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Prevalence of Malaria and Glucose-6-Phosphate Dehydrogenase Deficiency in Malaria-Endemic Southwestern Uganda: Implications for Primaquine Use

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May 1, 2015

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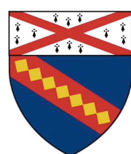

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Abstract

Introduction: Malaria transmission in Uganda is remarkably heterogeneous, and declines in prevalence have not been uniform. Previous surveys in southwestern Uganda have shown declines in parasite prevalence from 2004 to 2010. As malaria transmission continues to decline in southwestern Uganda, aggressive strategies, such as the addition of primaquine (PQ) to artemisinin-combination therapies (ACTs), are being considered in low transmission settings. Despite the potential benefit of PQ in reducing transmission, concerns over its safety and efficacy have hampered its deployment. In particular, those with glucose-6-phosphate dehydrogenase (G6PD) deficiency are at a higher risk of hemolytic toxicity.

Methods: To better assess how primaquine may impact upon southwestern Uganda, we conducted a cross sectional survey among 631 children under five years of age sampled from districts previously characterized as low (Mbarara), intermediate (Bushenyi), and high (Isingiro) transmission intensities. Blood samples were collected via capillary fingerprick to determine the current status of malaria control and the prevalence of G6PD deficiency. Parasite prevalence was determined using (1) a combined Plasmodium HRP-2/LDH rapid diagnostic test (RDT) (SD Bioline Malaria Ag P.f/Pan) and (2) light microscopy. G6PD deficiency was evaluated by: (1) quantitative G6PD deficiency by spectrophotometric assay (Trinity Biotech®), (2) qualitative G6PD deficiency assay by rapid diagnostic test (CareStart™ G6PD RDT), and (3) DNA was isolated to conduct PCR-RFLP analysis to detect the G6PD A- 202A/376G allele.

Results: Prevalence of parasitemia was higher by RDT compared to microscopy (6.2% (95% CI: 4.3-8.1) vs. 3.2% (95% CI: 1.8-4.5)). By district, parasitemia prevalence was 1.2% (3/242) in Mbarara, 3.2% (5/157) in Bushenyi, and 5.2% (12/232) in Isingiro. All 20 microscopy positive cases were detected by RDT. Of the 19 cases detected only by RDT, 7 (36.8%) reported having been treated for malaria within the past month. Notably, of the 20 microscopy positive children, 50% (10/20) were infected with *P. falciparum*, 40% (8/20) with *P. malariae*, and the remaining 2 children were *P. vivax* and *P. ovale* mono-infections. Knowledge, attitudes, and practice regarding malaria prevention were also assessed, revealing a high proportion of households reporting bednet use (91.6%), but only a small fraction of households participating in indoor residual spraying (0.8%). The prevalence of mild G6PD deficiency (defined as 10-60% of normal activity) was 13.8% (95% CI: 11.1-16.5) as compared to 8.6% (95% CI: 6.4-10.8) by RDT. No participants in our study exhibited severe G6PD deficiency (<10% enzyme activity). Of the 577/631 children considered normal by RDT, 37 were mildly deficient by quantitative assay. Of the 54 children found to be G6PD deficient by RDT, 4 were quantitatively normal. Performance characteristics of the CareStart™ G6PD RDT as compared with the Trinity Biotech® assay revealed low/moderate sensitivity and high specificity (57.5% and 99.3%, respectively). The currently recommended qualitative G6PD assay, the fluorescent spot test (FST), defines deficiency as 10% to 30% of normal G6PD activity. When compared to FST, the sensitivity of CareStart™ G6PD RDT increased to 94.7%, while specificity slightly decreased (94.1%). We found a lack of correlation between

genotypic and phenotypic assays. The sensitivity and specificity of the quantitative enzymatic assay to detect the G202A mutation was 29.8% and 87.8%.

Conclusion: Our preliminary findings indicate continued strides toward malaria control over the past 10 years in southwestern Uganda. Most notably, our survey reveals a striking shift in species prevalence in this region of Uganda, with nearly 50% of asymptomatic children infected with non-falciparum species. Furthermore, our results strongly suggest the need for better qualitative screening methods evaluating both the phenotype and genotype of G6PD deficiency.

Introduction

Background

Malaria remains one of the major public health problems worldwide. Every year, an estimated 3.3 billion people are at risk for malaria with a high burden of disease in the world's poorest countries. In 2013, 584,000 (95% CI: 367,000-755,000) deaths were attributable to malaria and mostly among African children under five years of age [1, 2]. However, since the initiation of a malaria elimination agenda in 2000, malaria incidence has helped to reduce malaria mortality rates by 47% and by 54% in Africa [2]. In recent years, key interventions such as the widespread deployment of long lasting insecticide-treated nets (LLINs), indoor residual spraying, use of malaria rapid diagnostic tests (RDTs), and prompt treatment with artemisinin-combination therapies (ACTs) have made major advances in malaria control [2, 3]. Despite significant reductions in several areas worldwide, malaria continues to be a huge burden on many countries [3].

In Uganda, heterogeneity of malaria transmission is considerable and changes in prevalence have not been uniform. In particular, declines in malaria parasite prevalence have been observed in southwestern Uganda whereas in eastern Uganda, malaria incidence appears to be rising [4-6]. In regions of low malaria transmission intensity, such as southwestern Uganda, monitoring malaria parasite prevalence and detecting sub-microscopic infections becomes increasingly important, as asymptomatic individuals may continue to produce gametocytes and thus be a silent reservoir for malaria transmission [7]. It has been reported that when transmission reaches very low levels, individuals who carry sub-microscopic infections are the source of 20-50% of all human-to-mosquito transmission [8]. Thus, in order to dramatically reduce the malaria burden in southwestern Uganda, aggressive strategies towards its elimination need to be considered. A critical antimalarial in the move towards malaria elimination is primaquine. Currently, the World Health Organization (WHO) has recommended the use of low-dose primaquine as a *Plasmodium falciparum* gametocytocide for mass drug administration, and as a single, low-dose of primaquine with ACTs for parasitologically confirmed *P. falciparum* malaria in malaria elimination settings. However, a significant concern with the widespread administration of primaquine is the risk posed to individuals with glucose-6-phosphate (G6PD) dehydrogenase deficiency. As such, these individuals are at risk of hemolytic anemia. Thus, the aim of this study was to evaluate the prevalence of malaria and G6PD deficiency in children under five years of age, which could impact the future use of primaquine in the low malaria-endemic setting of southwestern Uganda.

Malaria Pathogenesis

Malaria is a vector-borne disease caused by *Plasmodium* parasites and transmitted via the bites of infected female *Anopheles* mosquitoes. Sporozoites are injected into the human bloodstream and migrate to hepatocytes (liver) to mature and replicate in large numbers. At this stage, some species may differentiate into dormant hypnozoites for periods of weeks to years. After leaving the liver, clinical symptoms of malaria begin as parasites multiply in red blood cells. In some cases, however, parasites differentiate into

gametocytes, which can be taken up by female mosquitoes during a blood meal to complete the malaria transmission cycle. Mosquito to human malaria transmission is caused by one of 5 species of the *Plasmodium* protozoa: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and recently, *P. knowlesi*. *P. falciparum* malaria is associated with the highest rates of severe complications and mortality among the plasmodium species [9]. Additionally, *P. falciparum* is endemic to sub-Saharan Africa, especially in children under five years of age. Notably, *P. vivax* and *P. ovale*, are the species that form hypnozoites, which remain latent in the liver stages, and can contribute to ensuing relapses [9].

Rationale for the use of single dose primaquine

Resistance to first-line treatments against malaria continues to emerge, even in the era of combination therapy. An additional challenge is the lack of activity of first-line regimens against hypnozoites, leaving individuals at risk for relapses from *P. vivax* and *P. ovale*. Primaquine, a member of the 8-aminoquinoline group of drugs, is among the only commercially available antimalarial with activity against hypnozoites and has been shown to have gametocytocidal activity against *P. falciparum*. Due to its unique properties, it has received renewed attention in the eradication agenda [10, 11]. Indeed, primaquine is the only WHO recommended drug to:

1) Reduce the transmission potential of asymptomatic and symptomatic individuals [12, 13]
2) Reduce the transmission of *P. falciparum* infections by acting against mature gametocytes [12, 13]
3) Achieve “radical cure” of *P. vivax* by killing dormant sporozoites known as hypnozoites [12, 13]

Primaquine is included as a first-line treatment for *P. falciparum* in 20 countries across Southeast Asia and South America [14, 15]. At a dose of 30-45 mg in adults and 0.5-0.75 mg/kg in children, single primaquine dose in conjunction with ACTs was well tolerated and prior testing for G6PD deficiency was not required in these areas [15]. Despite its therapeutic advantages, primaquine is not currently being used in sub-Saharan Africa [14, 16]. One of the major reasons for the limited use of primaquine in Africa is the association between primaquine use and the risk of hemolytic anemia among G6PD deficient individuals, given the disproportionately high G6PD deficiency prevalence in sub-Saharan Africa compared to the rest of the world [14, 15].

G6PD deficiency

G6PD is an enzyme involved in the pentose phosphate pathway that catalyzes the oxidation of glucose-6-phosphogluconolactone. In this process, the coenzyme nicotinamide adenine dinucleotide phosphate (NADP) is reduced to NADPH. NADPH in turn protects cells from destruction caused by oxidative stress. Since erythrocytes lack mitochondria and do not generate NADPH in any other way, G6PD is the only source of protection against oxidative damage.

G6PD deficiency is the most common enzymopathy in humans, affecting 400 million people worldwide. It has been noted that higher frequencies of G6PD deficiency occur in malaria-endemic areas (Africa, Asia, Mediterranean, Middle East), which has led to the hypothesis that G6PD deficiency may confer partial protection to cerebral malaria, but increased the risk of severe malarial anemia [17-19].

G6PD deficiency is an X-linked, heredity defect of the *G6PD* gene, with over 160 known mutations leading to varying phenotypic severity [10]. G6PD variants arise from different point mutations within the *G6PD* gene and penetrance of the deficient gene varies by mutation, but is known to be exceedingly low [20]. Clinical manifestations of G6PD deficiency include neonatal jaundice, acute hemolytic anemia, and renal failure usually triggered by oxidative stressors, and in rare cases, chronic hemolysis leading to congenital non-spherocytic hemolytic anemia [21]. Typically asymptomatic, acute hemolysis occurs in G6PD deficiency individuals when they undergo oxidative stress (e.g. infection, certain medications, ingestion of fava beans). In sub-Saharan Africa, the most common allelic variant of G6PD deficiency is G6PD A-. Recent studies have shown that the *G6PD A- 202A/376G* allele, defined as having point mutations at 202G>A and 376A>G, is the predominant allele in exhibiting deficiency in East Africa [22, 23]. Hemizygous males and homozygous females with the *G6PD A- 202A/376G* allele is considered to exhibit approximately 8-20% of wildtype enzyme activity, while enzyme activity is highly variable in heterozygous females [24, 25].

Diagnostic issues that arise with G6PD screening tests further complicate G6PD deficiency status. The current gold standard of G6PD testing is an enzymatic test that can quantitatively measure G6PD activity. The WHO defines G6PD activity by five classes categorized by the severity of G6PD deficiency they cause (Appendix A) [26]. However, defining the boundary between normal and deficient G6PD activity is somewhat arbitrary and does not necessarily correlate with the risk of exposure to primaquine [26]. In addition, intermediate enzymatic activity, caused by heterozygous females or differential phenotypic presentation of G6PD deficiency variants, may be difficult to identify as either deficient or normal. Thus, this poses a significant safety issues in primaquine administration and challenges for developing a point-of-care test for mass screening of G6PD deficiency.

Methods

Study site and population

The study was conducted between August to early October 2014, at the end of dry season to the beginning of rainy season, in three districts of southwestern Uganda previously characterized as low, intermediate, or high malaria transmission intensities (Mbarara, Bushenyi, and Isingiro, respectively) (Figure 1) [27]. The area is mainly rural, with 23%, 12%, and 7% of its inhabitants living in urban areas within Mbarara, Bushenyi, and Isingiro Districts, respectively. In the southwestern highlands, malaria transmission is low and unstable (i.e. malaria is seasonal), with an annual entomological inoculation rate (EIR) rate of approximately 2-4 infective bites per year [28]. Rainfall is bimodal averaging 1,200 mm per annum, with rainy seasons occurring between September to January and March to May [4].

Study design and sample size

The study employed a stratified, two-stage cluster sampling method to select participants included in the survey. Data on the number of villages per district and household numbers per village was collected prior to sampling. A total of 20 villages within each district were first stratified by their urban and rural status and randomly sampled with probability proportionate to population size. The number of households that needed to be sampled per village cluster was determined from sample size weighted based on district population size in each district (Table 1). Households were then randomly selected from each village using the WHO's Expanded Program on Immunization (EPI) methodology [29]. Only one child under the age of five was eligible to participant in the survey per household. If there was more than one child under the age of five in the household, the participant was randomly selected.

Sample size was determined based on the minimum number of participants needed to obtain G6PD deficiency prevalence estimates with good precision. G6PD deficiency in Uganda was previously reported as 13% [30, 31]. Thus, assuming the population proportion under the null hypothesis is 13%, a sample of 384 achieves 80% power to detect a 5% difference using a two-sided binomial test. The sample size was increased to account for a design effect of 1.5 and subsequently by 10% to account for possible contingencies (e.g. nonresponse, recording error, sample loss) to reach 636. Our final sample size reached 660 to equalize the number of households selected per cluster, in each separate district (Appendix B).

Field procedure and sample collection

After obtaining parental informed consent, standardized questionnaires were administered to the head of household or parent/guardian as face-to-face interviews regarding their demographic information and malaria control measures. A blood sample from the participating child was collected by capillary fingerprick into an EDTA-coated microtainer (BD Diagnostics, Franklin Lakes, New Jersey). Rapid diagnostic tests and

preparation of thick and thin smears for microscopy were performed on site by field team laboratory technicians. G6PD enzymatic assays were processed within 1 day of sample collection. The remaining volume of blood was collected onto filter paper (Whatman® 903 Protein Saver Card, Sigma-Aldrich, St. Louis, MO) and stored at -80°C for molecular analysis. Samples were shipped at room temperature to New Haven, CT and DNA was extracted from dried blood spots using QIAamp DNA Mini Kit (Qiagen, Valencia, CA), according to manufacturer's instructions.

Parasite Detection

Parasite detection was assessed using the following measures: (1) a combined *P. falciparum HRP*-2/pLDH rapid diagnostic test (SD Bioline Malaria Ag P.f./Pan, Standard Diagnostics, Gyeonggi-do, Republic of Korea), (2) light microscopy, and (3) PCR for species confirmation. RDT was performed according to manufacturer's instructions. Thin and thick smears were prepared on the same slide and stained with 10% Giemsa (pH 7.2) for 15 min. Readings were independently read by trained microscopists as previously described [27]. Slides with discordant results were resolved by a third reader. Parasite and gametocyte densities, expressed as number of parasites/µl, were calculated by counting parasite numbers against 200 leukocytes and multiplied by an assumed standard leukocyte count of 8,000 leukocytes/µl.

Detection of G6PD Deficiency

G6PD deficiency was assessed by: (1) rapid diagnostic test (CareStart™ G6PD RDT, AccessBio, Inc., Somerset, New Jersey), (2) G6PD enzymatic activity by spectrophotometric assay (Trinity Biotech® G-6-PDH kit, Trinity Biotech®, Co Wicklow, Ireland), and (3) genotyping of the *G6PD A- 202A/376G* allele. RDT and G6PD enzymatic assay were performed according to manufacturer's instructions. Hemoglobin values (g/dL) were obtained by HemoCue® Hb 301 hemoglobin analyzer (HemoCue®, Brea, CA). A commercial set of known normal and no G6PD activity were used as controls (Trinity Biotech®, Co Wicklow, Ireland) to calculate % of G6PD activity.

Previous reports have elucidated strong linkage disequilibrium between G6PD 202A/376G mutations (D'=1) [18, 32]. Thus, identification of only the G202A mutation was used to detect the presence of the *G6PD 202A/376G* allele. PCR was performed using 5X GoTaq Flexi Buffer (Promega, Madison, WI), 1.5mM $MgCl_2$, 0.2mM dNTP, 0.2µM forward primer (5'-CCACCACTGCCCCTGTGACCT-3'), 0.2µM reverse primer (5'- GGCCCTGACACCACCCACCTT-3'), 1.25µM *GoTaq* polymerase, and approximately 10ng of DNA. Thermocycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 64°C for 1 min, 72°C for 30 sec, and a final extension at 72°C for 5 min. 10µl of PCR product was incubated with 1 U of *Nla*III at 37°C for 1 hr. Genotypes were identified based on digested products using 2.0% agarose gel electrophoresis (Appendix C). The overall call rate was 100%. For quality control, 3 samples were regenotyped and concordance rate was 100%.

Statistical analysis

Statistical analysis was performed using SAS 9.3 (SAS Institute, Cary, NC). Categorical variables were compared using the chi-square test. T-tests were performed when comparing continuous variables between two groups. If the variables contained more than two independent groups, one-way ANOVA was performed to calculate the difference between means or the Brown Mood test to compare the difference in medians. Kruskall-Wallis test was used when variables were non-parametric. Logistic regression was performed to test the association between outcome variable and covariates. P-values less than 0.05 were considered statistically significant.

Ethics approval

Written informed consent for study participation was obtained by the parent or guardian of each child. Ethics approval was obtained from the Uganda National Council of Science and Technology, the Mbarara University of Science and Technology Institutional Review Council, and the Yale University Human Investigation Committee.

Results

A total of 631 children were included in the study (Table 1). 242, 157, and 232 children were recruited from Mbarara, Bushenyi, and Isingiro, respectively. Overall, 50.1% of the children were males and the mean age at recruitment was 2.4 ± 1.3 years (range of 2.1 months to 5.0 years). 517 (81.9%) children were from rural villages and 114 (18.1%) lived in urban areas. Mbarara was found to have the highest proportion of urban areas (28.9%) compared to all three districts (p<0.001). The majority of the study population was of the Nyankore (84.2%) ethnic group. The overall mean hemoglobin level was 11.9 g/dL ± 1.3 in all three districts and no cases of severe anemia (defined as hemoglobin level <5 g/dL) were seen in children at the time of sample collection. Overall, bednet ownership was high (91.6%) among households, though coverage in urban households (95.6%, 95% CI: 91.8-99.4) was significantly higher than rural households (90.7%, 95% CI: 88.2-93.2; p=0.04). Importantly, consistent bednet use by participating child was higher than in 2010 where coverage was 78% (95% CI: 72-85) in urban areas and 57% in rural areas (95% CI: 46-67) [4]. However, indoor residual spraying (IRS) coverage was quite low, with a total of 5 (0.8%) households having reported utilizing IRS. While female to male ratio and mean age at recruitment were similar among all three districts, proportions of urban and rural villages, ethnic group, consistent bednet use by child, and mean hemoglobin level were significantly different (p<0.05; Table 1).

Epidemiology of malaria parasite prevalence

In our survey, prevalence of malaria infection across all three districts was 3.2% (95% CI: 1.8-4.5) by microscopy, indicating continued declines in malaria transmission intensity. In a similar study conducted in August 2010, DeBeaudrap and colleagues found malaria prevalence was relatively low and stable in urban areas (4%, 95% CI: 1-6) and unstable in rural areas (13%, 95% CI: 8-17) [4]. In our study, we found parasite prevalence in 2014 was 3.5% (95% CI: 0-6.9) and 3.1% (95% CI: 1.6-4.6) in urban and rural villages, respectively. As expected, parasite prevalence by microscopy was highest in Isingiro (5.2%) compared to Mbarara (1.2%) and Bushenyi (3.2%) (Table 1).

By SD Bioline Malaria P.f./Pan RDT diagnosis, overall parasite prevalence increased to 6.2% (95% CI: 4.3-8.1). By RDT, parasite prevalence was 12.1% in Isingiro, 5.1% in Bushenyi, and 1.2% in Mbarara (Table 1). The prevalence of malaria infection reported by RDT diagnosis was significantly different among districts, but did not differ by blood smear diagnosis (p<0.001 vs. p=0.051, respectively; Table 1).

Of the 20 children that were microscopy-positive, only half were *P. falciparum* infections. In Mbarara, all microscopy positive cases were due to *P. falciparum* infections (3/3; Figure 2). In Bushenyi, 80% (4/5) and 20% (1/5) of infections were caused by *P. malariae* (4/5) and *P. ovale*, respectively. We found that 58.33% (7/12), 33.33% (4/12), and 8.33% (1/12) of microscopy positive results in Isingiro were due to *P. falciparum, P. malariae*, and *P. vivax* infections, respectively (Figure 2). No mixed infections were observed in the study participants. Thin smears used to identify parasitemia elucidated that median parasite density across all three districts was 1,480 parasites/µl (range: 100-

31,960). No significant differences of median parasite density were observed across the three districts (p=0.05; Table 1). Additionally, gametocytes were detected in 35% (7/20) of children who were microscopy positive. Mean gametocyte density was 92.0 parasites/µl ± 55.8 across all three districts.

Parasite confirmation by SD Bioline Malaria Ag P.f./Pan RDT

Compared to microscopy results, the SD Bioline Malaria Ag P.f./Pan RDT detected 1.9 times the number of malaria cases. The sensitivity and specificity of SD Bioline Malaria Ag P.f./Pan RDT was 100.0% and 96.9%, respectively. PPV values and NPV values were 51.3% and 100.0%, respectively. All microscopy-positive individuals (n=20) were also positive by SD Bioline Malaria Ag P.f./Pan RDT (n=39). Of the individuals that were positive by SD Bioline Malaria Ag P.f./Pan RDT but were negative by microscopy, 7/19 were previously treated for malaria within the past month. 18 of the 19 children exhibited only one band for the *P. falciparum* HRP-2 antigen by SD Bioline Malaria Ag P.f./Pan RDT and 1 of the 19 children displayed a positive pLDH band.

Predictors of malaria infection

Factors associated with the risk of malaria infection in children less than five years of age was assessed by univariate analysis (Table 2). By univariate analysis, only hemoglobin level and consistent bednet use were associated with parasite prevalence (p<0.05). Multivariate analysis was not performed due to the low number of microscopy positive cases.

Epidemiology of G6PD Deficiency

In conjunction with evaluating malaria infection, study participants were screened for G6PD deficiency by (1) quantitative spectrophotometric assay, (2) qualitative RDT and (3) detection of the *G6PD A- 202A* mutation by PCR-RFLP analysis. Overall, G6PD enzymatic activity among ranged from 10.7% to 116.0% with a mean of 73.9% ± 16.7. No participant in our study exhibited severe G6PD deficiency (defined as <10% enzyme activity). 13.8% (87/631) of children were mildly deficient (defined as 10-60% enzyme activity) by spectrophotometric assay (Table 1) and no significant differences were found between districts (p=0.34). Gender, ethnicity, and malaria parasite density was not significantly associated with G6PD enzymatic activity or G6PD deficient RDT results (p>0.05).

Overall prevalence of G6PD deficiency by CareStart™ G6PD RDT was 8.6% (95% CI: 6.4-10.7), which was significantly lower than prevalence estimates by the gold standard spectrophotometric assay 13.8% (95% CI: 11.1-16.5) (p=0.003). The distribution of G6PD enzymatic activity of the study population by the CareStart™ G6PD RDT diagnosis is presented in Figure 3. Mean enzyme activity was significantly different between children characterized as G6PD deficient by CareStart™ G6PD RDT compared to participants who tested normal (37.18% ± 17.52, range: 10.66-96.34 vs. 77.35% ± 11.81, range: 15.63-116.00; p<0.001) (Figure 4). By RDT, more males were detected as

deficient than females, although this finding was not significant (59.3% vs. 40.7%; p=0.16). However, mean G6PD enzyme activity of G6PD deficient males by RDT was higher than RDT confirmed G6PD deficient females (38.6% ± 19.2 vs. 35.6% ± 14.9, respectively), but this finding was not statistically significant (p=0.46).

Among 630 individuals in our study, 23 (3.7%) females were heterozygous for the G6PD A- G202A mutation, 7 (1.1%) females were homozygous mutant, and 27 (4.3%) males were hemizygous (Table 1). The allele frequency was 5.9% among females, 8.6% among males and 6.8% overall, which is lower than previous reports of the allelic frequency of the G6PD 202A mutation in East Africa (12-19%) [22, 23, 33]. Presence of the G6PD G202A mutation was not associated with malaria parasitemia (p=0.87). Compared to the Nyankore ethnic group, ethnicity was significantly associated with the presence of the G202A mutation only when children were identified as belonging to an ethnicity other than Kiga or Ganda (OR=7.4; 95% CI: 2.5-21.6).

Correlation between G6PD A- genotype and phenotype

G6PD enzyme activity was stratified by both gender and genotype (Figure 5). In the female population, median enzyme activity was marginally lower (70%; range: 58.0-75.5) in homozygous mutant females compared to both wildtype and heterozygous females (72.0% (range: 12.4-112.0) and 76.5% (range: 10.6-116.0), respectively). Similarly, median enzyme activity was lower in hemizygous males as compared to wildtype males (74.0% (range: 12.9-96.5) vs. 76.4% (range: 11.2-112.4)). However, median enzyme activity did not fall below 60% enzyme activity in any of the groups associated with the G202A mutation (i.e. heterozygous females, homozygous mutant females, and hemizygous males) (Figure 5). Heterozygous females and hemizygous males exhibited a wide range of enzymatic activity (Figure 5). Additionally, we did not observe a bimodal distribution of enzyme activity typical of genetically deficient females or a right-skewed distribution typical of the hemizygous male population (Figure 6). Of particular note, 17/27 (63.0%) of hemizygous males and 6/7 (85.7%) of homozygous females exhibited over 60% enzymatic activity.

Sensitivity and specificity of the spectrophotometric assay was assessed in comparison the presence of the G202A mutation for all individuals, males and females (Tables 3-5). Sensitivity of the phenotypic test was higher in the male population compared in the female population (37.0% vs. 23.3%) (Tables 4-5). Overall, the phenotypic test exhibited low to moderately sensitivity, but was moderately high specificity (29.8% and 87.8%, respectively) (Table 3).

Performance of the CareStart™ G6PD RDT

Results of the CareStart™ G6PD RDT compared to the gold standard, quantitative analysis is shown in Table 6. The sensitivity and specificity of the CareStart™ G6PD RDT was 57.5% and 99.3%, respectively. PPV and NPV values were 92.6% and 93.6%, respectively. Of the 577 children that were classified as normal by the CareStart™ G6PD RDT, 37 children were falsely normal. 36 of these subjects had enzymatic activities that

ranged from 45.7-59.9%, while 1 subject exhibited a G6PD enzymatic activity of 15.6%. Of the 54 participants that were classified as deficient by CareStart™ G6PD RDT, 4 children were falsely deficient and had enzyme activities that ranged from 64.5-96.3%. Range of enzyme activity of the participants that were deficient by both CareStart™ G6PD RDT and quantitative analysis (<60% enzyme activity) was 10.7-59.5%.

The most currently widely used G6PD assay is the Beutler fluorescent spot test (FST) [14]. It is a rapid and inexpensive test that measures NADPH production by the G6PD enzyme, but it requires ultraviolet light, equipment, and poses challenges for a large-scale application. The FST has anecdotally set acceptable G6PD levels for primaquine administration as >30% enzyme activity, though there is no evidence to support these conclusions [26]. In order to determine whether the CareStart™ G6PD RDT yielded comparable sensitivity and specificity to the Beutler FST as a mass screening method, we adjusted our definition of 'G6PD deficiency' from <60% enzyme activity (as recommended by WHO) to 30%. Results of these values are presented in Table 7. Prevalence of G6PD deficiency, when adjusted to <30% enzymatic activity, dramatically decreased from 13.8% (87/631) to 3.0% (19/631). Sensitivity increased from 57.5% to 94.7%. Specificity of the CareStart™ G6PD RDT modestly decreased from 99.3% to 94.1%. PPV and NPV values changed to 33.3% and 99.8%, respectively.

Discussion

Epidemiology of malaria parasitemia in southwestern Uganda

Our study adds to the existing data on the continued decline in parasite prevalence in southwestern Uganda. Our findings suggest a 4-fold decrease in parasite prevalence in rural areas compared to a similar study conducted during the end of dry season in 2010 [4]. Parasite prevalence in urban areas has marginally decreased since 2010, but this finding was not significant. Increased bednet coverage could account for the decrease in rural areas. Since 2010, overall bednet ownership increased approximately 60% in rural areas and 20% in urban areas, meeting the target of the US President's Malaria Initiative (PMI) 2014 Plan for Uganda (above 85% coverage) [4, 34]. In our study population, the median time since bednet was purchased or provided was 8 months. As most conventional bednets LLINs will provide protection up to 3 to 5 years, bednet use may have had a strong protective effect [35]. However, our study is limited by its reported use and thus, actual presence and condition were not checked.

Of particular note is the changing dynamic of the plasmodium species population. In general, *P. malariae* is widespread throughout sub-Saharan Africa and in many instances, co-infections with *P. falciparum* are frequently found [36]. However, according to the WHO Malaria Report 2014, 100% of reported cases were infections from *P. falciparum*. Currently, diagnostic methods and experience in Uganda are generally focused on detecting *P. falciparum* infections, especially with the use of *P. falciparum*-specific RDTs. Thus, our data suggests the need for diagnostic capacity that allows for the detection of both *P. falciparum* and non-*P. falciparum* infections.

Importantly, infection with a non-falciparum species tend to be less symptomatic than *P. falciparum*, which may lead to a higher risk of undiagnosed cases. This becomes increasingly important to prolonging transmission time in untreated, asymptomatic individuals. Additionally, non-falciparum parasites transmit gametocytes more efficiently at low parasite densities compared to *P. falciparum [37]*. Thus, it may be that patients infected with *P. vivax*, *P. malariae*, or *P. ovale* infections will present without detectable gametocytemia and for longer periods of time, which may impact upon malaria transmission.

The current WHO recommended method of malaria diagnosis is by quality assured RDTs or light microscopy due to their good field performance, widespread availability, and relatively low cost [7]. Although the use of PCR and other nucleic acid amplification (NAA) assays are much more sensitive than both microscopy and RDT, widespread use in sub-Saharan Africa is often challenging due to the lack of resources and expertise, and time lag until diagnosis. In Uganda, the most widely used diagnostic tool is almost exclusively by RDT. However, sub-microscopic infections are frequently missed by microscopy and RDTs (<100 parasites/μl) [38, 39]. Several reviews of epidemiological surveys have found that at least half of all sub-microscopic malaria infections were missed by microscopy that were detected by PCR [8, 40]. As PCR provides the unique

ability to detect viable parasites following malarial infection at high sensitivity, further investigation of parasite prevalence by PCR is underway [41, 42].

A possible explanation of the false positive cases in our study population is the persistent antigen burden of HRP-2 within the host. During infection, HRP-2 is secreted from the parasite into the host bloodstream and has been widely found in the erythrocytes, serum, plasma, cerebrospinal fluid, and urine of the host. Notably, the HRP-2 antigen can persist in the blood after parasitemia has cleared or greatly reduced. Thus, even after successful treatment, these antigens may circulate in the bloodstream for two weeks, but may take as long as one month [43, 44]. Conversely, pLDH is found across all plasmodium species, including *P. falciparum*, but clears simultaneously with parasitemia after successful treatment [45]. In our study, we found that 19 children were microscopy negative, but positive by RDT. Seven of these children were treated for malaria within the past month and were positive for the P.f. HRP-2 antigen. Thus, in these children the HRP-2 antigen may be continuing to circulate in the blood, while remaining noninfectious.

A potential limitation of our study is that fieldwork proceeded from August to October 2014, during the end of dry season to the beginning of rainy season. As such, our estimates of parasite prevalence may be an underestimation of the peak malaria season.

Prevalence of G6PD deficiency in southwestern Uganda

Uganda is currently defined by the WHO as a country undergoing malaria control and malaria endemicity is highly variable by region. As shown in this survey, certain regions are experiencing dramatic reductions in prevalence, and thus alternate aggressive measures of control are being considered. One such strategy will be the introduction of primaquine. However, due to safety concerns in G6PD deficient individuals, the use of primaquine has not been widely adopted by policy makers. Data on the prevalence of G6PD deficiency and diagnostic tools used to characterize it are essential before the initiation of primaquine deployment.

To the best of our knowledge, this is the first study to establish the prevalence of G6PD deficiency in low malaria endemic southwestern Uganda using three different diagnostic methodologies. Our estimates of deficiency by spectrophotometric assay (<60% enzyme activity) are comparable to other published studies in East Africa (13.8% vs. 10-25%) [23, 33, 46]. By genotypic analysis, 9.0% of our study population carried the G6PD A-G202A mutation.

Similar to previous findings, we found high discordance between genotype and phenotype in our study [46]. However, while this lack of correlation may be typical for heterozygous females who exhibit variable expression of G6PD activity as per lyonization of the X chromosome, this cannot explain the wide distribution of enzyme activity in hemizygous males and high enzymatic activity of homozygous females we found in our study. This may be due to several factors. First, assays for the identification of G6PD deficiency may not have been performed accurately. Only three samples were regenotyped to check for concordance rate and further assessment is warranted. Also,

improper storage and handling of patient specimens may have had an affect on the quantitative assay, as samples were collected in variable weather conditions in the field. Second, exceeding variability in the biochemical and clinical penetrance of the *G6PD 202A/376* allele plays a significant role in an individual's G6PD deficiency status [23]. Third, as our study is limited to only the G202A mutation, it may be that other common G6PD A- SNPs can explain the variance in our data. Other G6PD A- alleles have been found to cause low enzyme activity in sub-Saharan Africa (e.g. G6PD A- 542A, G6PD A- 680T, G6PD A- 986C), yet their presence in East Africa has not been widely reported [22, 23].

Overall, we found the use of G6PD A- genotyping as a method of detecting deficiency may not entirely capture all individuals who are G6PD deficient in the population and conversely, enzymatic activity may miss carriers of the G202A mutation. This poses a stark challenge for the use of primaquine therapy as heterozygotes who exhibit normal enzyme activity can still present with hemolytic anemia when exposed to oxidative stressors [46, 47]. The low sensitivity of the spectrophotometric assay to detect G6PD A- genotypes may mean that carriers of the G202A mutation exhibiting over 60% enzymatic activity will be at risk for adverse effects when given primaquine therapy [46].

Testing for G6PD deficiency is not difficult, but they require expensive equipment, electricity, and reagents that are sensitive to light and heat [14]. As such, mass screening methods that are both sensitive and have a high detection threshold are urgently needed. In our study, we compared a newly commercially available RDT, CareStart™ G6PD RDT, to the gold standard, quantitative spectrophotometric assay. 37 out of 87 deficient individuals were falsely characterized as G6PD normal by RDT. Range of enzymatic activity of these individuals was 45.7%-59.9%, with the exception of one individual whose enzyme activity was 15.6%. These results require careful consideration when evaluating this RDT for widespread use. Moreover, further research is needed in identifying a G6PD enzymatic activity threshold for primaquine administration.

Albeit having a low sensitivity, the CareStart™ G6PD RDT was comparable to the FST. Use of the 30% enzymatic threshold is not research-driven, but people with severe deficiency are identified and can therefore be excluded from receiving primaquine. Individuals who are moderately deficient are classified as having normal G6PD activity. Our findings suggest that while the CareStart™ G6PD RDT cannot detect a deficiency threshold comparable to WHO's classification of deficiency, but it may be an ideal replacement for the FST, in regards to ease of widespread use and cost.

Conclusion

Here, we describe a comprehensive understanding of the epidemiological landscape of malaria and factors associated with its control in southwestern Uganda. While parasite prevalence has certainly reduced within the last decade, the prevalence of submicroscopic infections and increase in species diversity may impact upon our ability to accurately identify and treat malaria cases. The increase in *P. malariae* cases, accounting for nearly 50% of asymptomatic infections, has potential impacts on current diagnostic methods, transmission dynamics, and prompt treatment of malaria in southwestern Uganda. Mass drug administration of primaquine in combination with ACT, has the potential benefit of seriously reducing transmission potential in southwestern Uganda. Therefore, despite its proclivity to cause adverse effects in G6PD deficient individuals, efforts towards its use should not be quickly dismissed. Further studies investigating the optimal dosage of primaquine administration that is both safe and efficacious is warranted.

Future Direction

Over the last decade, significant progress toward malaria control has been noted in southwestern Uganda. As transmission intensity in this region continues to decline, additional approaches aimed at reducing transmission and to sustain current efforts should be investigated. Such interventions include detection of malaria transmission hot spots, targeted IRS, and notably, the addition of primaquine to ACTs as a transmission-blocking agent for *P. falciparum* infections.

Recently, the WHO has recommended a low dose of primaquine (0.25 mg/kg) for the treatment of falciparum malaria without prior G6PD deficiency testing and thus provides better coverage of the target population [12, 48, 49]. However, the use of suboptimal dosages may lead to drug resistance [48]. Furthermore, efficacy of primaquine is contingent upon its metabolization by human cytochrome P450*2D6 (CYP2D6). Recent studies have shown that low and intermediate metabolizer phenotypes were associated with primaquine failure [48, 50]. The prevalence of such phenotypes is unknown among many African populations. Thus, it will become increasingly important to identify the prevalence of CYP2D6 phenotypes in areas considering the widespread use of primaquine, as a significant proportion of the population may not respond to primaquine therapy [48].

Tables and Figures

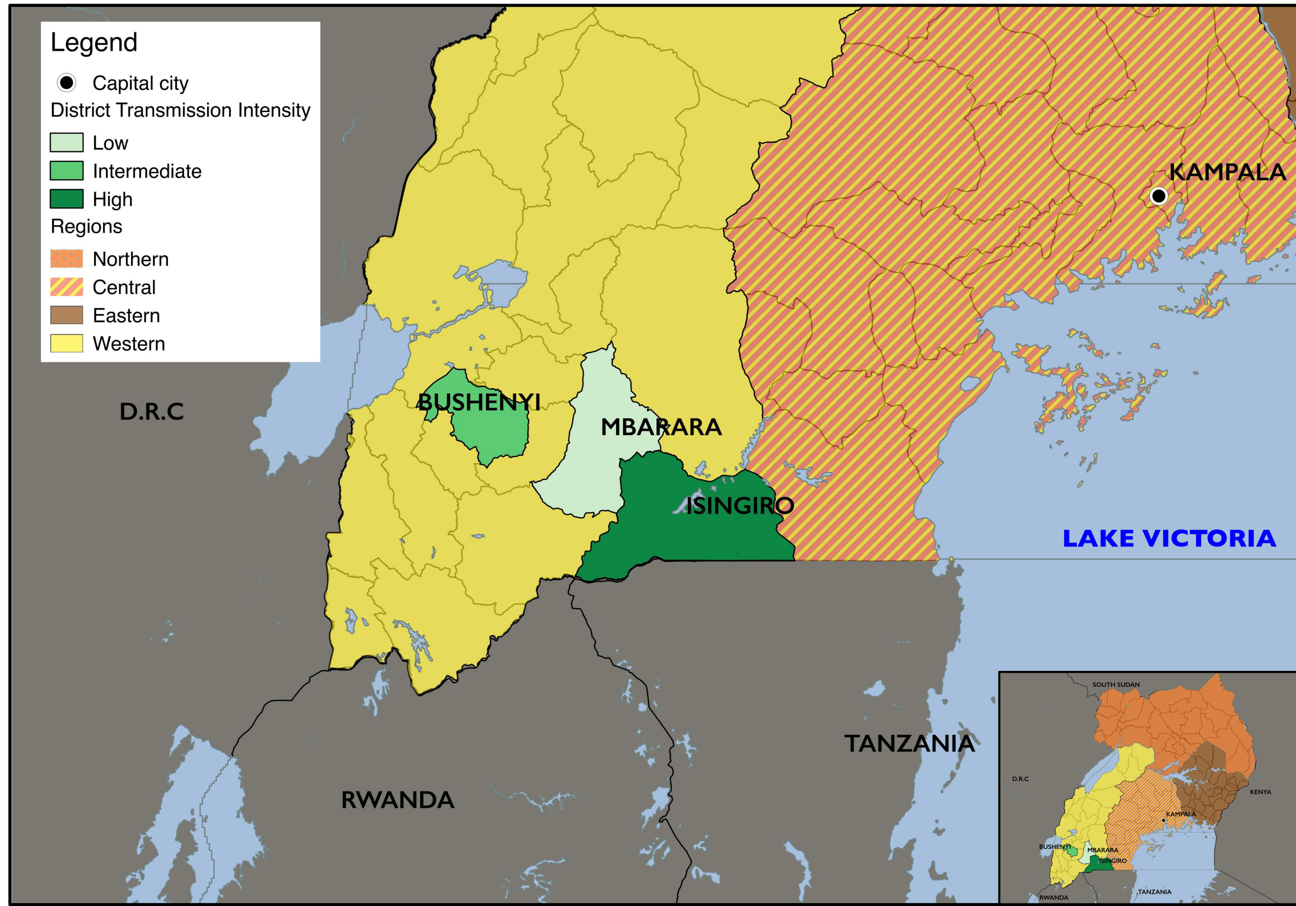


Figure 1. *Map of study site.*

Table 1. *Baseline characteristics of participants by district*[1]

Baseline characteristic	Mbarara (N=242)	Bushenyi (N=157)	Isingiro (N=232)	Total (N=631)	p-value[2]
Male	119 (49.2)	76 (48.4)	121 (52.2)	316 (50.1)	0.721
Mean Age (years), mean ± SD	2.4 ± 1.2	2.3 ± 1.2	2.4 ± 1.3	2.4 ± 1.3	0.787
Rural (%)	177 (73.1) ‡	134 (85.4) ‡	206 (88.8) ‡	517 (81.9)	<0.001 ***
Tribe/ethnic group					<0.001 ***
Nyankore	204 (84.3)	148 (94.3)	179 (77.2)	531 (84.2)	
Kiga	15 (6.2)	5 (3.2)	43 (18.5)	63 (10.0)	
Ganda	12 (5.0)	4 (2.6)	6 (2.6)	22 (3.5)	
Other	11 (4.6)	0 (0.0)	4 (1.7)	15 (2.4)	
Mean Hb count (g/dL)	11.9 ± 1.2	12.2 ± 1.2‡	11.8 ± 1.4‡	11.9 ± 1.3	0.013 *
Bednet use					<0.001 **
No	14 (5.8)	6 (3.8)	33 (14.3)	53 (8.4)	
Yes	228 (94.2)	151 (96.2)	198 (85.7)	577 (91.6)	
Indoor residual spraying (IRS)					0.124
No	238 (98.4)	146 (99.4)	230 (100.0)	624 (99.2)	
Yes	4 (1.7)	1 (0.6)	0 (0.00)	5 (0.8)	
Positive malaria status by microscopy	3 (1.2)	5 (3.2)	12 (5.2)	20 (3.2)	0.051
Median parasite density (parasites/µl)	2,080 (200-31,960)	460 (100-2,480)	2,270 (460-29,280)	1,480 (100-31,960)	0.051
P. falciparum	2,080 (200-31,960)	--	3,040 (500-29,280)	2,560 (200-31,960)	0.513
P. malariae	--	330 (100-2,480)	2,090 (720-3,820)	1,010 (100-3,820)	0.186
P. ovale	--	620 (620-620)	--	620 (620-620)	--
P. vivax	--	--	460 (460-460)	460 (460-460)	--
Mean gametocyte density (parasites/µl)	40.0[4]	--	100.7 ± 55.7	92.0 ± 55.8	0.360
Positive malaria status by RDT	3 (1.2)	8 (5.1)	28 (12.1)	39 (6.2)	<0.001 ***
Mean G6PD enzyme activity (%)	75.6 ± 14.8	72.8 ± 19.7	72.9 ± 16.3	73.9 ± 16.7	0.149
Class of G6PD deficiency[3]					0.342
Mild, 10-60% activity	25 (10.3)	26 (16.6)	36 (15.5)	87 (13.8)	

Normal, 60-100% activity	214 (88.4)	125 (79.6)	192 (82.8)	531 (84.2)	
Increased, 100%+	3 (1.2)	6 (3.8)	4 (1.7)	13 (2.1)	
Deficient G6PD status by RDT	16 (6.6)	19 (12.1)	19 (8.2)	54 (8.6)	0.155
G6PD 202A/376G genotype					0.713
Wildtype Female	110 (45.5)	75 (48.1)	100 (43.1)	285 (45.2)	
Heterozygous Female	9 (3.7)	5 (3.2)	9 (3.9)	23 (3.7)	
Homozygous Female	4 (1.7)	1 (0.6)	2 (0.9)	7 (1.1)	
Wildtype Male	106 (43.8)	67 (43.0)	115 (49.6)	288 (45.7)	
Hemizygous Male	13 (5.4)	8 (5.1)	6 (2.6)	27 (4.3)	

[1]Tables values are mean ± SD and median (range) for continuous variables and n (column %) for categorical variables.

[2]p-values represent probability that there is statistical difference between characteristic and district, unless otherwise stated.

[3]No cases of severe G6PD deficiency were found in our sample population.

[4]n=1 (no standard deviation)

[‡]Difference lies between these two districts

*Numbers may not sum to total due to missing data.

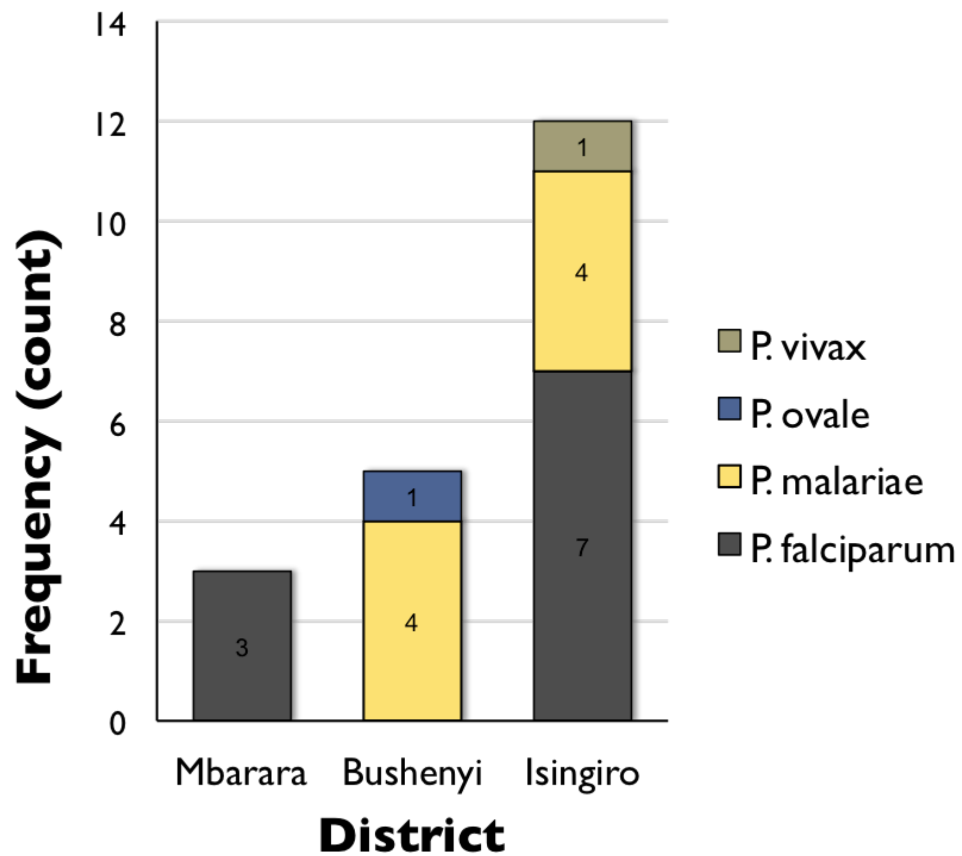


Figure 2. *Distribution of microscopy positive slides (n=20) by Plasmodium species in each district.* In Mbarara, 100% of all individuals with positive parasitemia were infected *by P. falciparum*. Microscopy positive individuals in Bushenyi were infected with *P. malariae* (80%) and *P. ovale* (20%). 58.3% of all microscopy positive individuals from Isingiro were infected with P. falciparum, 33.3% were infected with *P. malariae*, and 8.3% were infected with *P. vivax*.

Table 2. *Univariate analysis of factors associated with positive parasitemia (n=20)*[1]

Predictor variable	OR (95% CI)	p-value
Male	0.63 (0.25-1.58)	0.329
Age (years)	1.14 (0.81-1.62)	0.461
Rural	0.65 (0.21-2.05)	0.466
Hb count (g/dL)	0.76 (0.62-0.93)	0.007 **
Marital status of HoH[2]		
Married	1.00	--
Separated/divorced	2.58 (0.70-9.45)	0.154
Widowed	4.06 (0.47-35.13)	0.203
Never married	1.85 (0.23-15.00)	
Education level of HoH[2]		
None	1.00	--
Primary	0.52 (0.14-1.95)	0.336
Secondary/Tertiary	0.47 (0.11-2.11)	0.325
Type of roof		
Corrugated metal/other	1.00	--
Thatch leaf	0.29 (0.08-1.07)	0.064
Consistent bednet use		
No	1.00	--
Yes	0.31 (0.10-0.92)	0.034 *

[1]ORs adjusted for district selection
[2]HoH = Head of household

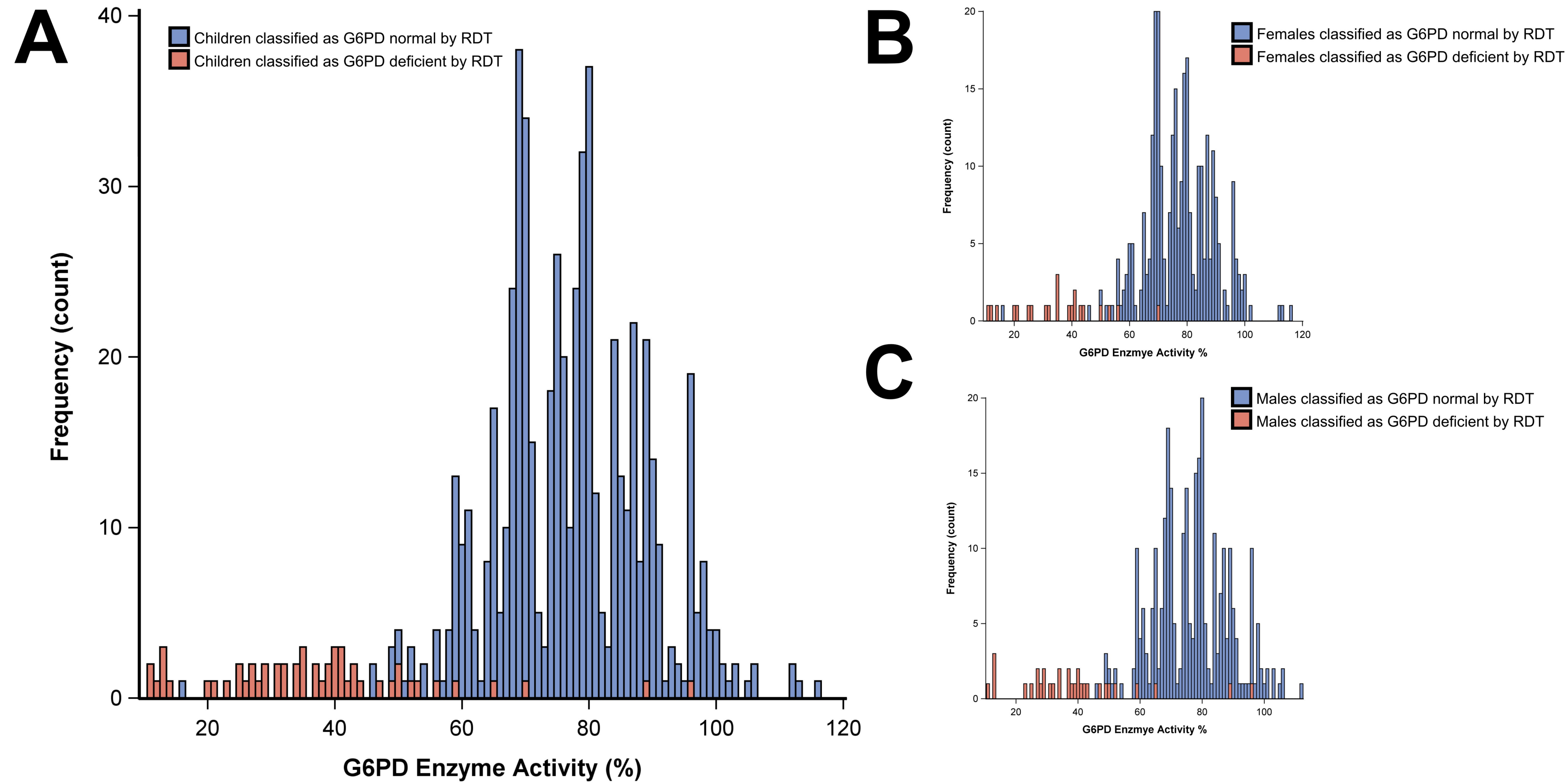


Figure 3. *G6PD enzymatic activity of all individuals, females, and males of study population.* Distribution of G6PD enzyme activity (expressed as a %) is displayed for total study participants (A), female (B), and male population (C). Individuals tested G6PD deficient by CareStart™ G6PD RDT are highlighted in red and individuals tested G6PD normal are highlighted in blue.

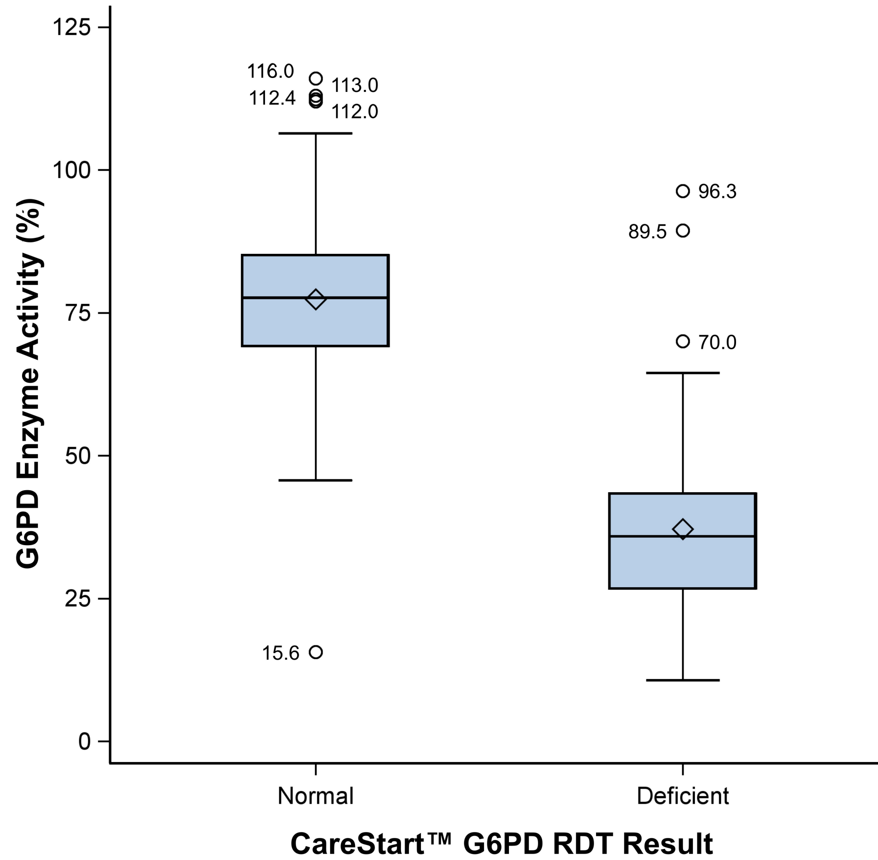


Figure 4. *G6PD Enzyme Activity (%) by CareStart™ G6PD RDT status.* Mean G6PD enzyme activity of individuals who tested G6PD normal was 77.3% ± 11.8. Range of activity was between 15.6% and 116.0%. Individuals who tested deficient by CareStart™ G6PD RDT had a mean enzyme activity of 37.2% ± 10.7 and activity ranged from 10.7% to 96.34%. Difference in mean enzyme activity between normal and deficient status was statistically significant ($p<0.001$).

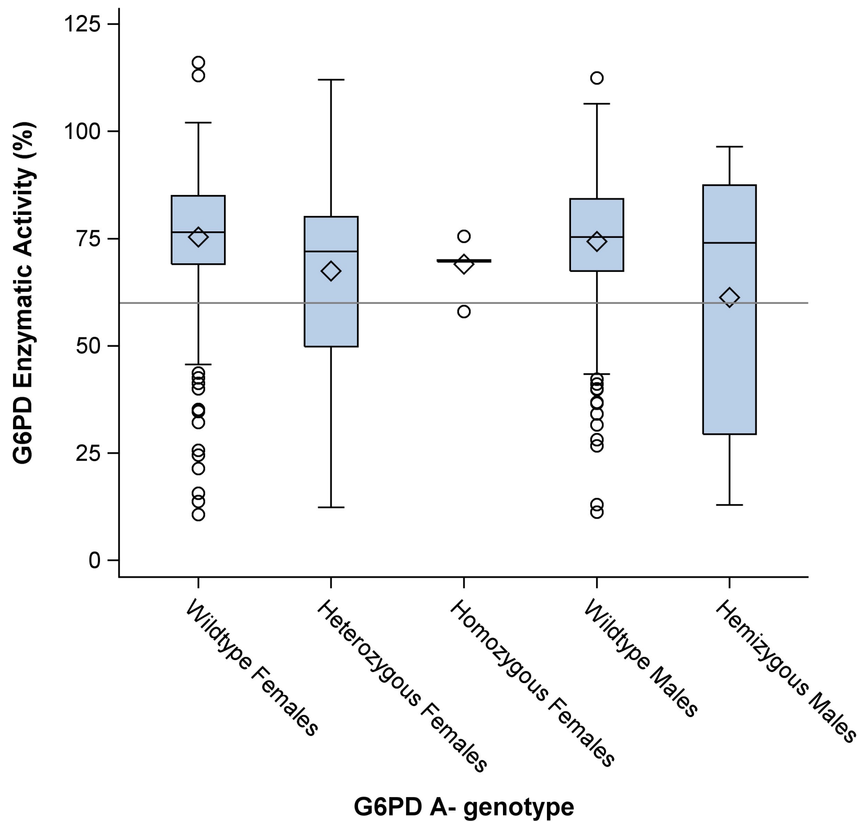


Figure 5. *G6PD Enzyme Activity (%) G6PD A- Genotype.* Median enzyme activity among wildtype females was 76.5% (Range: 10.6-116.0), 72.0% (Range: 12.4-112.0) in heterozygous females, and 70% (Range: 58.0-75.5) in homozygous mutant females. Median enzyme activity was 76.4% (Range: 11.2-112.4) in wildtype males and 74.0% (Range: 12.9-96.5) in hemizygous males. Gray bar indicates 60% enzyme activity cut-off.

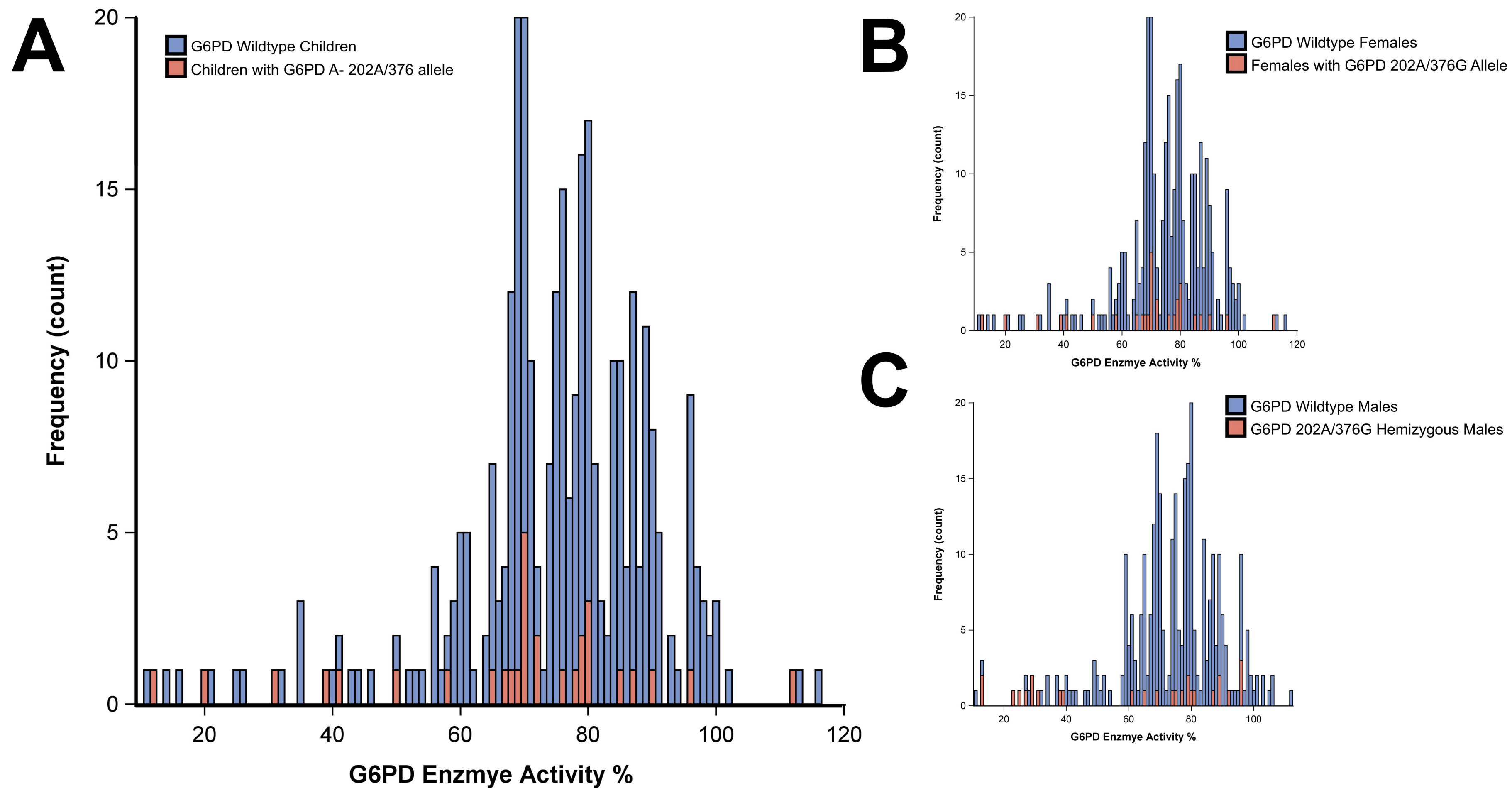


Figure 6. *G6PD enzymatic activity of all individuals, females, and males of study population by genotype.* Distribution of G6PD enzyme activity (expressed as a %) is displayed for total study participants (A), female (B), and male population (C). Heterozygous, homozygous, and hemizygous individuals are highlighted in red and individuals without the G6PD 202A mutation are highlighted in blue.

Table 3. Results of G6PD 202A/376 Allele and Trinity Biotech® quantitative spectrophotometric assay

		G6PD A- 202A mutant allele		**Total**
		At least 1 copy[2]	Wildtype	
Trinity Biotech spectrophotometer test	Deficient[1] (<60% enzyme activity)	17	70	87
	Normal	40	503	543
Total		57	573	630

[1] Defined by WHO classification of severe to mild deficiency (Class I-III)

[2] Defined as heterozygous, homozygous mutant, and hemizygous individuals

Sensitivity = 29.8%, specificity = 87.8%,PPV = 19.5%, NPV = 92.6%

Table 4. Results of G6PD 202A/376 Allele and Trinity Biotech® quantitative spectrophotometric assay in female population

		G6PD A- 202A mutant allele		**Total**
		At least 1 copy[2]	Wildtype	
Trinity Biotech spectrophotometer test	Deficient[1] (<60% enzyme activity)	7	31	38
	Normal	23	254	277
Total		30	285	315

[1] Defined by WHO classification of severe to mild deficiency (Class I-III)

[2] Defined as heterozygous and homozygous mutant females

Sensitivity = 23.3%, specificity = 89.1%, PPV = 18.4%, NPV = 91.7%

Table 5. Results of G6PD 202A/376 Allele and Trinity Biotech® quantitative spectrophotometric assay in male population

		G6PD A- 202A mutant allele		**Total**
		Hemizygous Male	Wildtype	
Trinity Biotech spectrophotometer test	Deficient[1] (<60% enzyme activity)	10	39	49
	Normal	17	249	266
Total		27	288	315

[1] Defined by WHO classification of severe to mild deficiency (Class I-III)

Sensitivity = 37.0%, specificity = 86.5%, PPV = 20.4%, NPV = 93.6%

Table 6. Results of CareStart™ G6PD RDT and Trinity Biotech® quantitative spectrophotometric assay

		Trinity Biotech® spectrophotometer test		**Total**
		Deficient[1] (<60% enzyme activity)	Normal	
CareStart™ G6PD RDT	Deficient[2]	50	4	54
	Normal	37	540	577
Total		87	544	631

[1] Defined by WHO classification of severe to mild deficiency (Class I-III)
[2] G6PD deficient = no background color in result window

Table 7. Results of CareStart™ G6PD RDT and Trinity Biotech® quantitative spectrophotometric assay

		Trinity Biotech spectrophotometer test		**Total**
		Deficient[1] (<30% enzyme activity)	Normal	
CareStart™ G6PD RDT[2]	Deficient[2]	18	36	54
	Normal	1	576	577
Total		19	612	631

[1] Defined by Beutler fluorescent spot test detection threshold
[2] G6PD deficient = no background color in result window

Appendix

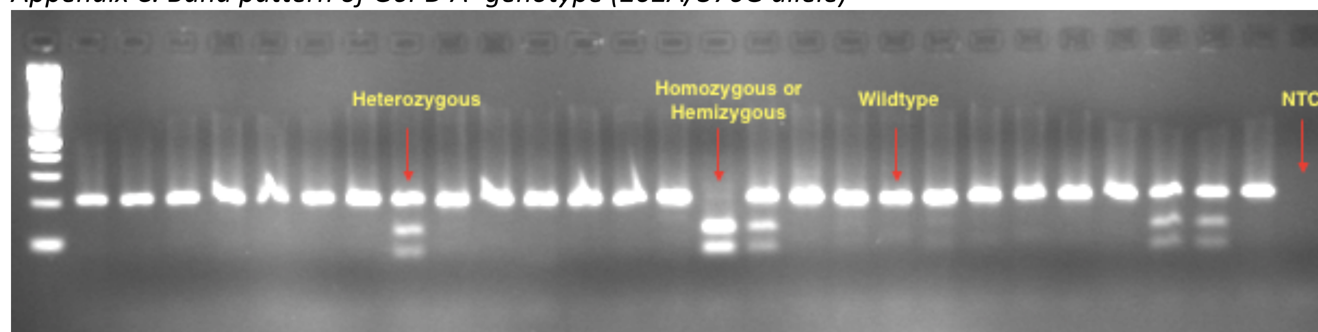
Appendix A. WHO Classification of G6PD Deficiency

Class	Level of Deficiency	Enzyme Activity
I	Severe	<10% activity; with chronic, nonspherocytic hemolytic anemia
II	Severe	<10% activity; with intermittent hemolysis
III	Mild	10-60% activity; hemolysis with stressors only
IV	Non-deficient	60-100% activity; no clinical sequelae
V	Increased enzyme activity	>100% activity, no clinical sequelae

Appendix B. Number of households required in each district

Study District	Sample Size	Cluster	# of Clusters	# of HH per Village
Mbarara	254	Rural	15	13
		Urban	5	
Bushenyi	143	Rural	17	8
		Urban	3	
Isingiro	239	Rural	18	12
		Urban	2	
				Final Sample Size
Total	636		60	**660**

Appendix C. Band pattern of G6PD A- genotype (202A/376G allele)



NTC = No template control

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