ARCHIVED GIEMSA-STAINED THICK-SMEAR BLOOD SAMPLES AS A SOURCE OF DNA FOR *PLASMODIUM FALCIPARUM* DETECTION BY THE POLYMERASE CHAIN REACTION (PCR): AN IDEA FOR LOW TRANSMISSION SETTINGS

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ABSTRACT

The absence of a reasonable number of cases of malaria in low transmission settings often presents problems for epidemiologic studies of antimalarial drug resistance. Whole blood from filter paper spots is normally used as a source of deoxyribonucleic acid (DNA). The number of samples available to derive statistical power is usually limited due to low transmission rates. This study suggests an alternative source of DNA from Giemsa-stained thick smears (GSTS) for epidemiological studies of Plasmodium falciparum drug resistance. A total of 73 archived GSTS and 6 whole blood filter paper samples were available for this analysis. DNA obtained from GSTS was successfully extracted, genotyped and sequenced for 64 (88%) samples for the Plasmodium falciparum chloroquine resistance transporter (pfcrt) gene, 51 (70%) samples for the Plasmodium falciparum multi-drug resistance 1 (pfmdr1) gene, 34 (47%) samples for the Plasmodium falciparum dihydrofolate reductase (pfdhfr) gene and 27 (37%) samples for the Plasmodium falciparum dihydropteroate synthase (pfdhps) gene. Whole blood from the 6 filter paper samples that were also run for comparison were all (100%) successfully genotyped and sequenced in a single attempt for all the four genes. The

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ease of the analysis of the filter paper DNA samples and the quality of the gel electrophoresis pictures suggests that filter paper DNA sources are much more sensitive than archived GSTS samples. Nonetheless, the results of this study suggest that safely stored and clearly labelled GSTS can provide a cheap and somewhat reliable alternative source of DNA for retrospective epidemiologic studies using the polymerase chain reaction (PCR) analyses where filter paper sources are either not available or insufficient. It is, therefore, strongly recommended that laboratories in malaria low transmission settings develop guidelines for safe storage of GSTS for future use in genotyping and other experiments. Protocols to refine extraction and PCR methods as well as the design of appropriate primer pairs may improve the sensitivity of current PCR methods to improve the results of GSTS.

Key words: Giemsa-stained thick smears, filter paper blood samples, *pfcrt*, *pfmdr1*, *pfdhfr*, *pfdhps*, polymerase chain reaction, DNA analysis.

INTRODUCTION

Microscopic examination of Giemsa-stained thick blood smears remains the method of choice for diagnosis malaria in endemic and non-endemic settings. Therefore, many laboratories generate large amounts of Giemsa-stained blood films that are stored either for future review or as a general practice. However, recently developed molecular techniques have gained importance particularly in reference laboratories and quality control settings. Whole blood provides a reliable source of high quality DNA for laboratory analysis (Zhong and Kain, 1999) whether microscopy or molecular methods are used. DNA analysis results are dependent mainly on the handling, storage and transportation of samples to prevent degradation of DNA quality or contamination. In low transmission settings, fewer people are

infected and obtaining a sufficient number of samples for DNA analysis is often problematic. Lengthy follow-ups are required in order to obtain a sufficient number of samples to derive statistical power during analysis of epidemiologic studies. This problem has resulted in the reduction of the number of important research agendas requiring DNA analysis such as those for the analysis of molecular markers of antimalarial drug resistance.

The polymerase chain reaction (PCR) has now revolutionized contemporaneous aetiologic diagnosis of infectious diseases and it provides a good standard for microscopic methods. However, the performance of the PCR method depends on the quality of DNA and the reagents used, as well as adequate DNA amplification conditions. Purchasing reagents from a reputable supplier and practising strict laboratory procedures when receiving, storing and using them ensures high performance of the PCR, leaving the quality of DNA as the only real problem towards achieving good PCR results. For a long time Giemsa-stained blood films were not considered as a possible source of DNA for PCR analysis because the quality was thought to be too low to guarantee good results. Previous studies had shown that DNA degradation may occur in Giemsa-stained blood films stored for greater than 4 years (Yokota *et al*., 1995). However, recent studies have showed success with blood films stored for more than 10 years in conditions not designed to protect DNA integrity, further suggesting that properly stored thick smears could remain useful for more than 10 years (Lee *et al*., 2009). Storage of thick smear in the open air was reported to cause DNA degradation through oxidative damage by atmospheric oxygen (Matsuo *et al*., 1995). However, the study by Matsuo and colleagues did not elaborate at what rate this degradation occurs and therefore, for how long thick smear blood could remain PCR sensitive on slides. None of these studies specifically report success of PCR analysis for *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene, *Plasmodium falciparum* multi-drug resistance 1 *(pfmdr1)*

gene, *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) gene *and Plasmodium falciparum* dihydropteroate synthase (*pfdhps*) gene from archived GSTS which is presented in the findings of this study.

Several reports have also indicated poor performance of the PCR method for low parasite densities (Scopel et al, 2004; Kimura et al., 1995; Xiao et al., 2006). Detection of low parasite densities is of particular importance since the recently developed and globally introduced rapid diagnostic tests (RDTs) have low sensitivities at parasite densities below 100/µl and 500µl for *Plasmodium falciparum and non-falciparum* species infections respectively. Detection of low parasite densities may be further enhanced by development of real-time PCR methods from the standard PCR methods. Real-time PCR assays are particularly attractive because of the short turn-over-time and the avoidance of post-PCR contamination (Klein, 2002; Mackay, 2004).

Microscopy is also important in defining the species of malaria responsible for an infection. A well trained, proficient microscopist should be able to recognise the *Plasmodium* species correctly in GSTS samples at relatively low parasite densities. However, species definition by microscopy still has errors and most endemic countries contain incomplete or inaccurate information. Most documented species-defining errors involve differentiating between human infections with simian plasmodia, in particular, *Plasmodium knowlesi*. Again, the recently improved molecular methods have contributed a lot in correction of errors emanating from use of microscopy for classification of plasmodia species.

This study describes the successful performance of PCR methods on GSTS samples derived from an area of low endemic setting such as Swaziland.

MATERIALS AND METHODS

Source of isolates:

Six filter paper blood samples were obtained from a 3-month cross-sectional survey in the Lowveld of Swaziland. The 6 cases were the only cases of malaria over the 3-month period of the survey. The number was too small for the analysis intended. So, an alternative source of DNA was sought from archived Giemsa-stained thick blood smears. Seventy-three thick smear slides were obtained from two centres in Swaziland (Ubombo Ranches Clinic and the Royal Swaziland Sugar Corporation [RSSC] Medical Centre). The analysis comprised only of slides prepared in 1999 were preferred because they were more in number than those of any other year which allowed confinement of the analysis to slides prepared in the same year.

Isolation of DNA:

The DNA extraction from slide samples was done at the London School of Hygiene and Tropical Medicine (LSHTM) laboratories using the QIAmp Mini Blood Purification kit (QIAGEN, UK) following the manufacturer's instructions. The extraction was based on lysis of the blood sample products with QIAGEN protease enzyme in the presence of lysis Buffer AL. Following release, the DNA binds to the QIAmp silica membrane on the spin column before it is washed with Buffers AW1 and AW2 and then eluted with Buffer AE. DNA from filter paper blood samples collected from patients that were positive for *Plasmodium falciparum* by microscopy was extracted using the Chelex method (Plowe *et al*., 1995). The pieces of filter paper impregnated with whole blood were cut with sterile scalpel blade and then lysed in 1ml of freshly made 0.5% Saponin (Sigma, Germany) in 1x Phosphate Buffer Solution (PBS). The samples were then incubated at 37^{o}C overnight to lyse the cell membranes of red blood cells. Haemoglobin was released into the PBS leaving the parasite

DNA on the paper. The samples were briefly centrifuged at 4 000g for 2 minutes and saponin solution and debris removed using tips attached to a vacuum pump. One millilitre of 1x PBS was added and the samples spun again at 4 000g for 2 minutes. The liquid and debris was again removed. 150µl of 6% Chelex®100 suspension (Sigma, Germany) were added to the samples, using trimmed or wide bore pipette, and then they were covered with foil and heat sealed. The plate was then placed in a water bath for 20 – 25 minutes on a heated magnetic stirrer. The samples were again centrifuged at 4000g for 2mins to spin down the Chelex. The supernatant containing the DNA in aqueous solution (approx. 100µl) was taken off to a new plate and stored at -20^0C.

Amplification and genotyping of the *pfcrt* gene:

Genotyping of the *pfcrt* gene for polymorphisms C72S, M74I, N75E, and K76T was done in at least two multiplex real-time PCR assays with full agreement using the Rotorgene 3000 (Corbett Research, Australia) and primer sets and cycling conditions described elsewhere (Pearce *et al*., 2003) in the presence of the double-labelled probes representing **CVIET** (5'-TGT GTA ATT GAA ACA ATT TTT GCT AA-3'), **CVMNK** (5'-TGT GTA ATG AAT AAA ATT TTT GCT AA-3') and **SVMNT** (5'-AGT GTA ATG AAT ACA ATT TTT GCT AA-3'). 3D7, Dd2 and 7G8 DNA obtained from the Malaria Reference Laboratory of the London School of Hygiene and Tropical Medicine (LSHTM) was used as positive controls and nuclease-free water as negative control.

Amplification of the *pfmdr1* loci:

Polymorphisms at codons 86, 184, 1034, 1042 and 1246 of the *pfmdr1* gene, associated with chloroquine resistance, were determined by PCR and direct sequencing. Amplification of the *pfmdr1* gene was performed in two fragments (FR1 and FR2) using primer pairs and cycling conditions described earlier (Humphreys *et al*., 2007). However, for poor quality DNA that could not be successfully amplified in the long FR2, the fragment was further analysed in two smaller fragments (FR3 and FR4) using primer pairs and cycling conditions designed at the LSHTM laboratories and described elsewhere (Dlamini *et al*., 2010). All fragments of the *pfmdr1* gene further involved a nested PCR amplification step using the primers and conditions described by Dlamini *et al*., (2010) for FR3 and FR4 or described earlier (Humphreys *et al*., 2007) for FR1 and FR2.

Amplification of the *dhps* and *dhfr* loci:

A PCR amplification of the 711bp *dhps* and 594bp *dhfr* proteins, associated with resistance to sulphadoxine-pyrimethamine (Fansidar®), containing single nucleotide polymorphisms (SNPs) at codons 50, 51, 59, 108, 164 and at codons 436, 437, 540, 581 and 613 respectively, was performed in two steps (primary and nested PCR) according to methods, primers and cycling conditions described previously (Pearce *et al*. 2003). The primary PCR mix comprised: 15.3µl Nuclease free water, 2.5µl 10x KCl Reaction Buffer (Bioline, UK), 0.5µl 10mM dNTPs (Bioline, UK), 1.0µl 10µM Forward Primer, 1.0µl 10µM Reverse Primer (MWG, Germany), 0.2µl 5U/µl BIOTAQ DNA Polymerase and 5.0µl DNA product. The DNA samples, 3D7 and Dd2, were used as positive controls and water as negative control. The reagents were mixed and run in a Thermal Cycler (Thermo Scientific, USA) using the primers and cycling conditions: at 93^{o}C for 5 min, 41 cycles at 94^{o}C for 30s, 54^{o}C for 60s, and 65^{o}C for 60s, and a final single cycle of 65^{o}C for 5 min. The nested PCR step contained:

18.8µl Nuclease free water, 2.5µl 10x KCl Reaction Buffer, 0.5µl 10mM dNTPs, 1.0µl 10µM Forward Nested Primer and 1.0µl 10µM Nested Reverse Primer, 0.2µl 5U/µl BIOTAQ DNA Polymerase and 1.0µl DNA primary PCR product to make a final volume of 25µl. The reactants were placed in a Thermo Cycler and run at: 95^{o}C for 5 min, 30 cycles of 93^{o}C for 30s, 56^{o}C for 30s and 68^{o}C for 75s and a final cycle of 75^{o}C for 5 min.

Purification of PCR products:

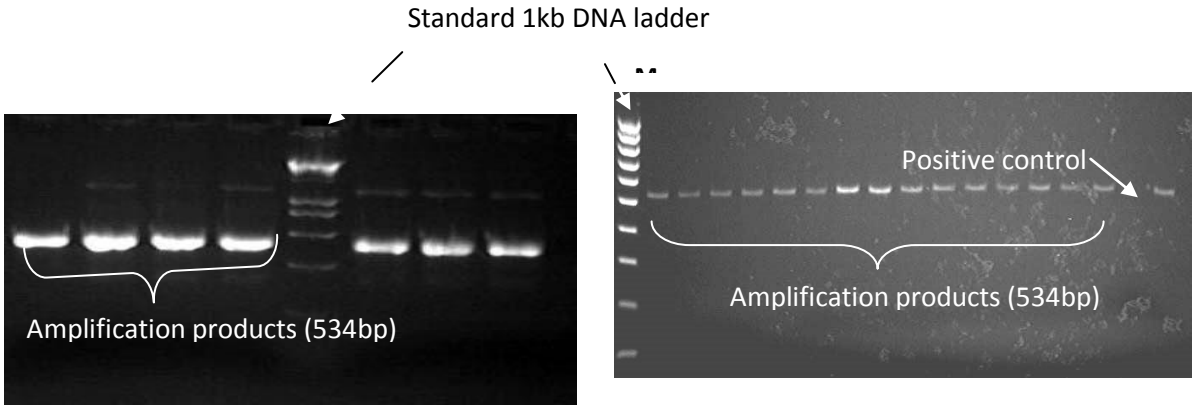
All PCR products of nested reactions were separated by gel electrophoresis on a 1.2% agarose gel stained with Ethidium bromide to identify amplified bands of DNA under ultra-violet illumination. Selected amplicons that were successfully amplified according to the gel electrophoresis result were purified using the QIAquick PCR Purification Kit (QIAGEN, UK) according to the manufacturer's instructions. Briefly, the QIAquick system is conveniently constructed to combine a spin-column and a selectively binding silica membrane in the presence of binding Buffer PBI. In the presence of high chaotropic salt concentrations, about 10µl of the DNA is adsorbed onto the silica membrane while contaminants pass through. Binding Buffer PBI provide correct chaotropic salt concentration and pH to optimise the recovery of DNA and removal of contaminants. The Buffer (PBI) contains a pH indicator (yellow) which allow easy determination of the optimal pH for DNA binding. Salts are quantitatively washed away by the ethanol-containing Buffer PE. Adsorption is about 95% when the pH is $\leq$7.5 and is reduced as the pH increases. DNA bound on the silica membrane was eluted with Tris buffer (Buffer EB) and the efficiency is determined by the salt concentration and pH of Buffer EB. Elution is most efficient at high pH and low salt concentrations.

Sequencing of purified PCR products:

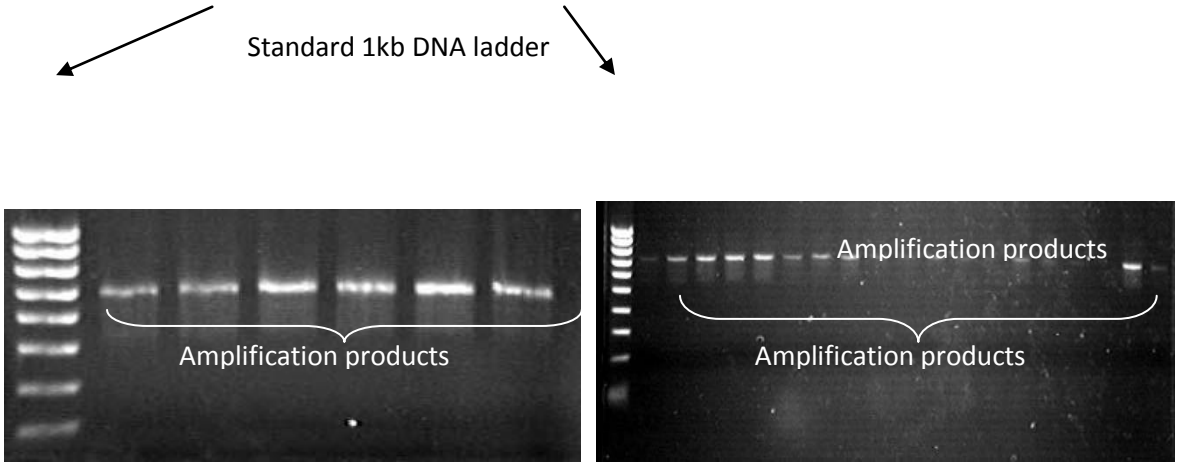
Purified PCR products were subjected to the BigDye Terminator sequencing using conditions and sequencing primer pairs discussed elsewhere (Dlamini *et al*., 2010).

RESULTS AND DISCUSSION

The presence and quality of amplified DNA is determined by running the amplified nested PCR products on a 1.2% agarose gel. The concentration of the DNA in the PCR product corresponds with the intensity of the band on the gel picture. The gel electrophoresis pictures below (Fig.1) compare, in terms of intensity, the quality of filter paper DNA PCR product with Giemsa-stained thick smear DNA PCR product.



Filter paper *pfmdr1* amplification products Giemsa-stained thick smear *pfmdr1* amplification products



Filter paper *pfdhps* amplification products Giemsa-stained thick smear *pfdhps* amplification products

Fig. 1 Gel electrophoresis pictures of PCR products

From the gel pictures, it can be deduced that good quality bands were obtained from Giemsa-stained thick smear sources of DNA, suggesting that good quality DNA was obtained from a majority of samples by the DNA extraction methods used. Some of the bands are comparable in terms of quality with those from whole blood filter paper sources. However, in a majority of samples, the filter paper samples show better quality of bands particularly for the *pfmdr1* gene.

Sixty-four (88%) of the thick-smear blood samples were successfully genotyped for polymorphisms at the 72-76 codons of the *pfcrt* gene using multiplex real-time PCR. Other genes were genotyped using standard PCR methods. The most difficult gene to genotype was the *pfdhps* gene, where only 27 (37%) samples were successfully genotyped. The complete results of all the genes are given below (Fig.2):

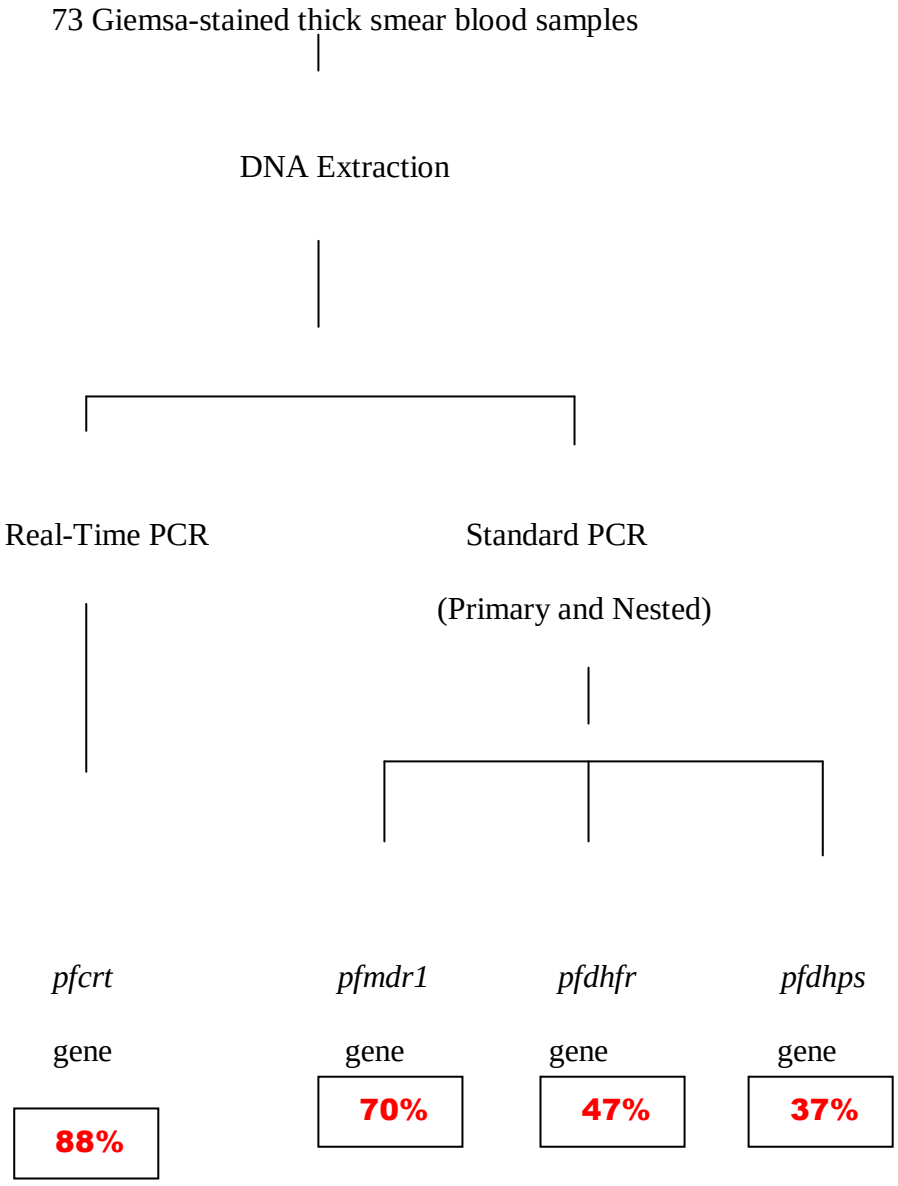


Fig. 2 Summary results of GSTS genotyping and sequencing experiments with *pfcrt*, *pfmdr1*, *pfdhfr* and *pfdhps* genes

The original amplification of Fragment 2 (FR2) of the *pfmdr1* gene yielded results for only 24/73 (33%) of the samples. The fragment was then analysed in a further two smaller

fragments (FR3 and FR4) and this resulted in an additional 27 to yield 51/73 (70%) of the samples being successfully genotyped. The *pfdhfr* and *pfdhps* genes were analysed in single fragments of 711bp and 594bp, respectively.

The PCR method is very useful for genetic studies on *Plasmodium* parasites, particularly those involving genetic markers of antimalarial resistance. The success of molecular analyses methods, including the PCR method, are influenced by the quality of DNA used for the analysis. This study has confirmed findings from earlier studies (Scopel *et al*., 2004) that filter paper DNA provides the best sensitiveness for PCR analysis. However, the analysis presented in this study raises an awareness of an alternative source of DNA that could yield useful results where filter paper samples cannot be obtained. This study has genotyped and sequenced DNA from GSTS that had been archived from routine practice i.e. without any intention to use in the future. Therefore, collection and storage of the samples may not have taken any precautions or followed specific guidelines to prevent DNA degradation and contamination. Preparation and storage of thick-smear blood samples is associated with the success of PCR amplification of a sample (Snounou *et al*., 1993; Barker *et al*., 1992). The success observed in this analysis is encouraging and suggests that countries with low or diminishing malaria transmission intensities can benefit from improving collection, labelling and storage of GSTS samples for further analysis in future to identify genetic changes in the parasite population.

This analysis performed genotyping experiments for polymorphisms in the *pfcrt*, *pfmdr1*, *pfdhfr* and *pfdhps* genes. The real-time PCR used for *pfcrt* analysis could be more sensitive than the nested PCR method, hence 88% of the blood smear samples were successfully analysed. Therefore, development of real-time PCR methods for the *pfmdr1* gene

could possibly improve the yield during analyses of this gene, probably resulting in improved yields even for Giemsa-stained samples. Recently, a real-time PCR was developed and evaluated on whole blood samples (Cnobs et al., 2010a) and Giemsa-stained thick blood samples (Cnobs et al., 2010b) that proved to be excellent in the detection of single and mixed species infections and showed a low detection limit. Scopel and colleagues (2004) compared the results of nested PCR using filter paper sources of DNA with GSTS sources. The study found 65% sensitivity and 93% specificity when DNA was obtained from GSTS, obviously showing lower sensitivity than DNA from filter paper sources. The variable sensitivity and high specificity of thick smear sources of DNA was further asserted to recently (Lee *et al*., 2009).

The *pfmdr1* gene achieved 70% success after Fragment 2 was split into two shorter fragments (FR3 and FR4). Unfortunately, the study reported here did not have the time and resources to design new primers for shorter portions of the *pfdhfr* and *pfdhps* genes, but the improvement achieved in the *pfmdr1* gene suggest that designing primer pairs that transcribe shorter regions of the *pfdhfr* and *pfdhps* genes has the potential to improve the result. Nonetheless, archived GSTS can reveal lots of information if re-analysed by PCR, suggesting that improved methods of collection, preparation and storage of thick blood films should be developed with the understanding that the films could be useful in the future when new diagnostic methods become available.

Recently in Malaysia, archived blood films that were diagnosed in 1996 as *Plasmodium malariae* using microscopy were again analysed using the nested PCR method (Lee *et al*., 2009). Out of 35 blood films that were positive with *Plasmodium*-specific primers, 35 (97.2%) were found to actually contain *P. knowlesi* DNA and only one contained *P.*

malariae DNA. Because microscopically diagnosed *P. knowlesi* infections are rare, prospective studies would not have revealed this important information which has also further strengthened the re-classification of *P. knowlesi* as also a human species of malaria. This Malaysian study further illustrates the volumes of information that may be revealed through analysis of carefully stored thick blood films. This study further strengthens the argument derived from the Malaysian study that stored GSTS samples offer material for retrospective studies from samples collected for routine diagnosis.

The effect of long-term storage of GSTS samples on the sensitivity of a PCR in detecting malaria is unknown. Chaorattanakawae *et al*., (2003) reported that the sensitivity of the PCR method increases with the length of time filter paper blood samples are stored. If this assertion is true, it suggests that there might similarly not be a significant reduction of the sensitivity of stored GSTS as previously thought by many researchers. Nonetheless, the samples in this study have been stored for 10 years and still yielded fairly satisfactory results, suggesting that DNA stored for 10 years can still yield good results particularly if cycling conditions and primers that transcribe shorter portions of the target genes are developed. Apart from the quantity of DNA yielded, the sensitivity of the PCR is also affected by the impurities in each sample (Panteleeff *et al*., 1999; Barker *et al*., 1992), particularly haeme which interferes with the PCR process (Akane *et al*., 1994). This study performed only a single purification step using the QIAquick PCR Purification Kit (QIAGEN, UK). This kit is widely used and yields good results from a single purification step. However, studies to determine the effects of repeated purification procedures are very few. Chaorattanakawae *et al*., (2003) reported significantly improved yields after three purification steps, suggesting that the results achieved during analyses of DNA from GSTS could possibly be further improved by increasing the number of purification steps.

CONCLUSION AND RECOMMENDATIONS

Despite the successful performance of the PCR on GSTS samples, whole blood samples remain the first choice for malaria diagnosis by PCR whenever possible. However, the results of this study suggest that GSTS samples could provide a solution for retrospective epidemiologic studies of drug resistance in situations where sufficient numbers of filter paper samples cannot be obtained or when not available at all. GSTS samples that are prepared for routine malaria diagnosis could be accumulated into large numbers to build statistical power during analyses. Laboratories should store their Giemsa-stained slides safely i.e. away from heat, direct sunlight and exposure to chemicals. Guidelines for proper collection and storage of GSTS slides should be developed in order to improve their usability for genotyping or other experiments many years later. Certainly, these slides should not be thrown away as was found to be the case with most laboratories in Swaziland. Protocols involving more robust and sensitive methods (such as multiplex real-time PCR) including appropriate primer pairs should be developed specifically to further improve the yield of genotyping experiments of GSTS samples. The advantage of real-time PCR is its higher analytical sensitivity when compared to standard PCR methods. Nonetheless, even with the existing methods, it has been proved that Giemsa-stained slides can provide lots of retrospective and accurate information where filter paper blood samples cannot be obtained.

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