Validation of the rapid test Carestart™ G6PD among malaria vivax-infected subjects in the Brazilian Amazon

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Abstract

Introduction: In the Brazilian Amazon, malaria infections are primarily caused by Plasmodium vivax. The only drug that kills the hypnozoite form of P. vivax is primaquine, thereby preventing relapse. However, treating glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals with primaquine can lead to severe hemolysis. G6PD deficiency (G6PDd) affects approximately 400 million people worldwide, most of whom live in malaria-endemic areas. Therefore, clinicians need tools that can easily and reliably identify individuals with G6PDd. This study estimated the accuracy of the Carestart™ G6PD rapid test (Access Bio) in the diagnosis of G6PDd in male participants with and without P. vivax acute malaria.

Methods: Male participants were recruited in Manaus. Malaria diagnosis was determined by thick blood smear. G6PD quantitative analysis was performed spectrophotometrically at a wavelength of 340nm. The Carestart™ G6PD test was performed using venous blood. Genotyping was performed for individuals whose samples had an enzyme activity less than 70% of the normal value. Results: Six hundred and seventy-four male participants were included in this study, of whom 320 had a diagnosis of P. vivax malaria. In individuals with enzyme activity lower than 30% (n=13), the sensitivity, specificity, positive predictive value, and negative predictive value of the Carestart™ G6PD test were as follows: 61.5% (95%CI: 35.5%-82.3%), 98.3% (95%CI: 97.0%-99.1%), 42.1% (95%CI: 23.1%-63.7%), and 99.2% (95%CI: 98.2%-99.7%), respectively. Increases in sensitivity were observed when increasing the cut-off value. Conclusions: Despite low sensitivity, Carestart™ G6PD remains a good alternative for rapid diagnosis of G6PDd in malaria-endemic regions.

Keywords: Malaria. Plasmodium vivax. Glucose-6-phosphate dehydrogenase deficiency. Care Start G6PD.

INTRODUCTION

Primaquine, an 8-aminoquinoline drug, is crucial to the control and elimination of Plasmodium vivax malaria. Presently, it is the only drug commercially available that offers a radical cure for this type of malaria. Since the 1940s, this drug has been prescribed to kill hypnozoites, the dormant hepatic stage of P. vivax, thereby preventing relapse[1][2][3]. However, the use of primaquine in glucose-6-phosphate dehydrogenase (G6PD)-deficient subjects can cause acute hemolytic events. These events can be fatal or lead to unnecessary hospital admissions that burden the public health system in Brazil[4][5][6][7][8][9].

G6PD deficiency (G6PDd) is a genetic disorder that affects approximately 400 million people worldwide[10][11]. It is defined by alteration in the protein structure of G6PD, and thereby its antioxidant activity. Most commonly, this alteration is due to a single amino acid substitution; double substitutions or amino acid depletions are rare[12].

In erythrocytes, this enzyme represents the only mechanism for neutralizing the oxidizing agents that cause hemolysis, by reducing nicotinamide adenine dinucleotide phosphate[13][14].

G6PDd genetic heritage has typical patterns linked to the X chromosome, and is more common in men. Males are hemizygous for the G6PD gene, and therefore may have either normal or deficient expression. Women, who have two X chromosomes and therefore two copies of the gene, either display the same expression in both X chromosomes (homozygous normal/deficient phenotype) or may be heterozygous. In some populations in which the defective allele
frequency is high, cases of homozygous deficient women are not uncommon. Heterozygous deficient women have a mixed population of erythrocytes (with normal or deficient expression of the G6PD enzyme), owing to random inactivation of one of the two X chromosomes (the ionization phenomenon)\(^{(13)(15)(16)}\).

In Brazil, no diagnostic method is implemented in the public health system to identify individuals with G6PDd before primaquine therapy\(^{(4)}\), and this situation is similar to that in many other vivax malaria-endemic countries. Peixoto et al.\(^{(4)}\) estimates that in the Brazilian Amazon there were 19,974 hospitalizations and 572 deaths of male G6Pd carriers between the years of 2009 and 2011. Between 2009 and 2010, the cost of severe adverse events among carriers of G6PDd varied from US$4,310,233.17 to US$5,487,124.14\(^{(4)}\). The risk of hemolysis may be even worse considering the complications of malaria and other co-morbidities found in these populations.

The current main diagnostic techniques used to assess G6PD status are molecular genotyping and quantitative spectrophotometry. These techniques are expensive, and need to be performed under good laboratory conditions by qualified technicians, which end to restrict access to these tests. The fluorescent spot test, the rapid screening test preconized from the World Health Organization (WHO), is able to identify severe deficiency, but has decreased sensitivity in cases of mild- and low-deficiency phenotypes\(^{(12)(13)(17)}\). To solve such problems, some point-of-care tests have emerged for screening G6PDd individuals.

The rapid test BinaxNow\(^{®}\) G6PD, produced by Inverness Medical, is sensitive in detecting severe deficiency. However, the test needs to be performed at temperatures between 18 and 25°C and requires venous blood\(^{(18)(19)}\), which limits its use under field conditions.

Another, and more promising, assay is Carestart\(^{™}\) G6PD, produced by AccessBio. It is a rapid test kit that has several advantages compared with other tests: it is easy to deploy, it is much cheaper than other G6PDd diagnostic tools, it does not have temperature range limitations, and it can be performed using capillary blood. Moreover, this rapid diagnostic test (RDT) has been shown to be accurate in detecting deficient phenotypes in previous studies\(^{(20)(21)(22)(23)(24)(25)}\).

The purpose of the present study was to validate the Carestart\(^{™}\) G6PD test, by comparing its performance with that of a reference standard technique, in populations living in malaria vivax-endemic areas in the Brazilian Amazon.

**METHODS**

**Study sites and population**

This study was conducted at the Tropical Medicine Foundation Dr. Heitor Vieira Dourado [Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD)] and in the peri-urban communities of Brasilieirinho, Ipiranga, and Puraquequara in Manaus, Amazonas State, Brazil. The City of Manaus has an estimated population of 1,832,423 inhabitants, most of whom live in urban and peri-urban areas. In this municipality, intense migration, combined with limited epidemiological and entomological surveillance, results in the active transmission of malaria in rural and peri-urban areas. The Annual Parasitic Incidence in the city ranges from low to medium risk in rural areas, whereas in urban/peri-urban areas, it varies from no risk to high risk.

Blood samples were collected, consecutively, from male participants who were competent to give consent. Due to limitations of the quantitative gold standard in detecting heterozygous females, owing to ionization, females were not included in this study. The sample size was calculated for a finite population, using the following parameters: 1) sensitivity estimate: 0.68\(^{(22)}\), 2) specificity estimate: 0.99\(^{(22)}\), 3) z value (alpha error of 5%): 1.96; 4) precision: 0.10 (for sensitivity) and 0.01 (for specificity); finite population: 1,500 men. Base parameters assumed the following: 4.5% prevalence of G6PDd in the study population, based on the study in 3 districts of Manaus, with 1,478 men\(^{(26)}\), a frequency of vivax malaria/mixed malaria of 3,000 cases/year (FMT-HVD 2012 data), and 50% of male individuals in the Brazilian population.

**Sample collection and storage**

All blood samples were obtained between August 2013 and October 2014. Four milliliters of venous blood were collected in Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) containing EDTA (K\(_2\) ethylenediaminetetraacetic acid) as an anticoagulant. All samples were maintained at 4°C until G6PD quantitative analysis was performed. From each sample, whole-blood aliquots were maintained at -20°C until DNA was extracted and genotyped. Peripheral blood samples were collected using a standard lancet to perform a thick blood smear for diagnosis of malaria. All collection procedures were performed according to biosafety standards.

**Data collection and storage**

Demographic data was gathered using standard questionnaires. All questionnaires were scanned and transferred to the automatic data insert software Cardiff Teleform v10.8.

**Hematological analysis**

All hematological parameters were measured using a Sysmex KX21N automated hematology analyzer (Sysmex Corporation, Kobe, Japan). A reticulocyte count was performed on the same day as blood collection using brilliant cresyl blue staining.

**Malaria diagnosis**

The parasite density was determined in thick blood smears. For malaria-positive individuals, total parasitemia was determined by a 500 leukocyte count, and the parasite density was calculated for a total leukocyte count. A result was considered negative if the parasite was absent in a thick blood smear 200 leukocyte count.

**Reference standard**

The G6PD spectrophotometric quantitative analysis was performed in a Shimadzu UV 1800 series spectrophotometer, using a Pointe G6PD reagent kit (Pointe Scientific, London-UK). All samples were analyzed in duplicate (5µL whole blood...
per replicate) and within 24 hours after blood collection. The manufacturer’s instructions were followed for reagent preparation. Absorbance of the NADPH produced was detected at a wavelength of 340nm, at 37°C. For analysis quality control, each sample was analyzed together with normal and deficient controls from lyophilized red blood cell samples provided by Pointe Scientific. The analyses were considered valid only if the results for the control samples were correct. If the control samples gave incorrect results, the analysis was repeated. G6PD activity was calculated as international units per gram of hemoglobin (IU/gHb).

G6PD variant genotyping

Deoxyribonucleic acid (DNA) was extracted from blood samples using a FavorPrep 96-well Genomic DNA (Favorgen®) kit, following the manufacturer’s instructions. Thereafter, the molecular characterization of the mutations 202 (A-), 376 (A+), 563 (Mediterranean), 143 (Aures), and 1,003 (Chatham) in the G6PD gene was performed by polymerase chain reaction (PCR) and restriction fragments length polymorphism (RFLP) analysis. After amplification of the regions containing mutations 202, 376, 563, 143, and 1003, the reaction products were restricted with NalIII, FokI, MboII, MboI, and BstXI enzymes (New England BioLabs Inc.), respectively. The PCR reaction was performed in a thermocycler with the amplification product stained with ethidium bromide, and analyzed by agarose gel electrophoresis (Sigma Aldrich) in 1.5% 1× TAE buffer pH 8.3 (40mM Tris base, NaOAc, 20mM 1mM EDTA). Analysis of the digested products was performed using 7% polyacrylamide gels (Sigma Aldrich), stained with 0.002% ethidium bromide, and visualized under ultraviolet light. Positive and negative controls were included to validate the analysis results.

Carestart™ G6PD screening test

The rapid test was performed within 10 minutes after venous blood collection, according the manufacturer’s instructions. Two microliters of venous blood was added to sample wells of the test platform, followed immediately by addition of two drops of the reagent buffer. The tests results were read visually after 10 minutes. All tests were read under the same luminosity conditions. Tests that developed a purple color in the reading window were considered G6PD normal. Tests with a pale purple color background were also classified as G6PD normal. Tests showing no color development were considered G6PD deficient. Results were read by two independent observers; in the case of disagreement, a third observer was called in. Rapid tests from the same batch (GP13E01) were used for all participants. All observers were blinded to the results from the spectrophotometric analysis.

Data analysis

For all mean values, we calculated the confidence interval and standard deviation. To calculate mean differences between two groups, we used Student’s t parametric test. All data analyses, including diagnostic test accuracy evaluation, were performed using the software EpiInfo v.6.

Ethical considerations

The study protocol was reviewed and approved by the Ethics Committee Board of the Tropical Medicine Foundation Dr. Heitor Vieira Dourado (approval number 282086/2013). Volunteers provided written consent before inclusion in this study. For those volunteers under 18 years old, parents were instructed on the study objectives and they signed the informed consent. All participants tested G6PD deficient (by the quantitative method) were informed of the risks of hemolysis due to primaquine uptake. Participants testing positive for malaria by thick blood smear (excluding G6PD-deficient participants) were treated in accordance with the malaria treatment guidelines from the Brazilian Ministry of Health.

RESULTS

From June 2013 to July 2014, 674 male participants were included in the study, among whom 320 were diagnosed with malaria vivax (by thick blood smear) (Figure 1). All malaria patients were recruited from the FMT-HVD malaria clinic. With regards to non-infected participants, 214 (individuals with febrile syndrome in search of diagnosis) were recruited from the FMT-HVD clinic and 140 were recruited from their homes or work places in the communities of Brasileirinho, Ipiranga, and Puruaquequara communities (Manaus, Amazonas State).

The age of individuals ranged from 12 to 66 years, with a mean age of 39 years. There was no significant difference between the ages of the two participant groups (malarial participants/non-malarial participants) (p>0.05). Among the participants, 82.6% were self-identified as mestizo, and 69.1% had presented with previous episodes of malaria. Of those with previous episodes, 26.3% reported symptoms related to hemolysis during antimalarial treatment, the most frequently reported of which was dark urine (11.4%). Among participants with G6PDd, 83.3% had, at most, experienced three previous episodes of malaria (Table 1).

Hematological parameters

In participants with malaria, the mean hemoglobin (Hb) concentration was 13.4g/dL, and in non-malarial participants, the mean concentration was 14.3g/dL. Participants with malaria had a significantly lower mean Hb concentration compared to non-malarial participants (p<0.001). Participants with malaria had a higher reticulocyte percentage count when compared with non-malarial participants (0.81% versus 0.56%). Participants with G6PDd had lower hemoglobin levels compared to normal participants (13.1 versus 13.7g/dL) (p<0.05). There was no significant difference between G6PDd and normal participants in reticulocyte count (p>0.05).

Enzymatic activity

G6PD enzymatic activity measured by spectrophotometry ranged from 0.4 to 13.7IU/gHb. The mean enzymatic activity was estimated in the population of non-malarial participants, with the exclusion of participants with <1% enzymatic activity (<1.0IU/gHb) (n=352). The mean activity estimated was 6.6IU/gHb and the range of normal enzyme activity (between
Study Participants (n=674)

Malaria patients (n=320)

UV-Vis Spectrophotometry (n=674)

Malaria patients (n=320)

Non-malaria patients (n=354)

Non-malaria patients (n=354)

Normal (n=340)

Deficient (n=14)

Carestart™ G6PD (n=674)

Malaria patients (n=320)

Non-malaria patients (n=354)

Normal (n=311)

Deficient (n=9)

Normal (n=344)

Deficient (n=10)

Genotyping (n=116)

Wild type (n=101)

A - variant (n=15)

FIGURE 1. Flow chart of study volunteers distribution.
TABLE 1
Characteristics of the study population - proportional distribution.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Malarial participants</th>
<th>Non-malarial participants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>normal</td>
<td>deficient*</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>white</td>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>mestizo</td>
<td>81.1</td>
<td>90.0</td>
</tr>
<tr>
<td>afrodescendant</td>
<td>8.9</td>
<td>5.0</td>
</tr>
<tr>
<td>Previous episodes of malaria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>21.1</td>
<td>35.0</td>
</tr>
<tr>
<td>1 to 3</td>
<td>31.1</td>
<td>52.5</td>
</tr>
<tr>
<td>&gt;3</td>
<td>47.8</td>
<td>12.5</td>
</tr>
<tr>
<td>Parasitemia/µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;500</td>
<td>18.2</td>
<td>17.5</td>
</tr>
<tr>
<td>from 501 to 2,000</td>
<td>26.8</td>
<td>40.0</td>
</tr>
<tr>
<td>&gt;2,000</td>
<td>55.0</td>
<td>42.5</td>
</tr>
<tr>
<td>Hemolysis symptoms**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>jaundice</td>
<td>3.2</td>
<td>8.0</td>
</tr>
<tr>
<td>black urine</td>
<td>12.8</td>
<td>8.0</td>
</tr>
<tr>
<td>both</td>
<td>7.3</td>
<td>16.0</td>
</tr>
<tr>
<td>none</td>
<td>76.7</td>
<td>68.0</td>
</tr>
</tbody>
</table>

NA: not applicable; *Participants with enzymatic activity ≤3.9 international units per gram of hemoglobin (IU/gHb). **Only participants with previous malaria vivax episodes.

60% and 150% activity) was estimated to be between 3.9 and 9.8 IU/gHb. There was no significant difference in mean enzymatic activity between malarial and non-malarial participants (p>0.05) (Figure 2).

**G6PDd frequency**

Using a cut-off value of ≤3.9 IU/gHb (≤60% of normal enzyme activity), 4% (14/354) of individuals were G6PDd among non-malarial participants and 12.5% (40/320) of individuals were G6PDd among malarial participants (Figure 3).

According to the WHO classification scheme, 0.0% (0/674) of participants were classified as Class I (enzyme activity ≤0.10), 0.5% (3/674) as Class II (>0.1 and ≤0.7 IU/gHb), 7.6% (51/674) as Class III (>0.7 and ≤3.9 IU/gHb), 90.1% as Class IV (>3.9 and ≤9.8 IU/gHb), and 1.9% (13/674) as Class V (>9.8 IU/gHb).

**G6PDd genotyping**

Among the participants, 116 were selected for genotyping diagnosis according their enzymatic activity (<70% of the normal value). Total DNA was extracted from whole blood samples for molecular diagnosis of G6PDd. The mean concentration of the extracted DNA was 21 ng/µL, ranging between 13 and 27 ng/µL. Five genotypic variants were investigated: A- (202 G→A); A+ (376 A→G); Mediterranean (563 C→T); Aures (143 T→C); and Chatham 1003 (G→A). A deficient allele was identified in 12.9% (15/116) of participants. Only African variants were identified: A- was found in 6% (7/116), 3.4% (4/116) were positive for the variant A+ (202 G→A), and 3.4% (4/116) were positive for the variant A+ (A→G 376). The enzymatic activity of these participants ranged from 0.4 to 7.1 IU/gHb, with a mean activity of 2.8 IU/gHb (95%CI 1.8-3.7 IU/gHb).

**Carestart™ G6PD validation**

In individuals with enzyme activity lower than 30% (n=13), the sensitivity, specificity, positive predictive value (PPV), and positive for the variant A+. The enzymatic activity of these participants ranged from 0.4 to 7.1 IU/gHb, with a mean activity of 2.8 IU/gHb (95%CI 1.8-3.7 IU/gHb).
negative predictive value (NPV) of the Carestart™ G6PD rapid test were: 61.5% (95% CI: 35.5%-82.3%), 98.3% (95% CI: 97.0%-99.1%), 42.1% (95% CI: 23.1%-63.7%), and 99.2% (95% CI: 98.2%-99.7%), respectively. At this cut-off, there were five false negatives. The rapid test showed a higher sensitivity among non-malarial participants when compared to malarial participants. There was no significant difference in specificity between the two groups of individuals, which, in all the cut-offs analyzed, varied between 97.7% and 99.0% (Table 2).

**DISCUSSION**

Because individuals with G6PDd are not routinely screened in Brazil, or in other malaria-endemic countries, primaquine continues to be used to treat P. vivax, regardless of its hemolysis risk.

It is noteworthy that, in Brazil, the diagnosis of G6PDd would also be important for predicting the risk of jaundice in infants, when performed during neonatal screening. It would similarly be important before starting treatment of hanseniasis with dapsone, since hanseniasis is an endemic disease in the Brazilian Amazon and dapsone is known to have hemolytic effects in G6PDd carriers.

It is known that approximately one-third of the world population lives in areas with active transmission of malaria vivax, and that approximately 400 million people that carry the G6PDd allele live in these areas. In the Amazonas State of Brazil, the only studies that have estimated the prevalence of G6PDd variants [A- (202 G→A) and A+ (376 A→G)] were identified. Santana et al. (26) (Manaus municipality) and Barraviera et al. (32) (Humaitá municipality), showed a G6PDd prevalence of 4.5% and 5%, respectively. In our study, we recorded a G6PDd frequency 4% among male participants (non-malarial infected), which is only slight less than that of previous estimates.

The mean blood hemoglobin level was lower among individuals with malaria than in non-malarial participants, which is a consequence of the pathophysiology of the disease. Participants with G6PDd showed lower levels of hemoglobin than participants with normal G6PD. Although a previous study on the Cambodian population found that G6PDd was associated with lower hemoglobin levels, we could discern no clear relationship between G6PDd and hemoglobin levels in the present study. However, in another study, Satyagraha et al. (34) found that G6PDd was associated with higher hemoglobin levels, whereas, in contrast, other studies have found no correlation between G6PDd and hemoglobin levels.

Our study recorded a mean G6PD enzyme activity of 6.6 IU/gHb [95% confidence interval (CI): 6.4-6.7], as determined spectrophotometrically, which is consistent with the results of the only previous study in which G6PD enzyme activity has been quantitatively measured in a local population. The slight difference in the results of the two studies may be related to the techniques used for the quantitative analysis, which is very sensitive to small changes in the protocol. On the basis of this finding in non-malarial participants (excluding all individuals with activity ≤1IU/gHb), it was possible to calculate the normal range of enzyme activity for the local male population, namely, 3.9 to 9.8 IU/gHb. These are the only data of this type available for the Brazilian population, using a quantitative laboratory technique as a reference standard for the analysis of enzyme activity.

Of the five genotypic variants investigated, only African variants [A- (202 G→A) and A+ (376 A→G)] were identified. Santana et al. (26) described a frequency of 0.7% for the Mediterranean variant (C→T 563) in the Manaus population, which was not replicated in our results. Our analysis was restricted to just 116 individuals, selected based on their G6PD enzymatic activity (<70% of the normal value). Regarding the Mediterranean variants, Chatham and Aures, there have been no reports of the presence of these variants in local populations.

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**TABLE 2**

Validation of the Carestart™ G6PD rapid diagnostic test according to different cut-offs values (proportional distribution).

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>20% activity ≤1.3IU/gHb</th>
<th>30% activity ≤2.0IU/gHb</th>
<th>40% activity ≤2.6IU/gHb</th>
<th>60% activity ≤3.9IU/gHb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>malaria</td>
<td>non-malaria</td>
<td>malaria</td>
<td>non-malaria</td>
</tr>
<tr>
<td>G6PDd Cases (%)</td>
<td>5 1.5</td>
<td>3 0.9</td>
<td>8 2.5</td>
<td>5 1.0</td>
</tr>
<tr>
<td>Se (%)</td>
<td>40.0</td>
<td>66.7</td>
<td>50.0</td>
<td>80.0</td>
</tr>
<tr>
<td>(97.2-99.7)</td>
<td>(20.8-93.9)</td>
<td>(21.5-78.5)</td>
<td>(37.3-96.4)</td>
<td>(21.3-72.0)</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>22.2</td>
<td>20</td>
<td>44.4</td>
<td>40.0</td>
</tr>
<tr>
<td>(6.3-54.7)</td>
<td>(5.7-51.0)</td>
<td>(18.9-73.3)</td>
<td>(16.8-68.7)</td>
<td>(16.8-68.7)</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>99.0</td>
<td>99.7</td>
<td>98.7</td>
<td>99.7</td>
</tr>
<tr>
<td>(97.4-100)</td>
<td>(96.7-99.5)</td>
<td>(98.4-99.6)</td>
<td>(95.9-99.0)</td>
<td>(97.9-99.8)</td>
</tr>
</tbody>
</table>

G6PD: glucose-6-phosphate dehydrogenase; IU/gHb: international units per gram of hemoglobin; Se: sensitivity; SP: specificity; PPV: predictive positive value; NPV: negative predictive value.
Among carriers of the African variants, there was a wide variation in enzyme activity measured. Only 2 of the 15 genetically identified individuals were self-identified as African descendants, a result that can be explained by the mixing that has occurred among local populations. Of these carriers, 60% (9/15) had experienced up to 3 previous episodes of malaria during their lifetime, whereas 33% (5/15) had never presented with a previous episode of malaria, a result similar to that obtained in previous studies\(^{(26)}\)\(^{(33)}\)\(^{(37)}\). Ten participants had a positive diagnosis of vivax malaria at the time of blood collection. The parasitemia among these participants also showed a wide range. The small number of variant carrier samples in our study is a potential limitation for this type of analysis. In Ghana, Amoako et al.\(^{(38)}\) showed no significant difference in parasitemia between individuals with G6PDd and normal individuals, even though parasitemia is highly associated with G6PDd. Some studies of African variant A- carriers with falciparum malaria have found no relationship between the presence of the mutant variant and African variant A- carriers with falciparum malaria have found no significant difference in parasitemia between individuals with G6PDd and normal individuals, even though parasitemia is highly associated with G6PDd. Some studies of African variant A- carriers with falciparum malaria have found no relationship between the presence of the mutant variant and malaria parasitemia\(^{(39)}\)\(^{(40)}\)\(^{(41)}\). In Sri Lanka, among individuals with falciparum malaria and vivax malaria, Dewasurendra et al.\(^{(42)}\) observed lower parasite density in male individuals with deficient mutant alleles, but did not observe the same phenomenon in female carriers of the deficient allele\(^{(42)}\).

In the present study, we selected a cut-off point of ≤3.9IU/gHb (determined spectrophotometrically) to define G6PDd, which is equivalent to 60% of the mean population enzyme activity and follows WHO recommendations that define individuals with an enzyme activity <60% of the local enzyme activity mean as enzymopathy, i.e., classes III, II, and I\(^{(43)}\). The results of our study show the high sensitivity of the Carestart™ G6PD rapid test in detecting individuals with severe deficiency of the enzyme, but with low sensitivity among individuals with intermediate activity and African mutant allele carriers. Carestart™ G6PD also showed lower sensitivity in detecting deficiency in participants with malaria when compared with the group of non-malarial individuals.

In comparison with the fluorescent spot test (FST), Carestart™ G6PD was easier to handle because it does not require the preparation of reagents or use of special equipment (e.g., UV light). However, like the FST, the Carestart™ G6PD assay has similar difficulty in identifying individuals with borderline enzyme activity. Our study estimated an assay sensitivity of 61.5% for individuals with ≤30% of activity, a range of activity in which FST also offers similar performance\(^{(42)}\). In a study published by Baird et al.\(^{(21)}\), the Carestart™ G6PD test showed non-inferiority in accuracy when compared to the FST.

Although in the present study we did not formally compare the Carestart™ G6PD assay with the BinaxNow G6PD assay (Inverness Medical), the accuracy estimates for Carestart™ G6PD in this study are similar to those of the BinaxNow G6PD test when used in patients without malaria infection and using high enzyme activity cut-offs, as in the studies published by La Brito MAM et al. Carestart™ G6PD was lower in malaria-infected individuals than in those without infection. Although the results of both BinaxNow G6PD and Carestart™ G6PD tests rely on relatively subjective interpretation, Carestart™ G6PD has an advantage over the BinaxNow G6PD test in that it is simpler to implement, does not rely on automatic pipettes, and is not limited by temperature, which are factors that limit the use of Binax Now G6PD under field conditions\(^{(18)}\)\(^{(19)}\)\(^{(23)}\)\(^{(31)}\).

The low sensitivity of the rapid test observed in the present study can be attributed to the high frequency of false-negative results among individuals with malaria and the small number of deficient individuals who participated in the study. There was a high frequency of false-negatives even when using cut-offs considered low, where one would expect greater sensitivity due to lower enzyme activity in the samples tested, which is a phenomenon also observed elsewhere\(^{(22)}\) and the main factor responsible for the poor performance of the Carestart™ G6PD rapid test in our study. The use of venous blood to perform the rapid test probably did not affect the sensitivity of the test. In this regard, a previous study by Bancone et al.\(^{(44)}\) showed high agreement between the results for venous and capillary blood when using the Carestart test\(^{(44)}\). There is no clear explanation for the low sensitivity to malaria infection. The level of enzyme activity does not seem to be the main factor responsible: this study and previous studies have shown no significant difference in enzyme activity between individuals with and without malaria infection\(^{(20)}\)\(^{(13)}\). This may be due to intrinsic characteristics of the parasite that somehow affect the chemistry of Carestart™ G6PD, leading to a decrease in sensitivity. This should be studied in further detail. In contrast, Carestart™ G6PD specificity remained high at all the cut-offs analyzed, ranging between 97.7% and 99.8%, with no difference between malaria status groups, corroborating the results obtained in previous studies\(^{(20)}\)\(^{(21)}\)\(^{(22)}\)\(^{(24)}\). To our study design, we did not examine the accuracy of the test among women. DNA sequencing or cytochemical assays can be useful for studying the heterozygosity phenomenon and make possible the determination of Carestart™ G6PD accuracy among the female population. Taking into account the wide range of G6PD activity in heterozygous females, owing to liconization, the detection of formazan formation (used by Carestart™ G6PD) does not seem to be the ideal method for confirming G6PDd in heterozygous females.

Among the few limitations of the Carestart™ G6PD assay are the facts that it does not show a normal control-window and that color development persists for only 15 minutes. Another important limitation is that the test window may display different shades of purple, introducing subjectivity into interpretation of the test results. This may lead to mislabeling those with intermediate and low enzyme activity. Despite these limitations, however, the Carestart™ G6PD test has considerably easier field handling compared to other G6PDd screening tests.

In conclusions, G6PD deficiency affects a clinically significant portion of Manaus population and their diagnosis before the administration of primaquine is required to guarantee the safety of these individuals. It is necessary to implement a
point-of-care screening test for G6PDd detection to avoid new hospitalizations related to primaquine antimalarial therapy in malaria-endemic regions. Our spectrophotometric findings showed no difference in G6PD enzyme activity related to vivax malaria, despite the greater G6PDd frequency found. Overall, we found that the Carestart™ G6PD RDT showed low sensitivity in detecting mild and intermediate G6PD deficiency, but high sensitivity in detecting severe deficiency.

Conflict of interests
The authors declare that there is no conflict of interest.

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