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The Effect of Storage and Extraction Methods on Amplification of *Plasmodium falciparum* DNA from Dried Blood Spots

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Abstract. Extraction and amplification of DNA from dried blood spots (DBS) collected in field studies is commonly used for detection of *Plasmodium falciparum*. However, there have been few systematic efforts to determine the effects of storage and extraction methods on the sensitivity of DNA amplification. We investigated the effects of storage conditions, length of storage, and DNA extraction methods on amplification via three PCR-based assays using field samples and laboratory controls. Samples stored as DBS for 2 or more years at ambient temperature showed a significant loss of sensitivity that increased with time; after 10 years only 10% samples with parasite densities > 1,000 parasites/μL were detectable by nested polymerase chain reaction (PCR). Conversely, DBS and extracted DNA stored at –20°C showed no loss of sensitivity with time. Samples with low parasite densities amplified more successfully with saponin/Chelex compared with spin-column-based extraction, though the latter method performed better on samples with higher parasite densities stored for 2 years at ambient temperature. DNA extracted via both methods was stable after 20 freeze-thaw cycles. Our results suggest that DBS should be stored at –20°C or extracted immediately, especially if anticipating 2 or more years of storage.

INTRODUCTION

Dried blood spots (DBS) offer a routine means of storing field-collected blood samples for subsequent analysis of plasmodial nucleic acids.^{1,2} Although it is appreciated that DNA degrades over time, there have been few systematic efforts to quantify this degradation, and there is a lack of agreed-upon standards for storage, extraction, and amplification of plasmodial DNA from DBS. Studies have shown that humidity negatively affects DNA quality and that Whatman 3MM and 903 filter paper (Whatman, Clifton, NJ) are ideal for long-term storage.³ However, it is unclear which other factors influence the sensitivity of subsequent DNA amplification.⁴ To systematically characterize factors affecting the sensitivity of amplification of *Plasmodium falciparum* DNA stored as DBS, we analyzed field samples spanning over a decade and well-characterized laboratory controls to measure the effects of storage conditions, length of storage, and DNA extraction methods on successful amplification of plasmodial DNA. Our results allow discrimination between DNA degradation before and after extraction, provide guidance on how to interpret the validity of existing results, and offer best practices for sample storage and extraction.

MATERIALS AND METHODS

Samples. For the determination of the sensitivity of nested polymerase chain reaction (PCR), the effect of storage of DBS at ambient temperature and –20°C and the effect of multiple freeze thaws on extracted DNA, we used control DBS containing *P. falciparum* strain W2 parasites at known parasite densities. Parasites were cultured and synchronized at the ring stage using standard methods.⁵ Parasitemias were

determined from Giemsa-stained thin smears. Positive controls were prepared by mixing infected red blood cells with uninfected whole blood to create parasite densities ranging from 0.1 to 100,000 parasites/μL. To simulate collection of dried blood spots, blood was spotted in 20 μL aliquots onto Whatman 3MM filter paper (with the exception of the DBS stored at two different temperatures, which were spotted on Whatman 903 filter paper), air-dried overnight, and stored at –20°C for up to 1 year in plastic bags sealed with desiccant. Clinical samples came from 3 longitudinal antimalarial drug efficacy trials performed between 2000 and 2011 in Kampala and Tororo, Uganda, and stored at ambient temperature in San Francisco, California. The details of these studies have been previously published.^{6–8} These samples were selected randomly for our study after stratifying based on parasite density, which was determined by thick smear.

Laboratory methods. DBS were cut into 6-mm diameter circles using a single-hole punch, and DNA was extracted by the saponin/Chelex method,⁹ yielding a final DNA volume of ~125 μL, or using the QIAamp DNA mini extraction spin column, yielding a final DNA volume of 100 μL, following the manufacturer's instructions (Qiagen, Hilden, Germany) with 5 μg of carrier RNA. Extracted DNA was stored at –20°C until use. Nested PCR for cytochrome B mitochondrial DNA using 5 μL template DNA followed previously published methods,¹⁰ except that for round one of amplification primers were CB1ab (5'-TTTAGCAAGTCGATATACACCAGA-3') and CB2ab (5'-CTTTAACTTGCCAACTCCCTATCA-3'); and temperature cycling conditions were: 5 minutes at 94°C, 40 cycles at 94°C for 30 seconds, 62.5°C for 90 seconds, 68°C for 90 seconds, and a final elongation at 68°C for 10 minutes, yielding an amplicon length of 1241 base pairs. Single round PCR of microsatellites was performed for two previously published loci (TA40 and PfG377) using 1 μL of template DNA in a 5 μL reaction volume.¹¹ All amplifications were performed on a Bio-Rad Thermal Cycler C1000 or S1000 (Bio-Rad Laboratories, Hercules, CA). Amplification products were detected by agarose gel electrophoresis for nested PCR and via capillary electrophoresis for microsatellites. Microsatellite results were

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TABLE 1

Effect of time of storage on success of PCR amplification from field samples stored as extracted DNA or DBS

Age of sample (years)		1,000–100,000 p/μL N = 10	100–1,000 p/μL N = 15	10–100 p/μL N = 20
2	Newly extracted	100%	80%	60%
	Previously extracted	100%	80%	70%
5	Newly extracted	100%	73%	30%
	Previously extracted	100%	73%	65%
10	Newly extracted	10%	NA	NA
	Previously extracted	100%	73%	50%*

DBS = dried blood spots; NA = not done due to poor sensitivity at higher density samples; PCR = polymerase chain reaction; p/μL = parasites/μL.

Percentages represent the percent of samples that tested positive by PCR.

*Only 6 blood spots could be located with correlating previously extracted DNA at this parasite density range.

deemed positive if peak height was above 250 relative fluorescent units.

Statistical analysis. All data were analyzed using 2011 Microsoft Excel (Redmond, WA) and R version 2.15.3 (www.r-project.org). To compare sensitivity of PCR between various groups of samples, the χ^2 test was used. To estimate the effect of length of storage time on the sensitivity of PCR to detect DNA in samples of various parasite densities, a binomial generalized additive model (R package “mgcv”) was used, including storage time and parasite density in the same tensor product smoothing function.

RESULTS

Sensitivity of nested PCR targeting cytochrome B mitochondrial DNA. To evaluate the sensitivity of nested PCR for amplifying this DNA target, we performed PCR on DBS spotted with 20 μL blood containing a range of parasite densities (0.1–100,000 p/μL), stored as DBS at –20°C, and extracted within 2 months of preparation. The cytochrome B PCR methods had 100% sensitivity for detecting *P. falciparum* in samples containing ≥ 10 p/μL, with 90% and 30% sensitivity for 1 and 0.1 p/μL, respectively.

Storage of DBS versus extracted DNA from clinical samples of various ages. To determine whether storing samples as DBS or extracted DNA better maintained the sensitivity of nested PCR, we evaluated a series of clinical samples collected over a 10-year period. Duplicate DBS samples were either stored at ambient temperature since the time of collection and extracted by the saponin/Chelex for this study or saponin/Chelex extracted near the time of collection and stored at –20°C until this study. For samples stored for only 2 years, sensitivity was similar between newly and previously extracted DNA (Table 1). For samples stored for 5 years,

however, sensitivity was lower for newly versus previously extracted DNA at the lowest parasite density range ($P = 0.04$). For samples stored for 10 years, there was a dramatic decrease in sensitivity for newly extracted samples, even at the highest parasite density range ($P < 0.001$). To evaluate whether an alternative extraction method would improve sensitivity for these older samples, a third DBS for 8 out of 10 of these samples was extracted using a spin-column-based extraction method. This method did not offer improvement in sensitivity (data not shown). Previously extracted samples did not show a significant decrease in sensitivity with age, though there was a trend toward lower sensitivity at the lowest parasite densities tested. These results indicate that long-term storage as extracted DNA at –20°C better maintains sensitivity for PCR compared with storage as DBS at ambient temperature.

Storage of DBS at ambient temperature versus –20°C.

To evaluate the effects of the temperature at which DBS are stored on the sensitivity of subsequent PCR amplification, we stored a series of duplicate control DBS containing known densities of parasites, including those below the limit of detection by microscopy, at ambient temperature and at –20°C for 2.5 years. These samples were then extracted using saponin/Chelex and spin-column methods, and amplified via nested PCR for cytochrome B. Less sensitive single-round PCR targeting two microsatellite loci were used as an alternative amplification method for comparison. Sensitivity of nested PCR was 100% for all samples containing ≥ 100 p/μL, but lower parasite densities showed a significantly higher positivity rate for samples stored at –20°C versus ambient temperature ($P < 0.001$) and extracted via saponin/Chelex versus spin-column methods ($P = 0.04$) (Table 2). Single-round microsatellite amplifications were less sensitive overall, but as with nested PCR, microsatellite PCR had higher success rates when the DBS was stored at –20°C before saponin/Chelex extraction ($P < 0.001$). Spin-column extraction performed no worse than saponin/Chelex when samples were stored at –20°C ($P = 0.3$), and significantly better when DBS samples were stored at ambient temperature ($P < 0.001$), although this relationship did not hold for low parasite densities. These results indicate that DBS stored for more than 2 years should ideally be kept at –20°C and suggest that saponin/Chelex extraction may provide better PCR sensitivity for samples with low parasite density (< 100 p/μL), whereas spin-column extraction may provide better sensitivity for samples with higher parasite densities stored for more than 2 years at ambient temperature.

Quantitative analysis of PCR detectability with DBS storage time. As described above, we found that the sensitivity of detecting parasites via PCR from DBS stored at ambient

TABLE 2

Effect of storage temperature and extraction method on PCR sensitivity

Assay (N = 10)	Storage temperature of DBS	Chelex				Qiagen			
		1,000 p/μL	100 p/μL	10 p/μL	1 p/μL	1,000 p/μL	100 p/μL	10 p/μL	1 p/μL
Cytochrome B	–20°C	100%	100%	100%	100%	100%	100%	100%	70%
	Ambient	100%	100%	90%	0%	100%	100%	30%	0%
TA40	–20°C	100%	100%	20%	0%	90%	80%	20%	0%
	Ambient	0%	0%	0%	0%	100%	90%	30%	0%
PfG377	–20°C	100%	100%	0%	0%	100%	90%	0%	0%
	Ambient	20%	10%	0%	0%	100%	90%	0%	0%

PCR = polymerase chain reaction; p/μL = parasites/μL.

TA40 and PfG377 are microsatellite loci; percentages represent the percent of samples that tested positive by PCR.

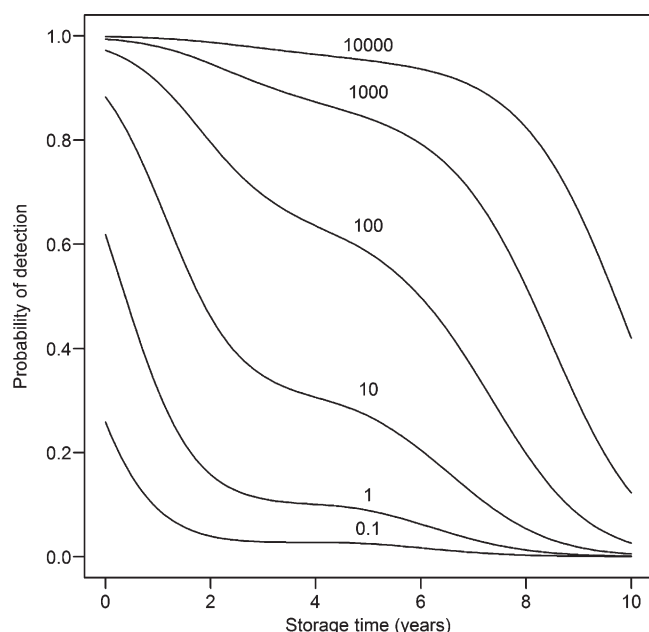


FIGURE 1. Statistically modeled probability of detection by cytochrome B polymerase chain reaction (PCR) by storage time for dried blood spots (DBS) stored at ambient temperature and extracted with saponin/Chelex. Labels on each curve represent parasite densities (parasites/ μ L).

temperature decreased with storage time for clinical samples and laboratory controls. To estimate the degree of this effect, we statistically modeled the detectability of parasites extracted via saponin/Chelex and amplified via cytochrome B PCR using data from all such samples included in Tables 2, 3, and 4 (Figure 1). As expected, sensitivity for very low parasite densities dropped off rapidly, but was maintained longer for higher parasite densities.

Multiple freeze thaws of extracted DNA. To determine whether multiple freeze-thaw cycles affected PCR sensitivity, we amplified known parasite density controls after extracting with saponin/Chelex or spin-column methods and freeze thawing duplicate sets of samples 1 versus 20 times over 2 months. There was no significant effect of freeze thawing on DNA extracted via either method, as measured with cytochrome B or microsatellite PCR (data not shown).

DISCUSSION

We showed that the choice of storage, extraction, and amplification methods all had a significant effect on the ability to detect *P. falciparum* DNA by PCR from DBS samples. Samples stored as DBS for 2 or more years at ambient temperature showed a loss of sensitivity that increased with time, whereas DBS and extracted DNA stored at -20°C did not. Experiments on laboratory controls demonstrated that multiple freeze-thaw cycles of extracted DNA did not significantly affect sensitivity, indicating that DNA within the supernatant of saponin/Chelex extraction does not degrade with repeated freeze thawing. DNA extraction via the saponin/Chelex method tended to outperform spin columns for low-density infections, whereas spin-column extraction had an advantage in extracting older samples with higher parasite densities.

Storage of DBS at ambient temperature compromised sensitivity, with amplification of samples with submicroscopic parasitemia noticeably diminished at 2 years and sensitivity for higher parasite densities compromised in samples stored for longer periods. Indeed, we were unable to detect even relatively high parasite densities ($\geq 1,000$ p/ μ L) by PCR in samples stored at ambient temperature for 10 years. Figure 1 provides an estimate of the loss of sensitivity over time, though results from other sites may vary based on storage conditions, DNA extraction methods, and PCR amplification assays. Our findings are in agreement with two prior studies that showed decreased PCR sensitivity for microscopically detectable infections after 4 and 7 years of storage.^{12,13} Thus, studies attempting to determine the prevalence of parasitemia by PCR, especially from subjects who are likely to have low parasite densities (e.g., asymptomatic subjects or those who are negative by microscopy or rapid diagnostic test), should be interpreted cautiously if DBS samples have been stored at ambient temperature for more than 2 years. On the basis of our results, if DBS samples are not analyzed promptly, they should be stored at -20°C or DNA should be extracted before storage.

It should be noted that while we stored all DBS samples with desiccant, in many cases samples stored at ambient temperature were noted to contain saturated desiccant. Although ideally desiccant should be changed frequently and DBS should be stored in completely airtight containers, in practice this is infrequently achieved.¹⁴ The benefit of DBS storage at -20°C may be from decreased humidity and/or colder temperatures inhibiting enzymatic activity that leads to DNA fragmentation and growth of molds. Of note, we have developed a protocol for inexpensive, compact storage of DBS cards, which facilitates storage of large numbers of DBS in freezers (Supplemental Material).

Regarding DNA extraction, we found significantly increased sensitivity for low parasite densities using the inexpensive saponin/Chelex method versus spin-column extraction, consistent with our prior findings.¹⁰ A likely explanation is that some DNA is lost during binding and elution in the spin column. A caveat to this finding is that we only evaluated three PCR assays; assays more sensitive to inhibitors may benefit from use of highly purified DNA obtained from the spin-column technique. On the other hand, spin-column extraction using protocols from Qiagen, which involves lysis with proteinase K instead of saponin, may have an advantage in obtaining DNA from older samples, which are difficult to lyse, especially when parasite densities are high such that inefficiencies of the column are relatively unimportant. Anecdotally, we have also found that spin-column extraction appears to work better than the saponin/Chelex method for DBS samples obtained from placentas,¹⁵ perhaps for the same reason. A positive finding in our study is that saponin/Chelex extracted DNA was remarkably stable during long-term storage at -20°C , and that the detectability of parasite DNA was not affected by repeated freeze thaws. Thus, if DNA can be extracted soon after DBS collection, stored DNA can be expected to remain stable.

The strength of this study was the use of laboratory controls, enabling us to test effects of storage, extraction, and amplification on DBS samples of known parasite densities that include submicroscopic densities, as well as the use of clinical samples of various ages. Clinical and laboratory samples each

have limitations. For clinical samples, parasite densities were measured via microscopy, which may have limited accuracy especially for low parasite densities. This may explain why our sensitivity for detecting low density infections was lower than expected. For laboratory controls, collection and storage may not reflect all sources of variation in field samples.

In conclusion, we have shown that sensitivity for detecting DNA from DBS stored at ambient temperature decreases with length of storage time, with decreases seen as soon as 2 years after collection. Studies using stored samples should take this into account, and when possible future studies should store DBS at -20°C or extract DNA soon after collection.

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