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ORIGINAL ARTICLE

Comparison of four methods for extracting DNA from dried blood on filter paper for PCR targeting the mitochondrial *Plasmodium* genome

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Background: Few studies comparing multiple methods for DNA extraction from dried blood spots (DBS) on filter paper for PCR targeting the *Plasmodium* genome have been done.

Methods: Frequently-used methods for DNA extraction from DBS using Chelex-100, InstaGene Matrix, QIAamp DNA Mini Kit and TE buffer were compared on a dilution series of a standardized *Plasmodium falciparum* positive sample. The two DNA extraction methods resulting in the lowest limits of detection were compared by testing both on 31 *P. falciparum* positive samples collected under field conditions and stored for 4 years.

Results: The Chelex-100, InstaGene Matrix and QIAamp DNA Mini Kit methods performed similarly, resulting in the detection of 0.5 to 2 parasites per microliter (p/μl). The same 13 clinical samples (13/31; 42%) were positive using both DNA extraction methods with the lowest limits of detection.

Conclusions: Simple and low-cost methods can be sensitive and useful in extracting DNA from DBS. Poor results on stored clinical DBS indicate that further studies on the impact of storage duration and conditions, and choice of filter paper should be performed.

Keywords: Chelex, Dried blood spot testing, Malaria, Mitochondrial genome, PCR

Introduction

Malaria took the lives of an estimated 627 000 people in 2012.[1] There are currently 97 malaria-endemic countries in the world with resource-poor countries in sub-Saharan Africa bearing the greatest burden.[1]

Polymerase chain reaction (PCR) for detection of malaria has become increasingly relevant for epidemiological research on malaria in endemic areas. With its high sensitivity and specificity PCR is increasingly being considered a gold standard method in research settings, although the clinical applicability of PCR results is not yet fully established.[2–4] Generally, DNA extracted from whole blood has been used for PCR, but dried blood spots (DBS) on filter paper have emerged as a convenient way to collect and transfer specimens for DNA extraction in studies in rural areas where effective cold-chains are lacking, transport is difficult and malaria flourishes. Dried blood spots do not require a cold chain and they allow for retrospective PCR analysis. A major limitation of using these methods is that PCR requires specialized expertise and relatively expensive equipment to perform.[5]

Studies on storage of DBS over longer periods of time have shown varying results. One study showed diminished sensitivity when stored beyond 5 years.[6] Another study showed increased sensitivity after 4 years, likely due to inhibitors being more easily eluted from newer rather than older samples.[7] Storage conditions including humidity and temperature, as well as the sort of filter paper used are likely to influence how well the DNA is preserved in the DBS.[8]

In research, in a resource-poor setting, a simple and inexpensive method of DNA extraction is desirable for the method to be feasible and sustainable. A simple boiling method using TE buffer was introduced by Bereczky et al. and was shown to have higher sensitivity compared to two established methods using Chelex and methanol.[9] Methods using Chelex[6,10–12] and InstaGene Matrix[7,13,14] are based on the use of a substance that removes PCR-inhibitors through a procedure including a boiling step. In addition to these methods commercial kits, such as QIAamp DNA Mini Kit, are also frequently used,[6,15,16] and are therefore relevant when comparing methods for DNA extraction from filter paper. Previous comparisons of DNA extraction methods from

DBS that have been published[6,9,13,17] have included only two to three methods that were tested on non-standardized reference or clinical samples. Research groups applying these and similar techniques are likely to have tested several methods of DNA extraction and optimized these before applying the methods to field samples. However, few studies are published illustrating this process of testing and justifying the choice of one DNA extraction method above another.

This study was performed to identify the method with the lowest limit of detection among several commonly used methods for DNA extraction from DBS, and to assess the cost-effectiveness of the methods. The purpose of the study was to find a method feasible for studies in resource-poor settings. In addition, to assess the performance on stored clinical samples, the two most sensitive DNA extraction methods were evaluated on a set of 4-year-old DBS from whole-blood malaria PCR-positive patients.

Materials and methods

Samples

A series of a total of 10 two- and five-fold dilutions of an external reference sample of *Plasmodium falciparum,* US 04 F Nigeria XII (WHO Specimen Bank at Centers for Disease Control and Prevention, Atlanta, GA, USA), with 2000 parasites per microliter (p/μl) in malaria-negative control blood were made. The dilutions ranged from 2000 to 0.25 p/μl. The DBS were prepared by dropping 50 μl blood on a segment of a Whatman Schleicher & Schuell filter paper, grade 589/2 (Whatman GmbH, Dassel, Germany). The DBS were air-dried completely before being analyzed within 1 week.

From 31 febrile patients that had previously tested positive by mitochondrial *Plasmodium* genus-specific PCR of DNA extracted from whole blood that had been stored at −20°C for 2–2.5 years before analysis, surplus DBS had been collected to perform the comparison of methods for DNA extraction from DBS. Of these, 26 had rapid diagnostic test (RDT) (First response malaria Ag pLDH/HRP2 Combo card test) results available, and 9 were RDT positive. All 31 samples were confirmed positive for *P. falciparum* either by species-specific PCR as described by Padley et al[18] or by sequencing,[3] in addition to being positive by the mitochondrial genus-specific malaria PCR (performed as described below). Samples were collected from admissions at the general paediatric wards at Muhimbili National Hospital (MNH) in Dar es Salaam, Tanzania as part of a study of febrile children.[3] The DBS were prepared by dropping two drops of venous blood, collected with a syringe at the same time as other blood tests were taken, on a segment of a Whatman Schleicher & Schuell filter paper, grade 589/2 (Whatman GmbH). The DBS were stored in sealed, airtight plastic pockets after air-drying completely. After storage at the ambient temperature of around 25°C for 3 to 9 months they were transported, protected from sunlight, and stored for approximately 3.5 years at −20°C before DNA extraction.[19,20]

DNA extraction

Harris Uni-Core puncher (Qiagen, Hilden, Germany) was used to punch out circles (3 mm in diameter) in the DBS. Between samples, the Harris Uni-Core was cleaned as described previously.[21] Care was taken to ensure that no liquid from the cleaning process carried over to the next DBS.

Chelex-100 Molecular Biology Grade Resin (Bio-Rad Laboratories, Hercules, CA, USA), InstaGene Matrix (Bio-Rad Laboratories), QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and TE buffer methods were tested on all DBS prepared from the dilutions of the reference blood sample to find the methods with the lowest limit of detection (LOD). The LOD was defined as a final sample dilution where at least two of three replicates were PCR-positive, and all preceding dilutions also had at least two of three replicates positive. For all methods, six punches of DBS (approximately 25 μl of blood) were used in the extraction of DNA from each dilution. Corrected LOD was found by dividing the estimated number of parasites in the 25 μl of blood, from the six punches of the DBS, by the final elution volume of each method. The Eppendorf Centrifuge 5417C (Eppendorf AG, Hamburg, Germany) was used for all methods. DNA extracts were stored at −20°C until PCR was performed. In addition, the two methods that resulted in the lowest corrected LOD when tested on the dilution series were compared using the same procedure on the clinical samples.

Three variants of the method for DNA extraction using Chelex-100 were tested (Box 1). For all three methods 5% Chelex-100 in distilled water was kept in suspension using a magnetic stirrer. In the first method, the DBS was soaked in saponin and this was a variant of a method described in several studies including one by Wooden et al.[19,22–25] A second version of the Chelex-100 method, which involved soaking of the DBS overnight in PBS, was based on a method described by Chaorattanakawee et al. using InstaGene Matrix.[7] A third Chelex-100 method, where the DBS punches were not soaked overnight, was also done as described by Kain et al.[26] The two first methods resulted in approximately 80 μl eluted DNA while the last method gave a volume of 150 μl.

Two methods were tested using 6% InstaGene Matrix in a pre-prepared solution for DNA extraction that was kept in suspension (Box 1). The first method, without soaking the DBS overnight, was done as described by Cox-Singh et al.[13] The second method (with overnight soaking of the punches in PBS) was performed as a combination of the methods described by Chaorattanakawee et al.[7] and Cox-Singh et al.[13] Both methods resulted in approximately 160 μl eluate. Extraction using QIAamp DNA Mini Kit was done according to the manufacturer's instructions and resulted in approximately 100 μl eluted DNA.

The TE buffer method was performed as described by Bereczky et al.[9] In brief, the punches from the DBS were placed in 65 μl TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA in distilled water) in a clean tube and incubated at 50°C for 15 min. The filter paper punches were then manually pushed towards the bottom of the tube several times with a pipette tip and then heated to 97°C for 15 min. The tube was centrifuged at high speed for 10 s before the supernatant (approximately 50 μl) was removed and placed in a clean tube.[9]

PCR

A genus-specific PCR targeting the *Plasmodium* mitochondrial genome, as described by Haanshuus et al.,[2] but with a primer concentration of 1 μM, was performed on DNA extracted from DBS. Primers PgMt19 F3 and PgMt19 B3 were used. The DNA template volume used in the PCR reaction was 2 μl, with a total volume of 25 μl. The amplification conditions were as follows: step 1, 95°C for 15 min; step 2, 95°C for 10 s; step 3, 62°C for 10 s;

Box 1. DNA extraction methods from dried blood on filter paper using Chelex-100 and InstaGene Matrix

Chelex-100			InstaGene Matrix	
Soaking in saponin[a]	Soaking in PBS	No soaking	Soaking in PBS	No soaking
• Place punches in 1 ml 0.5% saponin • Incubate at 4°C overnight • Remove saponin and add 1 ml PBS • Incubate at 4°C for 30 min • Remove PBS and place punches in 100 μl 5% Chelex-100 • Incubate at 100°C for 8 min • Centrifuge at 10 600 g for 2 min • Carefully remove and store supernatant at −20°C if the extract is not used promptly	• Place punches in 100 μl PBS • Incubate at 4°C overnight • Centrifuge at 18 000 g for 2 min • Remove supernatant and add 100 μl PBS • Centrifuge at 18 000 g for 2 min • Remove PBS and place punches in 100 μl 5% Chelex-100 • Incubate at 100°C for 8 min • Centrifuge at 10 600 g for 2 min • Carefully remove and store supernatant at −20°C if the extract is not used promptly	• Place punches in 180 μl 5% Chelex-100 already heated to 100°C • Vortex 30 s • Incubate at 99°C for 10 min • Centrifuge at 12 000 g for 1.5 min. • Remove the supernatant and transfer to a clean tube • Centrifuge the supernatant at 12 000 g for 1.5 min • Carefully remove and store supernatant at −20°C if the extract is not used promptly	• Place the punches in 100 μl PBS and incubate overnight at 4°C • Centrifuge at 18 000 g for 2 min • Remove and discard supernatant and add 100 μl PBS • Centrifuge at 18 000 g for 2 min • Remove and discard supernatant • Add 200 μl InstaGene Matrix • Incubate at 56°C for 30 min. Vortex carefully after 15 min and after completed incubation • Boil samples at 100°C for 8 min • Centrifuge at 15 000 g for 2 min • Carefully remove and store supernatant at −20°C if the extract is not used promptly	• Place the punches in a clean tube • Add 200 μl InstaGene Matrix • Incubate at 56°C for 30 min. Vortex carefully after 15 min and after completed incubation • Boil samples at 100°C for 8 min • Centrifuge at 15 000 g for 2 min • Carefully remove and store supernatant at −20°C if the extract is not used promptly

PBS: phosphate-buffered saline pH 7.4.
[a] Saponin, 0.5% saponin (Sigma-Aldrich Chemie, Stenheim, Germany) in phosphate-buffered saline (PBS) pH 7.4.

step 4, 72°C for 15 s. Steps 2–4 were repeated 50 times. This was then followed by incubation for 10 min at 72°C. Amplification was done using GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA). All samples were run in triplicate. PCR results were read blinded to other malaria test results, where relevant. Analysis of PCR products was done by electrophoresis using 2% SeaKem agarose gel (Lonza, Rockland, ME, USA) with 1X GelRed (Biotium, Hayward, CA, USA).

Cost assessment

The cost of reagents or kit per sample was calculated from the purchase price of reagents and kits and extrapolated to the mean price per test in US$ for extracting DNA from a total of 10 000 DBS.

Ethical considerations

For collection of patient samples, a research permit was obtained from the Tanzania Commission for Science and Technology (COSTECH), and ethical clearance was received from the appropriate bodies at Muhimbili University of Health and Allied Sciences (MUHAS), MNH and from the Regional Committee for Medical and Health Research Ethics, Western Norway. The collection of patient samples was done in collaboration between MUHAS/MNH and the University of Bergen/Haukeland University Hospital, Norway. Informed, written consent was obtained from the participants' parent or guardian by signature or thumbprint.

Results

The results for all methods for DNA extraction from filter paper, including LOD before and after correction for final elution volume, as well as the cost of reagent or kit per sample, are shown in Table 1. All the methods, except for the TE buffer method, performed well resulting in a limit of detection of 0.5 to 2 p/μl (0.16 to 0.63 p/μl after correcting for elution volume). The Chelex-100 method, with soaking in 0.5% saponin overnight, was positive for all dilutions up to 0.5 p/μl, which was the lowest

Table 1. Results of PCR targeting mitochondrial *Plasmodium* genome of DNA obtained with various extraction methods from dried blood spots (DBS) on filter paper with decreasing parasite densities, performed in triplicate

Parasite density (parasite/μl)	Chelex-100			QIAampDNA Mini Kit	TE buffer	Instagene Matrix	
	with saponin	with PBS	without soaking			with PBS	without soaking
2000	+	+	+	+	+N+	+	+
400	+	+	+	+	+	+	+
80	+	+	+	+	N	+	+
16	+	+	+	+	N	+	+
8	+	+	+	+	N	+	+
4	+	+	+	+	N	+	+
2	+	+	+	++N	N	+	N++
1	N++	+NN	N	N	N	N++	N
0.5	+	+N+	++N	N	N	N	N
0.25	N	+N+	N	N	N	N	N
LOD (p/μl)	0.5	2	2	2	400	1	2
Corrected LOD (p/μl)	0.16	0.63	0.33	0.5	200	0.16	0.31
Cost per sample (US$)[a]	0.16	0.14	0.27	4.69	0.01	1.23	1.23

Corrected LOD: limit of detection based on parasite concentration in eluate after correcting for each method's final elution volume; LOD: limit of detection; N: negative; p: parasites; +: positive (if only one + or N, positive or negative on all three replicates).
[a] Cost of reagents or kit per sample in US$, if reagent or kit is purchased for DNA extraction from a total of 10 000 dried blood spots.

LOD compared to the other methods. After correcting for elution volume the lowest LOD was 0.16 p/μl and was the same for both the Chelex-100 method with soaking in 0.5% saponin overnight and the InstaGene Matrix with soaking in PBS overnight. These two methods were therefore compared using the 31 DBS samples from whole-blood malaria PCR-positive patients. The same 13 samples (13/31; 42%) were positive using both of these methods. None of the clinical samples were positive using only one of the two DNA extraction methods. All clinical samples with positive RDT results were also positive by PCR of DNA extracted from DBS (Table 2).

Discussion

This study aimed to identify a cost-effective and sensitive method for extracting malarial DNA from filter paper with the intention of improving tools for malaria research in resource-constrained settings of malaria-endemic countries.

The parasite detection levels of several of the methods tested in this study were comparable to those found from PCR of DNA extracted from whole blood.[2] All extraction methods resulted in positive PCR results for all dilutions, with a lowest detection level of 0.5 to 2 p/μl (corrected LOD 0.16 to 0.63 p/μl), except for the method using TE buffer. This LOD-range is comparable to the range found when the same PCR methods were used on the reference whole blood sample (consistent parasite detection level of 0.5 p/μl).[2] It therefore appears that samples collected on filter paper can be useful for monitoring malaria epidemiology, giving similar results compared to when whole blood is used as the DNA template. In the current era with an increasing number of deaths and cases attributable to malaria,[1] tracking malaria epidemiology is increasingly important in order to monitor the effects of and appropriately target interventions.

Thirty-one clinical samples were also tested and the sensitivity of 42% (13/31) suggests that using PCR on stored clinical DBS is inferior to performing PCR on whole blood in detecting malaria parasitaemia. It appears that the LOD of 0.5 p/μl that was found using the reference *P. falciparum* positive sample does not apply to stored clinical DBS. This could indicate that many clinical samples positive by PCR on whole blood may have had very low parasitaemia that was undetectable when using filter paper. In one DBS of 50 μl there are on average 12 punches of 3 mm each. Thus, approximately 25 μl of dried blood were included in the DNA extraction methods when six punches from each DBS were used. With the final volumes of eluate from DBS varying from 50 to 160 μl depending on the DNA extraction method used, this results in 4–12 times lower malarial DNA concentration compared to the eluate from whole blood, where the extraction method used included 200 μl blood concentrated into 100 μl of eluate. This could explain the lower sensitivity of PCR on DBS compared to that on whole blood. The ranking of the methods based on LOD is similar even after LOD has been corrected for final elution volume (Table 1). The clinical samples negative by PCR using DNA extracted from DBS, for which the RDT results were available (n=17), were also all RDT negative, while PCR-positive DBS samples were also RDT positive (n=9) (Table 2). As the limit of detection of most RDTs is approximately 100–200 p/μl, it appears that for these clinical samples the PCR of DNA extracted from DBS using the Chelex-100 method (with soaking in saponin overnight) generally detected samples with higher parasitaemia compared to using PCR of DNA extracted from whole blood.

The clinical DBS had been stored for approximately 4 years, initially for 3 to 9 months at room temperature in a tropical

Table 2. Results of PCR using DNA from dried blood spots (DBS) extracted using Chelex and InstaGene Matrix, and rapid diagnostic tests (RDT) for 31 whole-blood mitochondrial PCR-positive samples

Patient no.	PCR of DNA extracted from DBS		RDT
	Chelex-100	InstaGene Matrix	
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	N	N	N
6	N	N	N
7	N	N	N
8	N	N	N
9	N	N	N
10	+	+	ND
11	N	N	N
12	N	N	N
13	N	N	N
14	N	N	ND
15	N	N	N
16	N	N	ND
17	N	N	N
18	N	N	N
19	+	+	ND
20	N	N	ND
21	N	N	N
22	+	+	ND
23	+	+	+
24	+	+	+
25	N	N	N
26	N	N	N
27	N	N	N
28	N	N	N
29	+	+	+
30	+	+	+
31	+	+	+

DBS: dried blood spot; +: positive; N: negative; ND: not done; RDT: rapid diagnostic test.

environment (generally above 25°C), followed by storage at −20°C until DNA extraction was performed. This has likely contributed to reducing the quality of the DNA on these DBS.[6] The transfer to storage at −20°C after several months at higher temperatures is common in field studies as cold-storage facilities are generally available for long-term storage of samples after transport from the site of field sample collection to the location of PCR analysis. Moll et al. recommend long-term storage of DBS at −20°C.[19] The filter paper used in this study has not been verified in previous studies as suitable for DBS. The test of the dilution series of samples confirms its general suitability, however its ability to preserve DNA over time compared to other filter papers needs further investigation. Other factors such as the exact pH, brand and type of polymerase used could also have influenced the results.

InstaGene Matrix was reported to be superior to Chelex-100 by Cox-Singh et al., as it is claimed to be more effective in removing PCR inhibitors than conventional Chelex-100 resin.[13] The findings of the current study contradict this, as the Chelex-100 and InstaGene Matrix methods performed similarly with the same corrected LOD of 0.16 p/μl, as opposed to the LOD of 30 and 6 p/μl, respectively, previously reported by Cox-Singh et al.[13] Chelex-100 is much cheaper with an estimated cost of less than 0.16 US$ per sample for the reagent compared to 1.23 US$ per sample for InstaGene Matrix (Table 2), and it therefore appears to be a useful, cost-effective and sensitive method for DNA extraction from DBS. In addition, the results of the testing of DBS from whole-blood PCR-positive patients were the same whether the most sensitive Chelex-100 or InstaGene Matrix method was used, indicating that they also perform similarly on stored clinical samples. The TE buffer method was the cheapest alternative but performed poorly in the current study, despite its very good performance in a previous study by Bereczky et al.[9] The QIAamp DNA Mini Kit cost approximately 4.50 US$ per sample and did not perform better than the other methods despite its higher cost. This kit was also one of the more labor-intensive methods tested in the current study as it included three different incubation temperatures and eight centrifugation steps.

The Chelex-100 method required only one heating step at 99°C, while the InstaGene Matrix method required two heating steps at 56°C and 100°C. The Chelex methods are therefore less labor-intensive than the InstaGene method. The Chelex method, which involved soaking in saponin overnight and had the lowest LOD in the current study, was also one of the most frequently used methods in other studies where DNA for malarial PCR was extracted from DBS.[19,22–25] InstaGene Matrix has also been used recently in several malaria studies using DBS.[7,27–30]

The limitations of the study include that the comparison of methods on clinical samples only included two of all the tested methods. The clinical samples examined had been stored for approximately the same length of time, which was longer than the storage time of the DBS made from the dilution series of the standard sample. A more reasonable comparison would have included clinical and standard samples stored for the same length of time. Another limitation of the study is that the filter paper used has not been validated or compared to other types of filter paper before being used in this study. The ability of the filter paper to preserve DNA over time is therefore uncertain. Also, the final elution volumes of the various methods were not standardized, however the same amount of DNA in each method was used. This complicated the comparison but was corrected for by using the corrected LOD.

Conclusions

Detecting *Plasmodium* DNA by PCR from DBS on filter paper is a minimally invasive, easy-to-use and cost-effective tool as a molecular diagnostic method in malaria research in endemic countries. With its low parasite detection level, cost-effectiveness and simple procedure, the Chelex-100 method with soaking in saponin solution overnight is a recommended method for DNA extraction from DBS. Furthermore, the method using Tris-EDTA, which has previously been reported to perform well, did not

perform well in this study and therefore should not be recommended or must be extensively tested before being used in future studies. The low sensitivity of PCR on DBS in malaria-positive clinical samples suggests that the sensitivity of DBS in clinical study settings can likely be significantly improved by optimizing storage and extraction methods.

Further studies on standardized reference samples comparing varying storage conditions (humidity and temperature), storage duration, various filter paper types and different PCR assays should be performed in order to identify the significance of various variables for the results of PCR using DNA extracted from DBS.

Authors' contributions: GEAS and BB were involved in all stages of this study; MGT and NL were involved in the study design; MGT selected methods for DNA extraction from DBS; GEAS, BB, KH and MGT contributed to the data interpretation and writing of the manuscript. All authors have read and approved the final manuscript. GEAS is the guarantor of the paper.

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Competing interests: None declared.

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