-tube kinetic method described by Glock and McLean.⁷ In Tube 1, 20 μ L of hemolysate was combined with 100 μ L of 1 M Tris-HCl buffer (containing 5 mM EDTA, pH 8.0), 100 μ L of 0.1 M magnesium chloride, 100 μ L of 2 mM NADP and 580 μ L of distilled water. After incubation at 37°C for 10 minutes, 100 μ L of 6 mM 6-phosphogluconic acid (6-PGA) was added. Tube 2 followed the same procedure as Tube 1 but included 480 μ L of distilled water along with 100 μ L each of 6-PGA and 6 mM G6P. A reaction blank was prepared similarly by replacing G6P with distilled water. NADPH production was monitored at 340 nm over 10 minutes at 37°C using a spectrophotometer (Evolution 600, Thermo, UK). Enzyme activity was calculated from the difference in absorbance (ΔA) between Tube 2 and Tube 1, in accordance with the G&Mc method. Internal quality control samples were included to ensure the accuracy and reproducibility of the results.

Qualitative G6PD Test (FST)

The G6PD FST (R&D Diagnostic, Greece) was used for screening G6PD deficiency. One microliter of whole blood was added to 50 μ L of G6P substrate solution, and the mixture was incubated for 10 minutes at room temperature. Ten microliters of the reaction mixture was then applied to filter paper, which was air-dried for approximately 30 minutes. The dried spots were visualized under ultraviolet (UV) light. Normal enzyme activity was indicated by strong fluorescence, while deficient samples showed weak or no fluorescence. Indeterminate samples demonstrated weak to moderate fluorescence. Each test was performed along with normal and deficient controls, and results were independently evaluated by two observers to minimize subjectivity.

Any discrepancies between observers were resolved by a third independent observer to ensure accuracy.

Statistical analysis

Statistical analysis was performed using MedCalc Statistical Software. The adjusted male median (AMM) was used to define 100% G6PD activity, and G6PD deficiency was categorized as any activity below 10%, 20%, 30%, 40%, 50% or 60% of the AMM. The performance of the FST was evaluated using spectrophotometry as the reference method, with sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) calculated for each activity threshold. Statistical significance was defined as $p < 0.05$, and confidence intervals were set at 95%.

Results

To calculate the AMM, representing 100% G6PD activity, G6PD enzyme activity values in the normal male population, (aged 1 to 40 years), were determined using the WHO and G&Mc quantitative tests, as previously described by Tachavanich, et al.⁸ A total of 55 males were analyzed to determine the median value. Seven males with activity levels below 10% of the population median were excluded from the calculation to avoid skewing the adjusted median G6PD activity value due to severely deficient samples. This adjusted value was then used to calculate various cutoff levels (Figures 1 and 2).

The median G6PD activity using the WHO method for the entire population was 12.5 IU/gHb (range: 0-17.08 IU/gHb), and the adjusted male median was 12.6 IU/gHb (range: 9.71-17.08 IU/gHb). For the G&Mc method, the median G6PD activity for the entire population was 8.6 IU/gHb (range: 0-11.9 IU/gHb), and the adjusted male median was 8.8 IU/gHb (range: 6.43-11.90 IU/gHb). Figures 1 and 2 illustrate the distribution of G6PD activities in the normal male population, indicating 100% activity level and various cutoff levels as percentages of the adjusted male median.

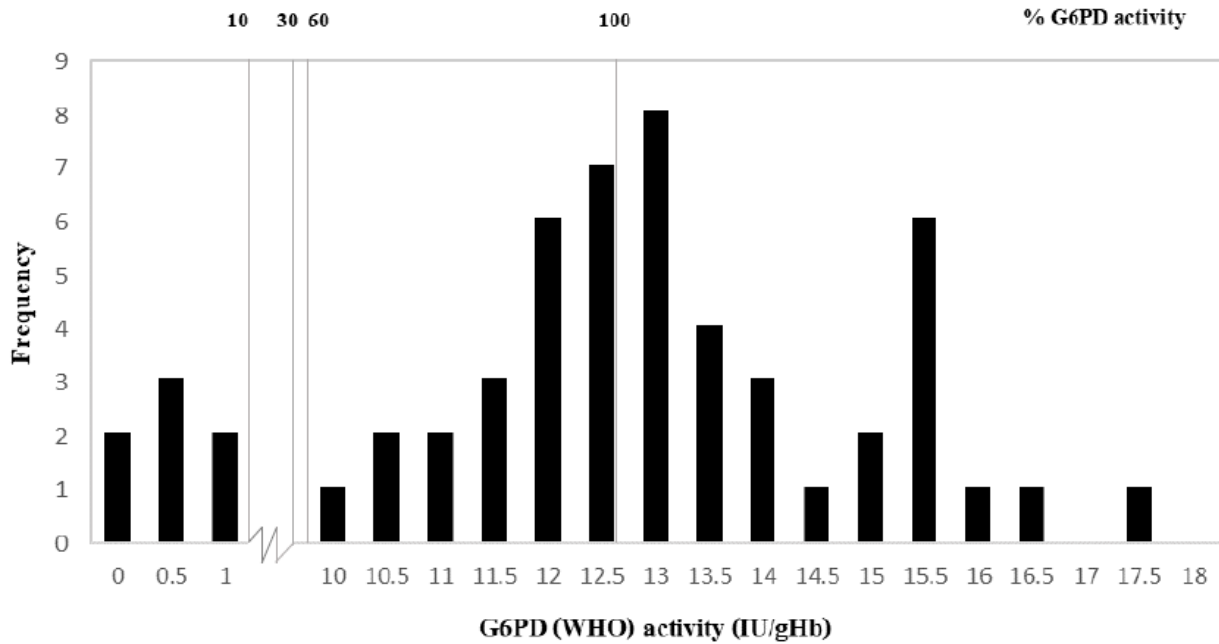


Figure 1 G6PD activity distribution in the normal male population using the WHO method. The figure indicates 10%, 30%, 60%, and 100% of normal activity for this population. Normal activity was defined as the adjusted G6PD median activity value for normal males

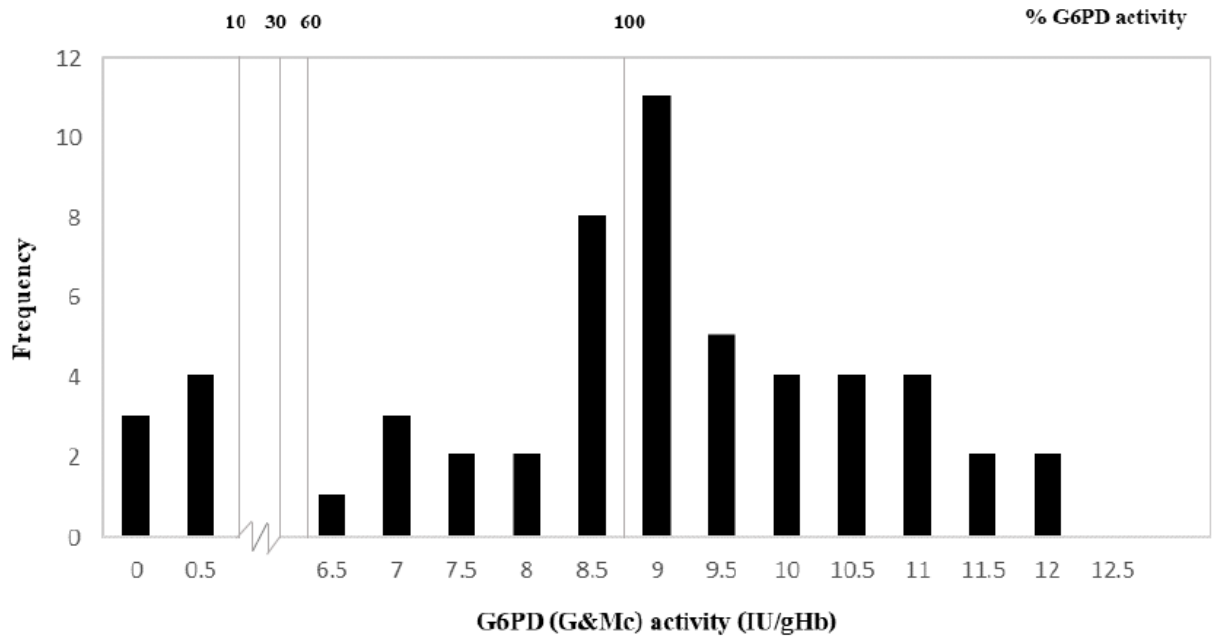


Figure 2 G6PD activity distribution in the normal male population using the G&Mc method. The figure indicates 10%, 30%, 60%, and 100% of normal activity for this population. Normal activity was defined as the adjusted G6PD median activity value for normal males

A total of 221 participants were tested using three diagnostic methods for G6PD deficiency: FST, WHO and G&Mc. The distribution of G6PD activity among males and females, as measured by the WHO and G&Mc methods, is illustrated in Figures 3 and 4. Among the FST samples, 76 (34.39%, 19 males and 57 females) were indeterminate and were excluded from the initial

analysis. The remaining 145 participants (116 males and 29 females) were included in further evaluations. The sensitivity, specificity, PPV and NPV of the FST, compared with the WHO and G&Mc quantitative methods are, detailed in Tables 1 and 2. Of the 76 initially excluded participants, 10 were reclassified as G6PD-deficient when using a 30% activity cutoff.

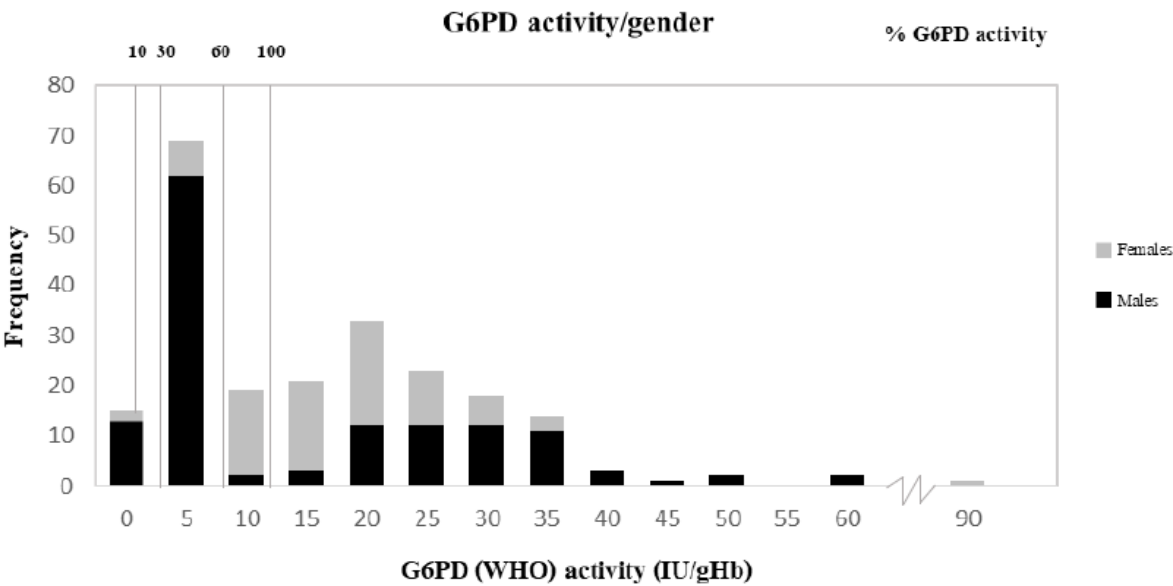


Figure 3 G6PD activity distribution in males and females using the WHO method. The figure indicates 10%, 30%, 60%, and 100% of normal activity for this population

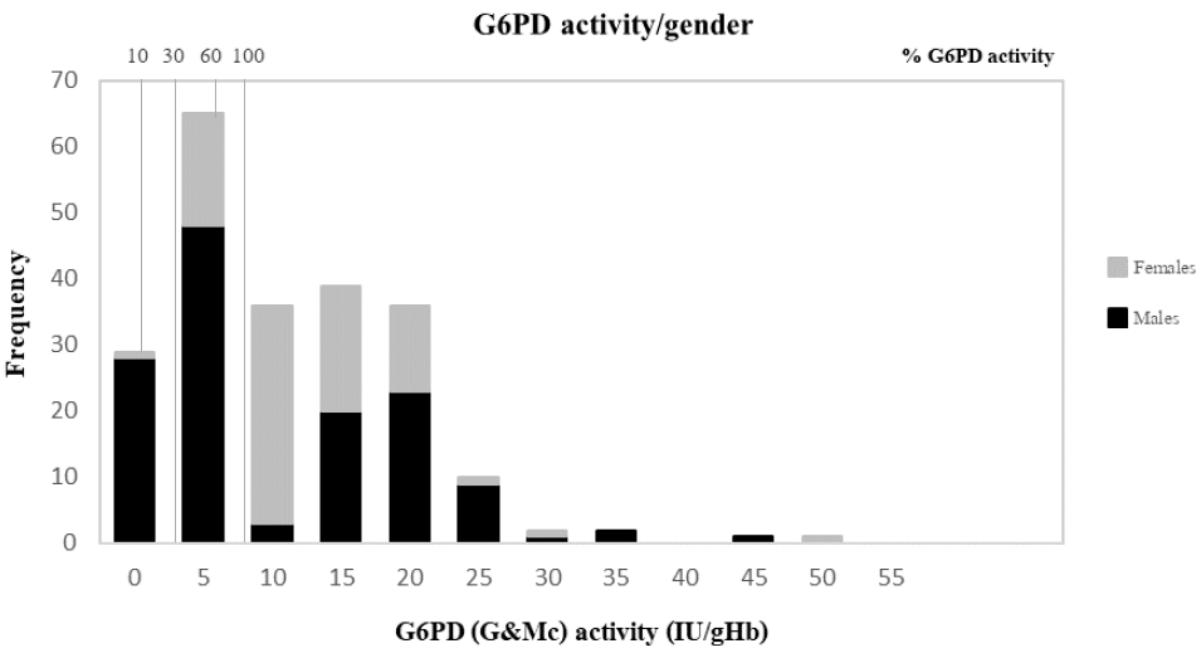


Figure 4 G6PD activity distribution among males and females using the G&Mc method. The figure indicates 10%, 30%, 60%, and 100% of normal activity for this population

Table 1 Performance test of the FST for detecting G6PD deficiency compared with the quantitative WHO test at 95%CI

Cutoff, IU/gHb (% of AMM value = 12.6)	10% Activity cutoff	20% Activity cutoff	30% Activity cutoff	40% Activity cutoff	50% Activity cutoff	60% Activity cutoff
Cutoff value, IU/gHb	1.26	2.52	3.78	5.04	6.30	7.56
No. of samples with G6PD levels below cutoff (M&F)	60	67	71	73	73	73
G6PD FST						
Sensitivity, %	100	100	100	100	100	100
Specificity, %	77.65	84.62	89.19	91.67	91.67	91.67
PPV, %	75.95	84.81	89.87	92.41	92.41	92.41
NPV, %	100	100	100	100	100	100

AMM, adjusted male median; CI, confident interval; F, female; FST, fluorescent spot test; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; IU, international unit; M, male; NPV, negative predictive value; PPV, positive predictive value

Table 2 Performance test of the FST for detecting G6PD deficiency compared with the quantitative G&Mc test at 95%CI

Cutoff, IU/gHb (% of AMM value = 8.8)	10% Activity cutoff	20% Activity cutoff	30% Activity cutoff	40% Activity cutoff	50% Activity cutoff	60% Activity cutoff
Cutoff value, IU/gHb	0.88	1.76	2.64	3.52	4.40	5.28
No. of samples with G6PD levels below cutoff (M&F)	61	69	71	73	73	75
G6PD FST						
Sensitivity, %	100	100	100	100	100	100
Specificity, %	78.57	86.84	89.19	91.67	91.67	92.29
PPV, %	77.22	87.34	89.87	92.41	92.41	94.94
NPV, %	100	100	100	100	100	100

AMM, adjusted male median; CI, confident interval; F, female; FST, fluorescent spot test; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; IU, international unit; M, male; NPV, negative predictive value; PPV, positive predictive value

Discussion

Although the WHO and G&Mc methods provide consistent and reliable measurements of G6PD activity, their reliance on specialized equipment, specific reagents and technical expertise limits their practicality in many endemic regions. Consequently, simpler and less labor-intensive methods are needed as effective diagnostic tools for G6PD deficiency screening. Qualitative methods, such as the FST, offer a favorable alternative due to their simplicity, cost-effectiveness and reduced labor requirements, making them particularly valuable in low-resource settings.

In terms of accuracy and sensitivity, the FST demonstrated excellent performance, especially at activity cutoffs above 30% of the AMM. Achieving 100% sensitivity and nearly 90% specificity when compared with the WHO and G&Mc methods, the FST effectively distinguishes G6PD-deficient homozygous females and hemizygous males from individuals with normal enzyme activity. These findings are consistent with earlier evaluations in the literature,^{1,9} reinforcing the FST's reliability as a screening tool. However, the inability of the FST to quantify enzyme activity limits its utility for identifying intermediate deficiency levels, which are critical for

clinical decision-making in certain populations, such as heterozygous females, who often exhibit a wide spectrum of enzyme activity due to X-chromosome inactivation.

The reclassification of ten individuals as G6PD-deficient using a cutoff above 30% of the AMM highlights the importance of quantitative thresholds in accurately identifying cases of intermediate deficiency. This demonstrates the limitations of qualitative methods like the FST, which can fail to capture individuals with clinically significant indeterminate results. The inclusion of a cutoff above 30% of the AMM provides a more precise diagnostic framework, particularly for identifying borderline cases and heterozygous females in genetically diverse populations. These findings underscore the need for an integrated approach that combines qualitative methods for large-scale screening with quantitative methods for detailed evaluations, ensuring accurate diagnosis and appropriate management of G6PD deficiency across diverse populations.

In conclusion, while the FST is an excellent screening tool due to its simplicity and efficiency—particularly in resource-limited settings—quantitative assays remain the gold standard for precise diagnosis and clinical management. The choice of diagnostic method should depend on the specific context, balancing the need for accuracy with available resources. This study provides valuable insights to guide the development and implementation of G6PD diagnostic strategies worldwide, ensuring effective screening and management of this common enzymopathy.

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