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The integrity and stability of specimens under different storage conditions for glucose-6-phosphate dehydrogenase deficiency screening using WST-8

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ABSTRACT

Accurate measurement of glucose-6-phosphate dehydrogenase (G6PD) activity is critical for malaria treatment as misclassification of G6PD deficiency could cause serious harm to patients. G6PD activity should be assessed in blood samples on the day of collection. Otherwise, specimens should be stored under suitable conditions to prevent loss of G6PD activity. Here, we assessed stability and integrity of G6PD testing in samples from normal controls, heterozygous females, and G6PD deficient individuals using water-soluble tetrazolium salts (WST-8) assay. Specimens were stored as ethylenediaminetetraacetic acid (EDTA) whole blood and dried blood spots (DBS) at various temperatures (37 °C, room temperature, 4 °C and -20 °C) and under different humidity conditions (with and without desiccant). G6PD normal samples were stable for up to 1 year when stored at -20 °C under controlled conditions, with 85% and 91% G6PD activity in EDTA whole blood and DBS in the presence of desiccant, respectively. Specimens from heterozygous females showed greater G6PD activity when stored as DBS, with 85% enzyme activity after 1 year of storage at -20 °C under controlled conditions in the presence of desiccant. G6PD deficient samples rapidly lost enzyme activity in all storage conditions tested. However, the reduction in G6PD enzyme activity in G6PD deficient samples did not interfere with G6PD classification. Samples stored under suitable conditions for G6PD testing will allow accurate measurement of enzyme activity, prevent misclassification of G6PD deficiency and enable safe and effective use of antimalarial drugs such as primaquine and tafenoquine.

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked genetic defect and the most common enzymopathy, affecting more than 400 million people worldwide (Luzzatto et al., 2020). G6PD deficiency is highly prevalent in tropical and sub-tropical regions where malaria is endemic, including Africa and Asia (Nkhoma et al., 2009, Ganczakowski et al., 1995, Jalloh et al., 2004, Nuchprayoon et al., 2002, Phompradit et al., 2011). It has been suggested that G6PD deficiency arises through natural selection and confers protection against malaria (Ruwende and Hill, 1998, Louicharoen et al., 2009). However, antimalarial drugs, such as 8-aminoquinolines (primaquine and tafenoquine) that play critical roles in malaria elimination and control, can cause drug-induced hemolysis in individuals with G6PD deficiency (Rueangweerayut et al., 2017, Taylor et al., 2019, Chu et al., 2017, Beutler, 2008). To ensure safe and effective treatment, testing for G6PD deficiency before administration of 8-aminoquinolines is recommended (Domingo et al., 2013, W.H. Organization 2016). However, using point of care devices for G6PD testing may not be sufficient for medical deciding on malaria treatment, especially in heterozygous females. This is because heterozygous females showed variation in the pattern of X-chromosome inactivation and they are usually reported as G6PD normal by current point of care tests. Clinically significant hemolysis was reported in G6PD heterozygous females receiving high doses of primaquine (1 mg base/kg/day for 7 days) who are reported as G6PD normal by phenotypic screening tests (Chu et al., 2017). Quantitative

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G6PD testing is, therefore, critical for malaria therapy. According to World Health Organization (WHO), samples with less than 30% enzyme activity are classified as G6PD deficiency. Samples showing G6PD activity in the range of 30 to 80% are classified as intermediate deficiency and the samples with greater than 80% enzyme activity are regarded as G6PD normal (W.H. Organization 2016). Nonetheless, G6PD activity of 70% was used as a threshold for tafenoquine prescription (Lacerda et al., 2019, Llanos-Cuentas et al., 2019). The standard method for G6PD activity measurement is spectrophotometry in which the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) is monitored at 340 nm. Recently, a colorimetric, sensitive, and rapid test for G6PD deficiency was developed using water-soluble tetrazolium salts (WST-8) (Tantular and Kawamoto, 2003). This test has been applied, in a 96-well plate platform, to screen G6PD deficiency and was found to have a comparable performance to that of the current quantitative tests (De Niz et al., 2013, Kuwahata et al., 2010).

Testing of G6PD deficiency immediately after blood collection is desirable as it will provide accurate enzyme activity information and allow for correct classification. However, when on-site diagnosis is not possible, or a substantial number of samples are screened, completion of G6PD activity measurement on the day of blood collection might be impractical. In such cases, blood samples must be stored or transferred to the laboratory. To ensure accurate interpretation of G6PD activity readings and to prevent misclassification of G6PD deficiency, it is essential that blood samples are properly stored. Previous reports have shown that storage time, temperature and, humidity affect G6PD activity (Castro et al., 2005, Kahn et al., 2015, Jalil et al., 2016, Flores et al., 2017). However, the results across individual studies vary widely and there is no report that compares the stability of specimens stored as ethylenediaminetetraacetic acid (EDTA) whole blood and dried blood spots (DBS). Moreover, the stability of blood samples from heterozygous females has not been reported before.

This study was designed to assess the integrity and stability of blood samples (EDTA whole blood and DBS) stored under different temperature and humidity conditions. Blood samples were stored at 37 °C and room temperature (25–29 °C), simulating the conditions in tropical regions where G6PD deficiency and malaria are widespread. Stability of blood samples stored at 4 °C and −20 °C, which are general conditions for preserving biospecimens, was also assessed. In addition to providing the guideline to handle blood samples in a context of clinical diagnosis, the stability of blood samples stored under both short-term (4 months) and long-term (1 year) conditions was examined for research purposes. Furthermore, to investigate whether repeated freeze-thaw cycles influence G6PD stability, samples were also stored under controlled and cool-warm/freeze-thaw cycled conditions and enzyme activity was routinely measured using the WST-8 assay at various time intervals over 16 (37 °C and room temperature) or 52 weeks (4 °C and −20 °C). G6PD activity was measured in samples with G6PD normal, and in those from heterozygous females and G6PD deficient samples.

2. Materials and methods

2.1. Materials

Oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) and cell counting kit-8 containing WST-8/1-methoxy-5-methyl-phenazinium methyl sulfate (1-mPMS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were obtained from Merck (Darmstadt, Germany).

2.2. Study design

Blood samples from healthy volunteers were collected in EDTA tubes at Ramathibodi Hospital, Mahidol University. All samples were spectrophotometrically tested for G6PD activity and genotyped by DNA sequencing. A total of 63 blood samples, including G6PD normal controls (30 samples: 13 males and 17 females), heterozygous females (15 samples), and deficient individuals (18 samples: 14 hemizygous males and 4 homozygous females), were subjected to stability test under different storage conditions. G6PD activity and hemoglobin concentration were measured on the day of blood collection (Day 0) and the samples were then divided for storage as whole blood or DBS (Fig. 1). For whole blood storage, samples were aliquot into tubes and stored under four different conditions (controlled: 4 °C and −20 °C, cool-warm cycled: 4 °C and freeze-thaw cycled: −20 °C). In controlled groups, blood samples were stored in individual tubes and were only removed on the day of the G6PD activity assay. For cool-warm/freeze-thaw cycled groups, the samples were stored in a single tube. On the day of assay, specimens were removed from storage, placed at room temperature, G6PD activity was determined and the single tube returned to storage at 4 °C or −20 °C. The specimens were subjected to cool-warm/freeze-thaw cycles until the end of the study.

For DBS, blood samples were spotted onto Whatman™ 1 filter paper (GE Healthcare, UK) and allowed to dry overnight at ambient temperature. Samples were punched out as 3 mm discs and stored in zip-lock bags. Zip-lock bags were placed in a plastic container in the presence or absence of silica desiccant beads. DBS samples were stored under different conditions (controlled: 37 °C, room temperature (25–29 °C), 4 °C and −20 °C and cool-warm/freeze-thaw cycled: 37 °C, room temperature, 4 °C and −20 °C). For controlled groups, blood spots were stored in individual zip-lock bags and removed from storage on the day of the G6PD activity assay. For cool-warm/freeze-thaw cycled samples, specimens were stored in a single zip-lock bag. On the day of the G6PD activity assay, samples were removed from storage, placed at room temperature, G6PD activity determined and remaining samples were returned to 37 °C, room temperature, 4 °C or −20 °C. The specimens were subjected to cool-warm/freeze-thaw cycle until the end of the study.

2.3. Hemoglobin estimation

Measurement of hemoglobin was done using Drabkin's reagent (Sigma Aldrich, Darmstadt, Germany), following manufacturer's protocol. The absorbance of cyano-derivative was determined at 540 nm in 96-well plate using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland). Total hemoglobin concentration was calculated from the calibration curve. Experiments were performed in triplicate.

2.4. G6PD activity assays

G6PD activity assays using WST-8 /1-mPMS were performed in triplicate, in 96-well plates at different time intervals. Formazan formation was monitored at 450 nm using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland). For whole blood storage (4 °C and −20 °C), G6PD activity was measured weekly to 4 months then every 4-week to 1 year. For DBS stored at 37 °C and room temperature, G6PD activity was measured weekly for 4 months. For DBS stored at 4 °C and −20 °C, G6PD activity was measured weekly to 4 months then every 4-week to 1 year. The reaction mixtures contained 20 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 500 μM glucose-6-phosphate, 100 μM $NADP^+$ and WST-8/1-mPMS. For whole blood samples, 2 μL was added into 96-well plates, mixed with 100 μL of reaction mixture, and absorbance was monitored for 2 h at 450 nm. For DBS, blood spots were placed in 96-well plates, mixed with 100 μL of reaction mixture, incubated at room temperature for 2 h and absorbance was monitored at 450 nm.

Enzyme activity assessed on Day 0 was reported as units (U) per gram of hemoglobin (gHb) while enzyme activity of stored samples was expressed as a percentage of the activity measured on Day 0 (% residual activity).

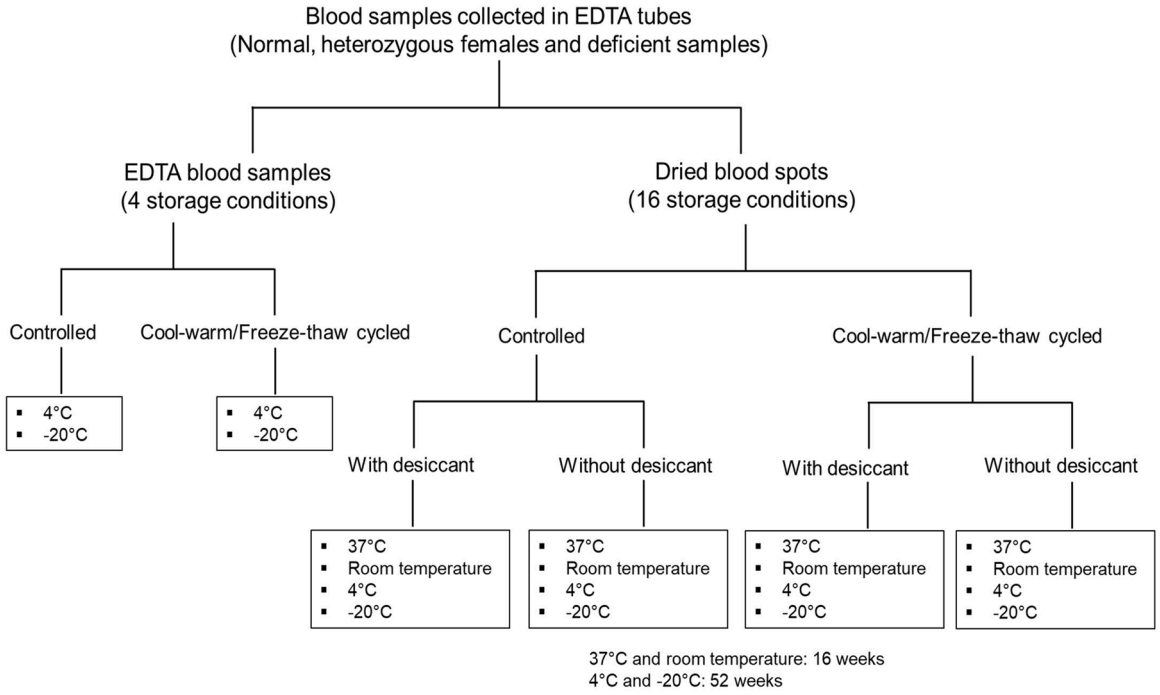

Fig. 1. Study design.

2.5. Ethics

Ethical approval for the study was provided by Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand, No. MURA2018/252 and the Human Ethics Committee, Faculty of Tropical Medicine, Mahidol University, under the study number MUTM 2019–016–01.

3. Results

3.1. G6PD activity of blood samples

G6PD enzyme activity measured on the day of blood collection (Day 0) is shown in Fig. 2. The mean G6PD enzyme activity of samples from people with normal G6PD activity, heterozygous females and deficient individuals was 13.1 ± 1.1, 12.8 ± 1.4 and 2.6 ± 1.4 U/gHb, respectively.

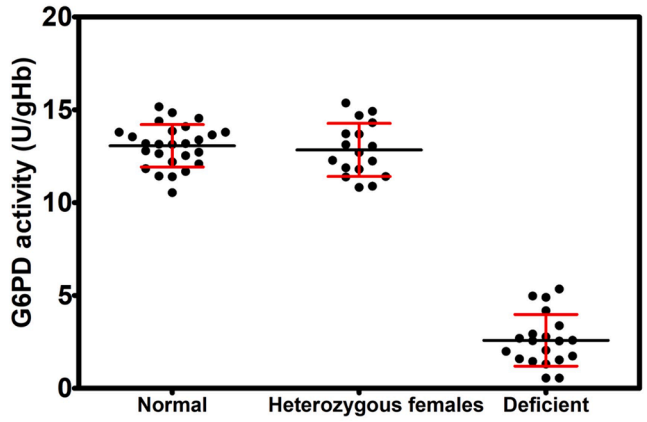

Fig. 2. G6PD enzyme activity of specimens measured on the day of collection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Stability of EDTA blood samples

3.2.1. G6PD normal

Under controlled conditions, specimens stored at 4 °C for 1 week lost approximately 16% of G6PD activity (Fig. 3A and Supplementary file 1). Residual G6PD activity was between 60 and 82% during 6 months of storage. After that, G6PD activity gradually decreased and only 15% G6PD activity was retained after 1 year of storage. When stored at −20 °C, G6PD activity was effectively retained for 8 months during which the residual G6PD activity fluctuated between 96 and 105%. A small decrease in enzyme activity was observed at month 9 and 85% G6PD activity was maintained after 1 year of storage. For samples stored under cool-warm cycled condition, residual G6PD enzyme activity decreased to 87% after storage at 4 °C for 1 week. A decline in G6PD activity was observed after 1 month of storage, with residual enzyme activity measuring 45%. At the end of the study (1 year of storage), 10% G6PD activity was retained. Normal G6PD samples stored under freeze-thaw cycled condition at −20 °C maintained enzyme activity over 90% for 1 month. After 2 months of storage, a significant decrease in G6PD activity was observed and enzyme activity fluctuated between 30% and 52%. During the last 4 months of storage, a further decrease in G6PD activity was observed and residual enzyme activity ranged between 16% and 19%.

3.2.2. Heterozygous females

Under controlled conditions, samples stored at 4 °C lost 20% of G6PD enzyme activity by the end of the first week (Fig. 3B and Supplementary file 2). G6PD activity fluctuated between 59% and 71% during 2 months of storage. Thereafter, G6PD activity continuously decreased and 9% residual enzyme activity was measured at the end of 1-year storage. When stored at −20 °C, residual G6PD activity was over 80% for 6 months. Afterward, G6PD enzyme activity gradually decreased and 36% activity was maintained after 1 year of storage. For samples subjected to cool-warm cycles, storage at 4 °C resulted in a steady decline in G6PD enzyme activity and only 5% enzyme activity was retained at the end of the study. Samples stored at −20 °C under freeze-thaw condition maintained 78% G6PD activity after 1 month of storage. After that, G6PD enzyme activity was moderately decreased and G6PD activity was

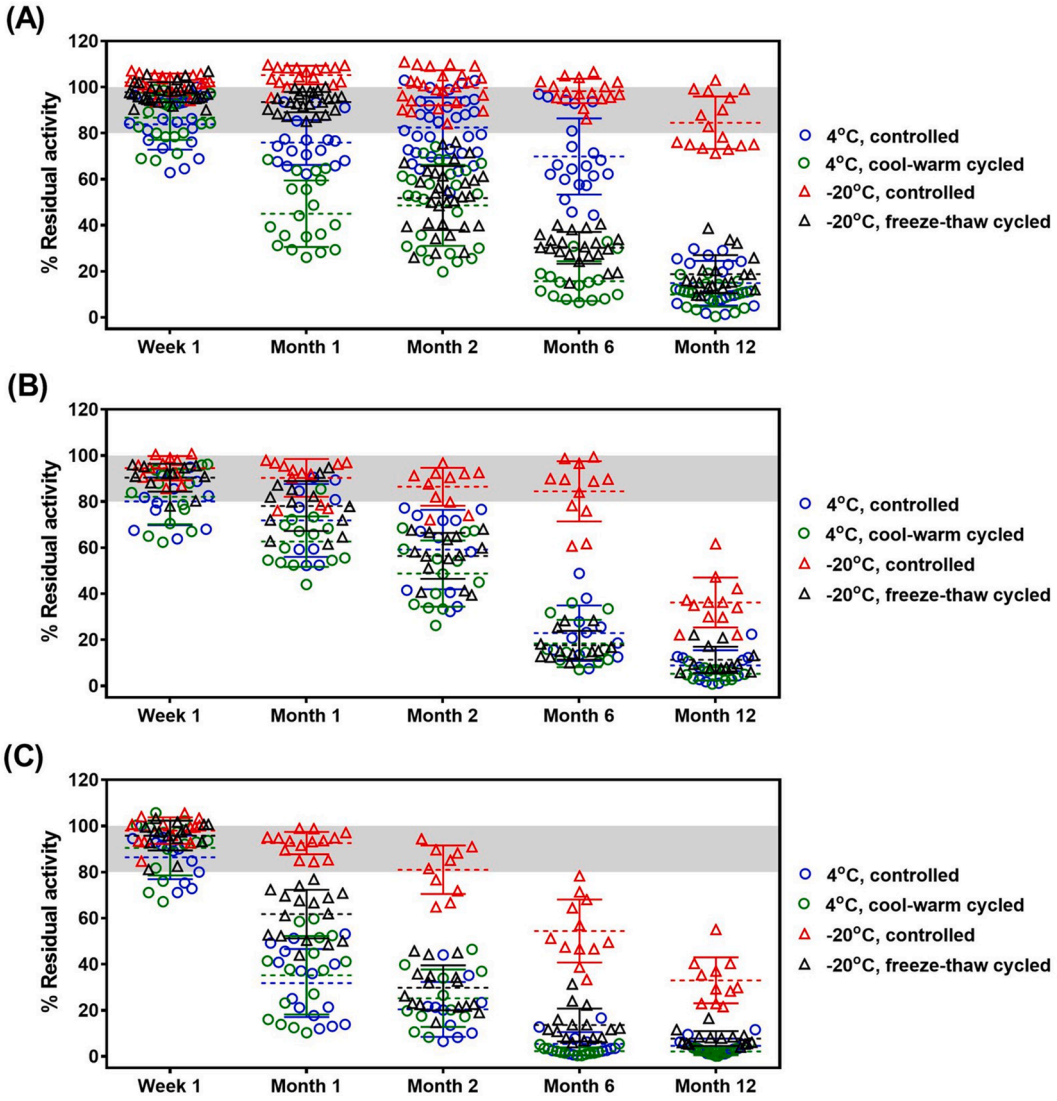

Fig. 3. Stability analysis of (A) G6PD normal, (B) heterozygous females, and (C) deficient samples stored as whole blood in EDTA tubes for 52 weeks. Samples were stored under controlled conditions at 4 °C and −20 °C, cool-warm cycled condition at 4 °C, and freeze-thaw cycled condition at −20 °C. Gray horizontal bar indicates normal G6PD activity (80–100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluctuated between 11% and 15% during the last 6 months of storage.

3.2.3. G6PD deficient

Specimens stored under controlled condition at 4 °C maintained 86% G6PD activity after 1 week of storage (Fig. 3C and Supplementary file 3). However, G6PD enzyme activity declined to 44% in the second week of storage and was only 5% after 1 year of storage. Blood samples stored at −20 °C retained over 80% G6PD activity after 2 months of storage. Residual G6PD activity slowly decreased over time and was 33% at the end of the study. For cool-warm samples, storage at 4 °C caused an approximate 9% reduction in enzyme activity by the end of week 1. By month 1, G6PD activity had dramatically decreased to 35% and continuously declined during storage. By the end of the study, only 2% residual enzyme activity was retained. For samples stored at −20 °C under freeze-thaw condition, G6PD activity slightly decreased to 95% during the first 2 weeks of storage. G6PD enzyme activity steadily declined over time and only 15% G6PD activity was retained by the end of 4 months of storage, following which residual G6PD activity was between 8% and 14%.

3.3. Stability of DBS samples

3.3.1. G6PD normal

Under controlled conditions, normal G6PD samples stored at 37 °C in the presence of desiccant dramatically lost G6PD activity (Fig. 4A and Supplementary file 4). After 1 week of storage, 87% G6PD activity was retained and only 10% G6PD activity remained by the end of the study. Similar results were observed for samples stored under the same conditions in the absence of desiccant (Fig. 4B). Though residual G6PD activity in DBS samples stored in the presence of desiccant was higher than that measured in samples stored without desiccant, the presence of desiccant did not significantly improve G6PD stability. Storage at 25 °C was more effective at maintaining G6PD activity than was storage at 37 °C (Fig. 4A). G6PD activity in samples stored at 25 °C in the presence of desiccant gradually declined and the residual G6PD activity was 31% by the end of month 4. The effect of desiccant was not significant (Fig. 4B). DBS stored at 4 °C in the presence of desiccant maintained over 80% G6PD activity after 8 months of storage, after which G6PD enzyme activity fluctuated between 74% and 80% until the end of 1 year of storage (Fig. 4A). A similar trend in enzyme activity reduction was seen when samples were stored at 4 °C in the absence of desiccant

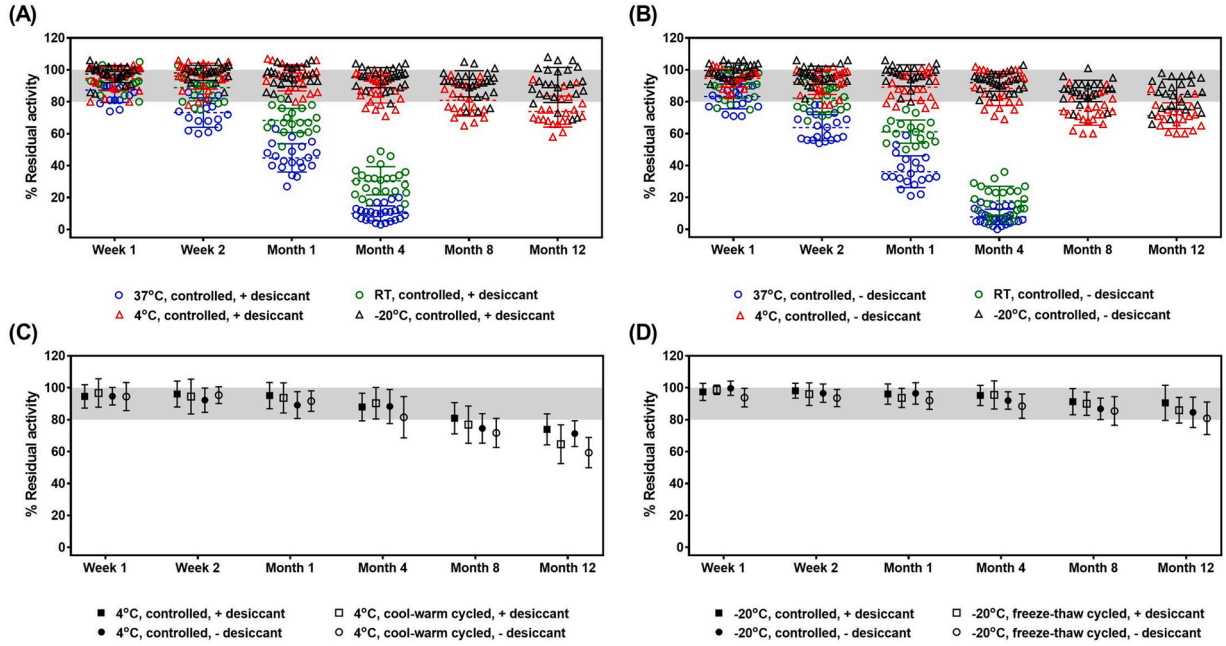

Fig. 4. Stability analysis of blood samples from normal controls stored as DBS. Samples were stored under controlled conditions at various temperatures in the presence (A) and absence (B) of desiccant. Storage under (C) cool-warm cycled condition at 4 °C and (D) freeze-thaw cycled conditions at −20 °C in the presence and absence of desiccant. Gray horizontal bar indicates normal G6PD activity (80–100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4C). However, slightly lower G6PD activity was observed in samples stored in the absence of desiccant when compared with those stored in the presence of desiccant over the same time. DBS samples stored at −20 °C in the presence of desiccant maintained over 90% G6PD activity after 1 year of storage (Figs. 4A and 4D). DBS samples stored at −20 °C in the absence of desiccant maintained approximately 85% of G6PD activity at the end of the study.

A greater reduction in G6PD activity was observed in DBS samples subjected to cool-warm/freeze-thaw cycles than in those stored under controlled conditions (Figs. 4C and 4D). Considering storages at 4 °C and −20 °C, by the end of the first week, at least 95% G6PD activity was retained and the presence of desiccant improved G6PD stability. DBS stored under cool-warm condition at 4 °C in the presence of desiccant maintained over 80% G6PD activity for 7 months which is longer than those stored in the absence of desiccant (4 months, Fig. 4C and Supplementary file 4). After 1 year of storage, samples stored at 4 °C had

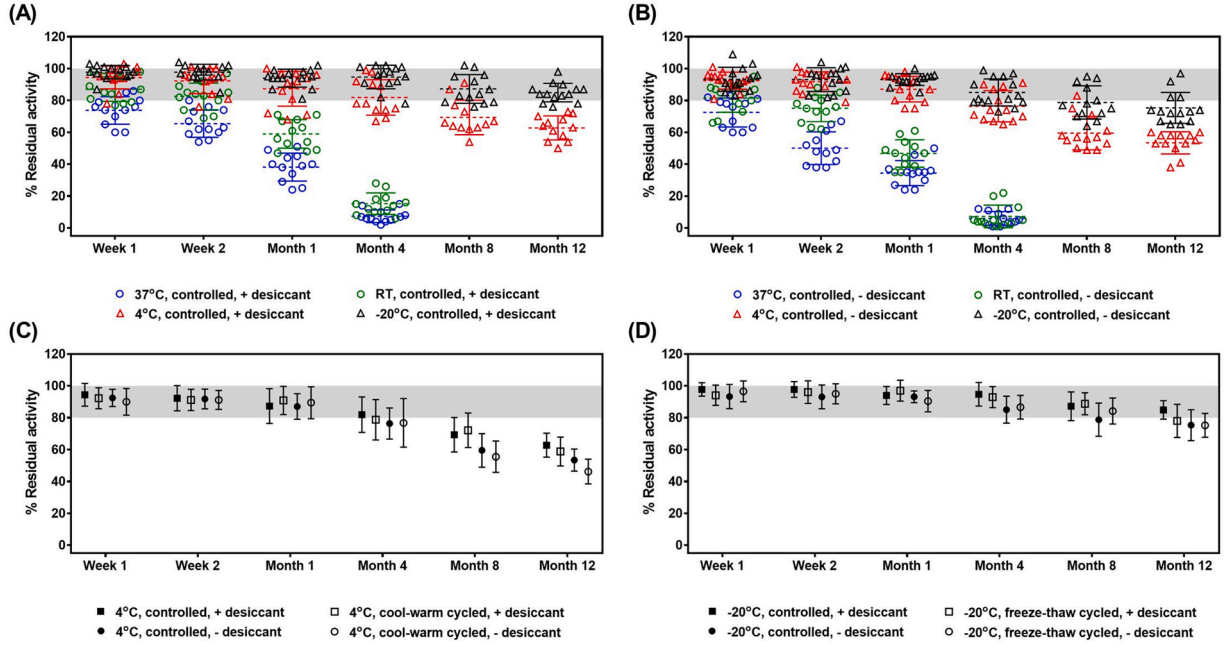

Fig. 5. Stability analysis of blood samples from heterozygous females stored as DBS. Storage under controlled conditions at various temperatures in the presence (A) and absence (B) of desiccant. Storage under (C) cool-warm cycled condition at 4 °C and (D) freeze-thaw cycled conditions at −20 °C in the presence and absence of desiccant. Gray horizontal bar indicates normal G6PD activity (80–100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

65% and 59% residual G6PD activity in the presence and absence of desiccant, respectively. At the end of the study, samples stored at −20 °C retained over 80% of G6PD enzyme activity in the presence and absence of desiccant (Fig. 4D).

3.3.2. *Heterozygous females*

DBS samples from heterozygous females were less stable than normal G6PD samples. Under controlled conditions, samples lost 50% G6PD activity after 2 weeks of storage at 37 °C and retained only 6%–7% G6PD activity after 16 weeks of storage (Figs. 5A and 5B and Supplementary file 5). Samples stored at room temperature retained greater G6PD activity than did those stored at 37 °C. In the presence of desiccant, 15% G6PD activity was retained after 16 weeks of storage at room temperature (Fig. 5A). In the absence of desiccant, samples lost more than 90% of G6PD activity by the end of the study (Fig. 5B). G6PD activity gradually declined in samples stored at 4 °C and residual G6PD activity was 63% and 53% at the end of the study in the presence and absence of desiccant, respectively (Figs. 5A and 5B). A greater G6PD activity was retained in samples stored at −20 °C. After 1 year, 85% and 75% enzyme activity was retained in the presence and absence of desiccant, respectively (Figs. 5A and 5B).

Samples stored under cool-warm/freeze-thaw cycled conditions retained less G6PD activity than did those stored under controlled conditions. Storage at 4 °C resulted in a steady decline in G6PD activity and 59% and 46% of enzyme activity was retained after 1 year of storage in the presence and absence of desiccant, respectively (Fig. 5C). Samples were more stable when stored at −20 °C for 1 year and, under these conditions, 78% and 75% G6PD activity was retained in the presence and absence of desiccant, respectively (Fig. 5D). Importantly, the presence of desiccant improved G6PD stability of heterozygous samples, comparing to those stored in the absence of desiccant (Supplementary file 5).

3.3.3. *G6PD deficient*

G6PD deficient samples were the least stable of all three sample types tested. The influence of storage time, temperature, and humidity was remarkable. Under controlled conditions, storage at 37 °C and room temperature resulted in a large decrease in G6PD activity of approximately 85% and 80% after 16 weeks of storage at 37 °C and room temperature, respectively (Figs. 6A and 6B and Supplementary file 6). Decreased storage temperature improved G6PD stability and residual enzyme activities of 43% and 51% were observed in samples stored at 4 °C and −20 °C for 1 year, respectively (Figs. 6A and 6B).

For samples stored under cool-warm/freeze-thaw cycled conditions, G6PD activity decreased in similar manner to that observed for specimens stored under controlled conditions. After 1 year of storage in the presence of desiccant, 39% and 54% G6PD activity was retained in samples stored at 4 °C and −20 °C, respectively (Figs. 6C and 6D). At all storage conditions, only a slight increase in G6PD activity was observed when G6PD deficient specimens were stored in the presence of desiccant.

4. Discussion

G6PD deficient individuals are susceptible to drug-induced hemolytic anemia caused by antimalarial drugs such as 8-aminoquinolines, which is a concern for malaria elimination and control (Rueangweerayut et al., 2017, Taylor et al., 2019, Chu et al., 2017, Chu et al., 2018). The WHO recommends that diagnosis of G6PD deficiency should be performed before administration of 8-aminoquinolines (W.H. Organization 2016). G6PD deficiency is common in regions with high temperatures and humidity and these two factors have been reported to have adverse effect on G6PD enzyme activity during storage (Nkhoma et al., 2009, De Niz et al., 2013, Castro et al., 2005, Flores et al., 2017). Generally, in the laboratory, blood samples are collected in EDTA tubes, stored at 4 °C, and G6PD activity is assessed from whole blood samples. However, in the field, it is easier and more convenient to collect and store blood on filter paper. Shipments of blood samples can be made with or without cold storage (ambient temperature vs. 4 °C or −20 °C). Storage conditions influence the integrity and stability of blood samples where specimen mishandling could have a significant effect on G6PD activity. Specimens for G6PD testing should be stored under proper conditions to maintain G6PD stability, enable accurate measurement of enzyme activity and prevent misclassification of G6PD deficiency.

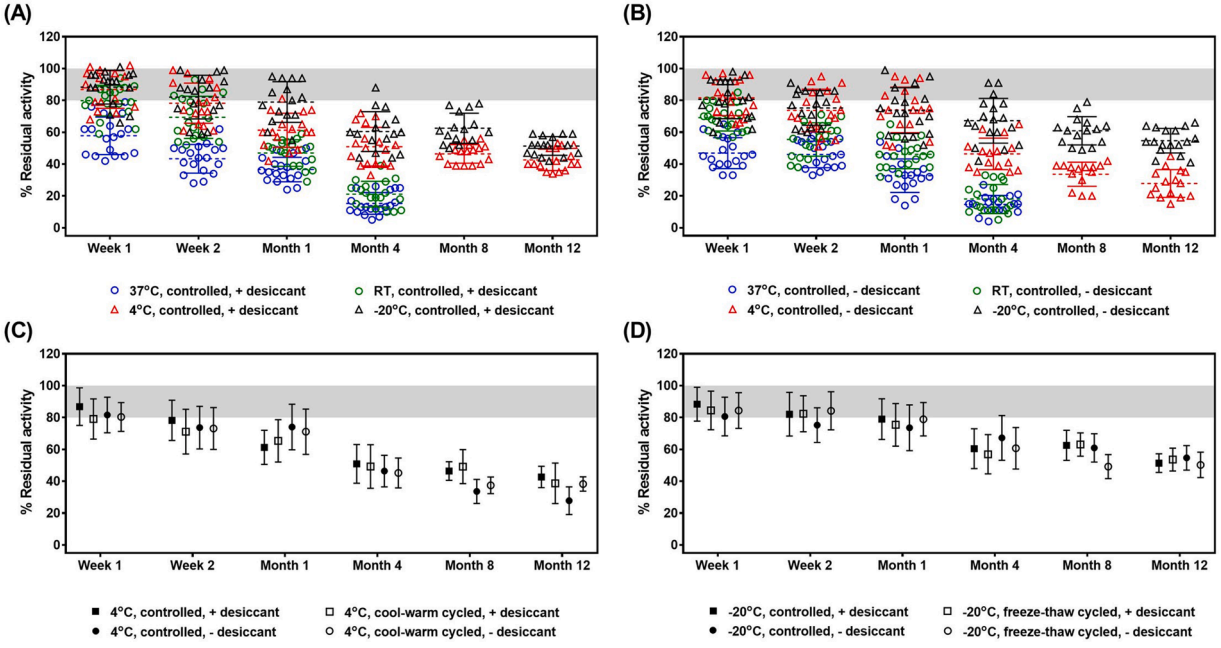

Fig. 6. Stability analysis of G6PD deficient samples stored as DBS. Storage under controlled conditions at various temperatures in the presence (A) and absence (B) of desiccant. Storage under (C) cool-warm cycled condition at 4 °C and (D) freeze-thaw cycled conditions at −20 °C in the presence and absence of desiccant. Gray horizontal bar indicates normal G6PD activity (80–100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In this study, G6PD activity was determined on the day of blood collection (Day 0) using WST-8 assay. The mean G6PD enzyme activity of samples from heterozygous females was 12.8 ± 1.4 U/gHb, which is indistinguishable from that of samples from G6PD normal (13.1 ± 1.1 U/gHb). Since G6PD deficiency is an X-linked genetic disorder, heterozygous females can have a wide range of G6PD activities as a result of random X-chromosome inactivation or lyonization. The mean G6PD enzyme activity of deficient samples was 2.6 ± 1.4 U/gHb which is approximately 20% of normal controls.

According to WHO, samples with enzyme activity over 80% are classified as G6PD normal (W.H. Organization 2016). We, therefore, summarized acceptable specimen storage conditions for samples stored as whole blood in EDTA tubes in Table 1. Of the three sample types assessed, G6PD normal samples retained the greatest G6PD activity at both 4 °C and −20 °C, followed by samples from heterozygous females and then G6PD deficient samples. Furthermore, G6PD activity in samples subjected to cool-warm/freeze-thaw cycle was significantly lower than that in samples stored under controlled conditions. These data suggest that repeated cool-warm/freeze-thaw cycles should be avoided for samples subjected to G6PD testing. Our results indicate that G6PD normal samples stored as EDTA whole blood are stable and can be stored at −20 °C for up to 1 year. It is also suggested that samples for G6PD screening can be stored at 4 °C under controlled conditions but for a much shorter time period of only 2 weeks. This is consistent with results presented by Kahn et al. which indicated that almost 100% G6PD activity was retained in EDTA blood specimens stored at 4 °C for 14 days (Kahn et al., 2015). Jalil et al. also showed that EDTA blood from neonates with normal G6PD activity maintained 93% G6PD enzyme activity after storage at 4 °C for 7 days (Jalil et al., 2016). For heterozygous females, to prevent G6PD misclassification, samples should be stored at −20 °C and 4 °C under controlled conditions for 4 months and 1 week, respectively. In G6PD deficient samples, enzyme activity rapidly decreased over the 1 year of storage. However, because the G6PD activity in deficient samples was already low (mean activity = 2.6 ± 1.4 U/gHb, about 20% of normal activity), further reduction in enzyme activity over the storage time did not interfere with G6PD classification.

Results obtained from specimens stored as DBS are in agreement with those from whole blood samples. Acceptable specimen storage conditions for samples stored as DBS were summarized in Table 2. At the end of the study, G6PD normal samples retained the highest G6PD activity of the three different sample types. Storage of samples at elevated temperatures significantly decreased G6PD activity. Residual G6PD activity was highest when specimens were stored at −20 °C, followed by 4 °C, room temperature and 37 °C, respectively. Our results suggest that storage at −20 °C in controlled conditions in the presence of desiccant is the most suitable storage condition for DBS. Repeated cool-warm/freeze-thaw cycles caused a reduction in enzyme activity and, therefore, are not recommended for blood storage. Samples stored as DBS were more tolerant to cool-warm/freeze-thaw cycles than were those stored as EDTA blood specimens. Improvement in the retention of G6PD activity was mild in the presence of desiccant. However, it was evident that DBS stored in the presence of desiccant maintained G6PD activity for a longer period of time, compared with that stored in the absence of desiccant. Hence, we recommend that DBS be stored in zip-locked bags and placed in a closed container in the presence of desiccant. Flores et al. measured G6PD activity with >90% G6PD activity retained after DBS were stored at −20 °C for 12 months (Flores et al., 2017). Consistent with our results, they observed 20%, 50%, and 87% reductions in G6PD activity when G6PD normal samples were stored at 4 °C, room temperature and 37 °C, respectively. They also described the adverse effect of humidity on G6PD activity when specimens were stored under extreme conditions (samples placed in zip-sealed bags and stored in plastic containers containing damp paper towel). Conditions of high humidity (>50%) resulted in > 50% activity loss after storage at 37 °C for 2 weeks. In our study, humidity only had mild effect on G6PD activity. This could be attributed to the fact that our DBS were kept in zip-lock bags which were stored in a closed container, which would minimize DBS exposure to humidity even in the absence of desiccant. Previously, based on WST-8 assay data, it was suggested that DBS for G6PD testing can be stored at 4 °C for up to 2 weeks (De Niz et al., 2013, Arai et al., 2006). However, in these studies, DBS were stored in containers instead of zip-lock bags. Therefore, it is likely that their samples were exposed to high levels of humidity, leading to a rapid decline in G6PD activity.

As summarized in Tables 1 and 2, normal G6PD samples maintained over 80% G6PD activity and can be stored as EDTA whole blood and DBS at −20 °C for up to 1 year under controlled conditions. Likewise, blood samples from heterozygous females can be stored as DBS at −20 °C under controlled conditions for 1 year and 85% G6PD activity was retained. For blood samples from G6PD deficient individuals, storage conditions did not interfere with G6PD classification because the enzyme activity in these samples was already low. The long-term storage (1 year) at −20 °C for G6PD testing is useful, especially for research purposes using archiving or banking of specimens. In different situations e.g. field test, it is recommended that blood samples be collected and stored as DBS. If G6PD testing can not be completed on the day of blood collection, samples should be kept in zip-lock bags and stored at either 4 °C or −20 °C where storage at −20 °C is preferred. However, when freezer for storage at −20 °C is not available, DBS samples can be stored at 4 °C in which G6PD testing should be done within 15 weeks of storage. In case where samples must be transferred to laboratory, shipment with cold storage (4 °C or −20 °C) is recommended. Transportation of samples at ambient temperature (e.g. 25 °C or higher) should be avoided to prevent loss of G6PD activity which could lead to misclassification of G6PD status.

Since classification of G6PD deficiency is done based on residual G6PD activity in blood samples, suitable storage conditions will maintain G6PD activity and enable accurate identification of G6PD status. On the contrary, inappropriate storage conditions will result in loss of G6PD activity which could lead to misidentification of G6PD status, regardless of method used. However, one should keep in mind of the sensitivity and specificity of the method used for testing G6PD. WST-8 is a quantitative colorimetric test for G6PD measurement. The performance of WST-8 assay is comparable to the standard spectrophotometric method in which the absorbance of NADPH was measured at 340 nm. Hence, optimal conditions for blood storage obtained from this study are likely to be applicable to other G6PD tests as well. However, to ensure reliable and valid results, further investigation might be required.

Table 1
Acceptable specimen storage conditions for samples stored as whole blood in EDTA tubes. .

G6PS status	Controlled		Cool-warm/Freeze-thaw cycled	
	4 °C	−20 °C	4 °C	−20 °C
Normal	2 weeks	1 year	2 weeks	6 weeks
Heterozygote	1 week	28 weeks	1 week	3 weeks
Deficient	1 week	9 weeks	2 week	2 weeks

5. Conclusions

In this study, we thoroughly assessed the stability and integrity of three groups of blood samples, normal controls, heterozygous females and G6PD deficient, for G6PD testing using the WST-8 assay. To our knowledge, this is the first report comparing the stability of three different G6PD sample types under different storage conditions. To maintain integrity and accuracy of G6PD testing, specimens with normal G6PD activity, both EDTA blood and DBS, can be stored at −20 °C for up to 1 year under controlled conditions. Samples from heterozygous females should be stored as DBS at −20 °C under controlled conditions in which 85% G6PD activity was retained at the end of the study. G6PD

Table 2
Acceptable specimen storage conditions for samples stored as DBS.

G6PS status		Controlled				Cool-warm/Freeze-thaw cycled	
		37 °C	RT	4 °C	−20 °C	4 °C	−20 °C
Normal	+*D*	1 week	2 weeks	32 weeks	1 year	28 weeks	1 year
	−*D*	1 week	2 weeks	28 weeks	1 year	16 weeks	1 year
Heterozygote	+*D*	NR	2 weeks	20 weeks	1 year	14 weeks	48 weeks
	−*D*	NR	1 week	15 weeks	40 weeks	13 weeks	40 weeks
Deficient	+*D*	NR	NR	1 week	1 week	1 week	2 weeks
	−*D*	NR	NR	NR	1 week	1 week	2 weeks

NR: not recommended; +*D*: in the presence of desiccant; −*D*: in the absence of desiccant.

activity in deficient samples significantly decreased under all conditions studied. However, the reduction in G6PD enzyme activity of deficient samples did not interfere with G6PD classification because the enzyme activity in these samples was already low. Accurate G6PD testing will allow safe and effective treatment using 8-aminoquinolines, such as primaquine and tafenoquine, to support global goals for malaria control and elimination.

Author contributions

Study design and conceptualization: KC and UB. Collection of blood samples: DS and SC. Acquisition of data: KC, AP, PP, SS, TS and UB. Data analysis: KC, AP, SS and UB. Interpretation of data: MI and UB. Writing and editing manuscript: KC and UB. Funding acquisition: UB, DS, SC and MI. All authors contributed to and approved the final version of manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2021.105864.

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