

Full Length Research Paper

A PCR screen for malaria carrier infections using human saliva samples

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With endemic countries now aiming for elimination, the detection of malaria infections, with or without symptoms, is increasingly important for monitoring and evaluation programmes. Current malaria screening methods necessitate blood withdrawal. This invasive approach is constrained, especially for identifying the asymptomatic carrier reservoir, since segments of communities with blood taboos avoid participating. Proof of concept has previously been shown for molecular detection of malaria infection using human saliva samples. The current study optimized saliva-based malaria detection in an area of southern Zambia. Saliva pellet fractions proved a more reliable source of amplifiable parasite DNA compared to the soluble fraction. After optimizing DNA extraction and amplification, saliva-based PCR showed 94.1% sensitivity and 97% specificity, using nested PCR on blood samples as gold standard. This study demonstrates that saliva samples are a reliable non-invasive alternative to blood for the PCR detection of asymptomatic and submicroscopic malaria reservoirs.

Key words: Malaria, saliva, polymerase chain reaction.

INTRODUCTION

Malaria remains a major killer disease accounting for nearly 800,000 deaths in 2009 (WHO, 2010). The development of accurate diagnostic tests that are appropriate for use in under-developed countries ravaged by malaria is important. Traditional diagnostic methods such as microscopy are beset with limitations. For example, experienced microscopists tend to detect malaria at a density not less than 500 parasites/ μ l in routine laboratory tests (Milne et al., 1994). The advent of polymerase chain reaction (PCR) based methods for the detection of malaria has seen a huge improvement in malaria detection levels 1000 times higher than data from microscopy (Schoone et al., 2000). Indeed, PCR conducted on blood samples also reportedly detects the parasite 5 days before the parasite can be detected by

experienced microscopists (Andrews et al., 2005). Some of the disadvantages of PCR based methods in malaria diagnosis include risk of contamination, the requirement of a thermal cycler and electricity to conduct the test. However, the advent of loop-mediated isothermal amplification (LAMP) which has capability of amplifying DNA at a constant temperature holds promise as a useful field technique for application in the diagnosis of malaria (Polley et al., 2010).

The use of blood in the diagnosis of malaria is problematic due to several factors. Amongst these is the fact that infected erythrocytes tend to be sequestered away from circulation at certain stages in the development of malaria (Delley et al., 2000). Further-more, drawing of blood for clinical purposes is fraught with challenges as this procedure must be conducted by skilled personnel who are not always readily available in remote locations of most countries affected by malaria. In addition, subjects tend to shy away from donating blood for clinical purposes due to fear of accidentally

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contracting infectious diseases such as HIV/AIDS and in some cultures donating blood is a cultural and religious taboo. As endemic countries are moving towards malaria elimination, this poses a major constraint, since measurement of malaria infections, with or without symptoms is now increasingly important in monitoring and evaluation programmes. Blood-based malaria testing approaches tend to miss asymptomatic, submicroscopic infections that are capable of sustaining transmission (Schneider et al., 2007; Gahutu et al., 2011), since individuals that are not sick are even less likely to participate in invasive surveys. For this reason, several studies have of late been focusing on detecting malaria in saliva, urine and surface mucosa by amplifying parasite DNA in these fluids (Mharakurwa et al., 2006; Nwakanma et al., 2009; A-Elgayoum et al., 2010).

Data based on PCR conducted on saliva show more reliability in malaria detection than tests conducted on urine samples (Mharakurwa et al., 2006; Nwakanma et al., 2009). Saliva is a fluid that occurs in the oral cavity and is made up of numerous constituents, mostly secretory products from various sources such as salivary glands, as well as blood-derived compounds (Lima et al., 2010). Water constitutes 90% of saliva, and the content of the dissolved components in saliva vary from time to time in the same individual and indeed these constituents vary across individuals. As testimony to its richness in organic constituents, saliva is known to contain at least 400 proteins (Lima et al., 2010). The use of saliva as material for disease diagnosis lends itself to the fact that saliva glands occur at interfaces that connect them with blood vessels, thus facilitating exchange of materials with the circulatory system.

Therefore, it is not surprising that residues of material from human pathogens end up in saliva. One of the first studies to demonstrate the availability of DNA from human pathogens in saliva involved the detection of hepatitis B virus DNA in saliva at a concentration comparable to levels in blood (van der Eijk et al., 2004). It is still unknown how DNA from malaria parasites ends up in saliva. It is further unclear whether whole parasite cells debris or parasite cells end up in saliva or whether it is merely parasite DNA that diffuses from blood into human saliva. In addition, the reliability of PCR as a tool for the detection of malaria in saliva needs to be optimized and to be demonstrated to work consistently across various populations.

Furthermore, the conditions under which the saliva samples are stored from point of source seem to influence the sensitivity of the test (Buppan et al., 2010). Additionally, inconsistent data where PCR conducted on malaria DNA derived from saliva, urine and buccal mucosa could not be used to distinguish between mixed parasites that were confirmed present by PCR conducted on parasite DNA derived from blood samples (El-Rayah et al., 2010). It has previously been suggested that the sensitivity of PCR for the detection of malaria in saliva could be improved by using primers targeting short

amplicons (300 bp or less) as the DNA template is less prone to degradation (Mharakurwa et al., 2006). This study sought to establish the optimal fraction of saliva (between the pellet and soluble fractions) for sourcing reliable template DNA for use in PCR. Furthermore, the study also established whether shorter amplicon primers targeting the 'pfdhfr' gene (Mharakurwa et al., 2006) would yield different PCR detection sensitivity to longer amplicon primers.

MATERIALS AND METHODS

Study area and population

The study samples were collected from the vicinity of Macha, located in the Zambian southern province. Natural malaria transmission at Macha is hyperendemic, the major vectors being *Anopheles arabiensis* and *Anopheles funestus*. In the study area the collection of blood samples during major malaria surveys or research, frequently led to tension and reduced community participation, due to recurrent suspicion, usually about Satanism (associated with blood). The development of simple bloodless alternative sampling approaches would lead to greater community participation for research and control of the disease.

Sample collection

Samples used in this study were collected during peak malaria transmission seasons between year 2008 and 2009. Willing participants from headmen areas in the vicinity of Macha were screened for malaria by microscopy. A total of 88 participants were recruited for this study, 41% males and 59% females with an age range between 3 months and 99 years (mean = 29.6 years; median = 18 years), following full explanation of the objectives, procedures, risks and benefits of the study. In the case of young children, informed consent was sought from the parents or guardians. The study excluded individuals with severe and complicated malaria or complicated medical conditions as recommended (WHO, 2003). Data collection was conducted using structured questionnaires. Axillary temperature was taken to monitor if the participants had pyrexia. The history of any disease symptoms and drug intake in the past 48 h for all adult participants were recorded. For active recruitment, only individuals resident in the selected headman area were enrolled. In passive enrolment, only individuals living within walking distance from the hospital, who were able and willing to return for study follow-up were included. Thick films and filter paper (Whatman® No 3 MM) blood blots were collected. Microscopic slides were examined at MIAM by an experienced microscopist followed by feedback to the community and treatment for confirmed malaria cases. On the day of feedback, whole saliva samples (5 ml) was collected from all willing positive and negative individuals in sterile tubes.

The saliva specimens were later aliquoted into 1 ml replicate amounts in microcentrifuge tubes at the laboratory and either immediately extracted or stored at -20°C for later extractions (Figure 1).

DNA extraction

Whole saliva samples of 500 µl were centrifuged in 1.5 ml microcentrifuge tube for 3 min at 20,000 g. The entire soluble saliva fraction was aspirated out without disturbing the pellet; the supernatant was collected into a new sterile tube. DNA was extracted from the pellet fraction and the soluble fraction of saliva

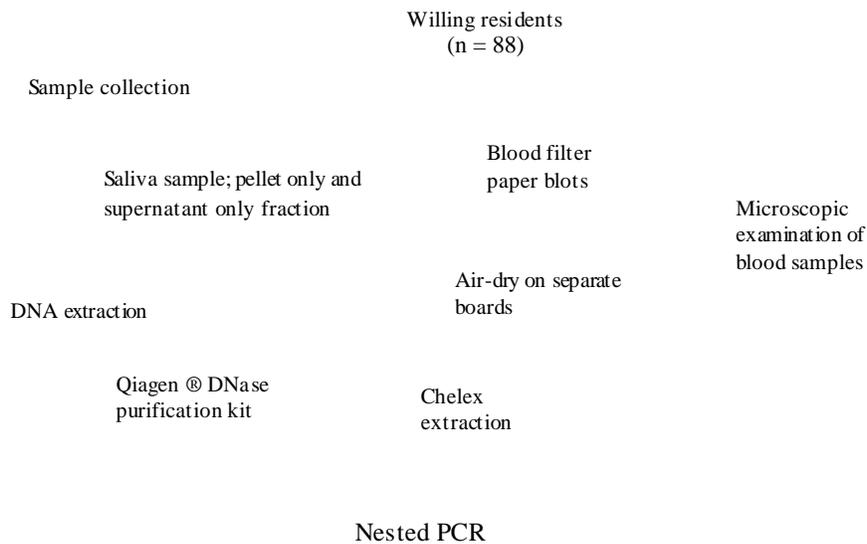


Figure 1. Overall project design; the flow chart shows the data and sample collection methods followed.

respectively using the crude cell lysates protocol following the manufacturer's (Qiagen DNeasy® purification kit) instructions. The final DNA elution was carried out in 50 µl volumes. Blood spots collected on filter paper were extracted using the Chelex method (Plowe et al., 1995).

Nested PCR amplification

Plasmodium falciparum was detected from blood and saliva DNA extracts by nested PCR following a previously described procedure (Mharakurwa et al., 2006). Briefly all PCR 'pfdhfr' amplifications consisted of initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 43.4°C for 45 s and extension at 65°C for 1 min. The final extension step was at 65°C for 2 min. Both primary and secondary PCR reactions comprised 1.2 µl template, 0.25 µM primers, 1.5 mM magnesium chloride, 200 µM dNTP's, 1X PCR Buffer and 0.6 U of Taq DNA polymerase in 15 µl volumes. Two sets of PCR primers were used that define fragments on the 'pfdhfr' domain. The first set were standard primers (M1[3..23]: 5'-TTTATGATGGAAACAAGTCTGC-3'/M5[625-645]: 5'-AGTATA CATCGCTAACAGA-3') for the primary round (643 bp product), followed by (F [144..172]: 5'-GAAATGTAATCCCTAGATATGGAATAAT-3'/M4 [439..469]: 5'-TTAATTTCCCAAGTAAACTATTAGAGCTT-3') for the secondary amplification (326 bp product) as described by Duraisingh et al. (1998). The second set of primers, defining shorter amplicon fragments were (U1[121..143]: 5'-GGAAATAAAGGAGTATTA CCA TG-3'/U2[372..393]: 5'-TAAGGTTCTAGACAA TATAACA-3') for the primary round (273 bp product), followed by (U3 [144..172]: 5'-GAAATGTA TTTCCCTAGATATGgAATATT-3'/U4 [351..372]: 5'-ATTTA TCCTA TTGCTTAAAGGT-3') for the secondary amplification (229 bp product).

The PCR amplifications were performed in a Thermo Electron® PX2 (HBPX2) thermal cycler. The PCR amplicons obtained were resolved by electrophoresis on 1.5% agarose gels stained in ethidium bromide and visualised by UV transillumination on a 1D

Kodak (EDAS 290) imaging system.

Data analysis

Diagnostic performance was measured by calculating sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV) and receiver operating characteristic (ROC) area under the curve (AUC) calculated using MedCalc® version 9.6.2.0 (MedCalc Software, Mariakerke, Belgium). Pearson's chi-square (χ^2) test of association was used to evaluate the strength of association between various tests. The degree of agreement was interpreted as follows: poor (<0.20) to very good (0.81 to 1) based on Kappa interpretation (Cohen, 1968).

Ethics

The study was approved by University of Zambia research ethics committee and University of Zululand ethics committee. Study permission was sought from local chiefs and headmen in whose area the study was conducted. Patient participation was obtained through the consent of the patients themselves or guardians where minors were concerned.

RESULTS

A total of 88 samples (blood and saliva) were collected and analyzed from willing individuals who participated in this study. The age range was from 3 months to 99 years old (mean = 29.6 years; median = 18 years). All the participants of this study were asymptomatic, none of the examined volunteers had a body temperature reading above 38°C nor was there any below 35°C on the day of examination. When the blood extracts were subjected to

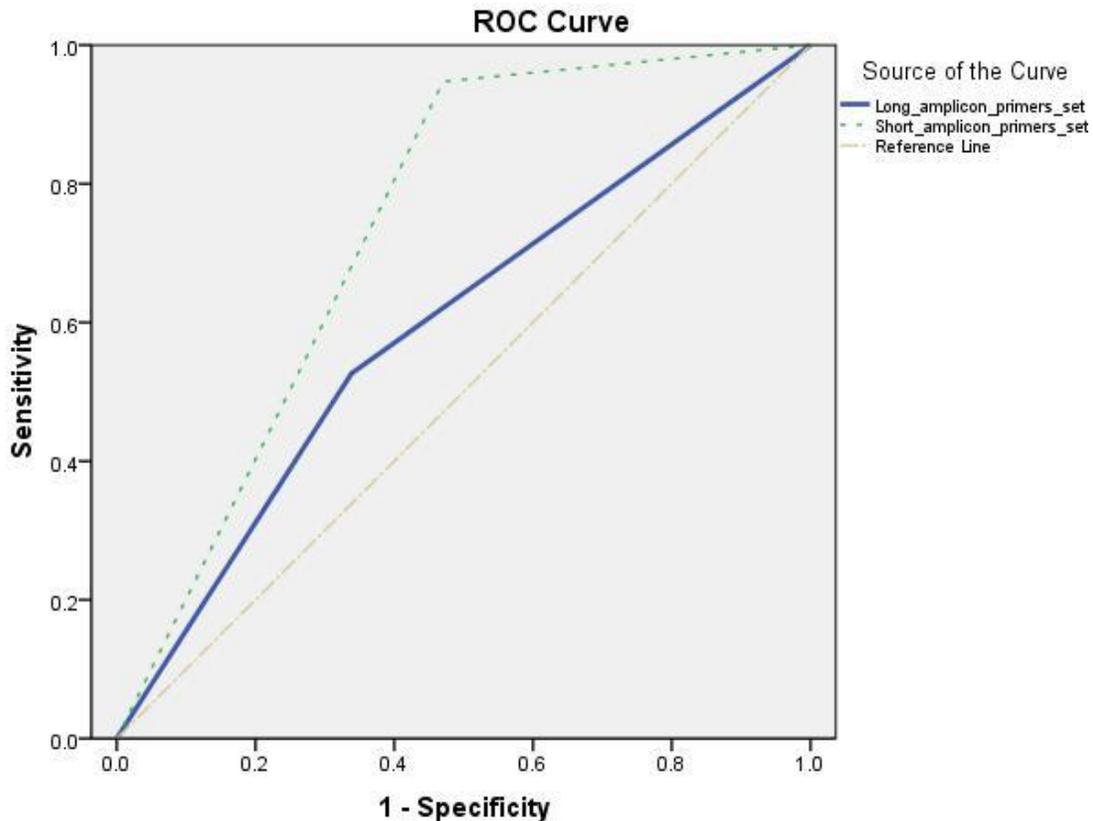


Figure 2. ROC curve on long amplicon and short amplicon primers. The curves in the graph were generated from nested PCR amplifications with long (solid bold line) and short (dotted line) amplicon primers and the diagonal reference line (broken line).

PCR amplification with the long amplicon primers, a total of 33 (37.5%) samples were confirmed to be *P. falciparum* positive. As could be expected, PCR had a higher detection sensitivity than microscopy which confirmed only 13 (14.7%) positives. However, short amplicon primers were able to detect 51 (57.95%) *P. falciparum* infections, 31 (35.23%) were submicroscopic and 18 (20.45%) were below long amplicon primer detection limit. Short amplicon primers were more sensitive (95%) as compared to long amplicon primers (52.63%) in amplifying *P. falciparum* DNA, using microscopy results as reference standard. AUC and ROC curve analysis were conducted to determine the visual indices for the accuracy of conducting PCR using the long primers compared to the short primers (Figure 2). The further the curve lies above the diagonal reference line, the greater the accuracy of the primer set used, the short amplicon primer set curve lies the furthest from the reference line as compared to the long amplicon primer set curve (Figure 2). Short amplicon primers had a greater AUC and therefore were more accurate when compared to long amplicon primers; 0.740, $p = 0.0005$ and 0.594, $p = 0.2176$ respectively. These results support claims previously noted by Mharakurwa et al. (2006) that

short amplicon primers enhance the sensitivity for *P. falciparum* DNA amplification.

Human saliva can be used as a source of amplifiable malaria parasite DNA in infected patients as noted by previous investigators (Mharakurwa et al., 2006; Nwakanma et al., 2009; Al-Elgayoum et al., 2010). To determine the fraction of saliva harbouring the malaria amplifiable DNA, saliva samples obtained from the 42 *P. falciparum* positive human participants and three negative cases, all samples were separated into two distinct fractions, the soluble and insoluble fractions. After centrifugation, DNA was extracted from the saliva insoluble and soluble fractions and then subjected to nested PCR in separate batches. The amplified products were resolved by gel electrophoresis (Figure 3). The short amplicon primers were subsequently used for all PCR amplifications. Furthermore, we separated the pellet fraction of saliva from the soluble fraction. We then purified DNA from these fractions using the Qiagen kit. DNA amplified from the pellet only fraction had higher amplification success than DNA isolated from the soluble fraction of saliva. The sensitivity and specificity values achieved using DNA derived from saliva pellet fraction compared to DNA isolated from blood were 94.12 and

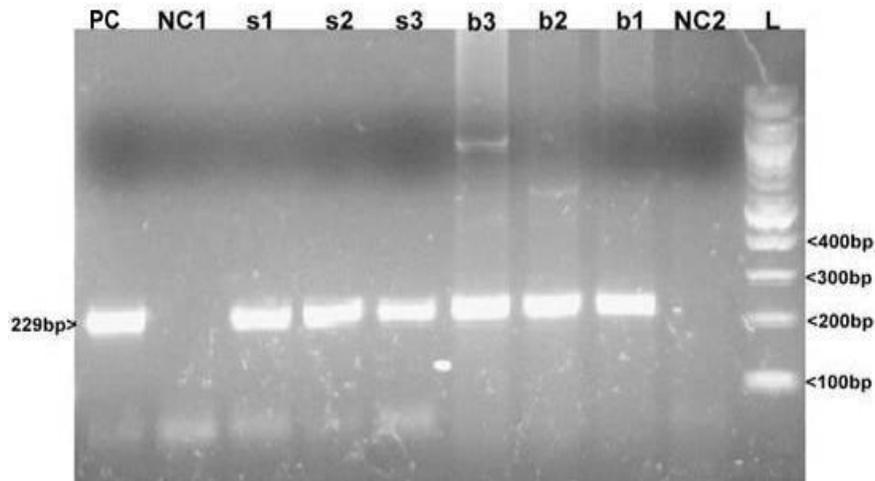


Figure 3. Amplified *Plasmodium falciparum* DNA extracted from saliva and blood. Saliva and blood DNA extracts from three individuals subjected to nested PCR amplification. Amplicons from saliva (s1, 2 and 3) and blood (b1, 2 and 3) samples obtained from three different individuals. Lane PC represents positive laboratory control (pfdhfr gene amplicon), 229 bp; NC1 and 2 lanes represents negative controls for saliva and blood amplicons respectively.

Table 1. Diagnostic performance comparing nested PCR results for saliva with blood samples as reference standard.

	PCR on DNA derived from pellet fraction
Sensitivity	94.12%
Specificity	97.3%
Positive predictive value	97.96%
Negative predictive value	92.31%
Kappa value (κ)	0.907

97.3%, respectively (Table 1).

DISCUSSION

PCR is expected to generally have an elevated sensitivity when compared to microscopy especially in detecting parasitemia at levels undetectable to microscopy (Snounou et al., 1993). Results obtained from PCR amplification on blood samples were superior to those obtained by thick film microscopy. Some of the samples that were thick film-negative were reported to be positive by PCR for *P. falciparum* infection. In the current study at least 35.23% of the samples were reported as positive for malaria infection using PCR (based on short amplicon primers), compared to 14.7% reported as positive using microscopy. A similar finding was reported where microscopy detected a total of 350 *P. falciparum* infections while PCR detected a further 331 *P. falciparum* submicroscopic infections (Zurovac et al., 2006). According to Bejon et al. (2006), true parasite counts are likely to be under-estimated by microscopy due to the

staining procedure, which may account for poor microscopy performance. In order for endemic countries to effectively manage malaria interventions, asymptomatic infection and latent disease reservoirs need to be closely monitored so as to avoid potential resurgences. The nested PCR diagnostic method can be useful for detection of latent malaria carrier infections. As previously proposed by Mharakurwa et al. (2006), short amplicon primers used in this study had a higher amplification rate as compared to long amplicon primers targeting the same region on the 'pfdhfr' domain. Consequently, the former were more reliable for detecting latent infections. It might be possible that at low level of parasite density, long amplicon primers were less reliable than short amplicon primers because of the poor recovery of good quality parasite DNA.

Parasite DNA may be degraded during storage and transportation of samples leading to low amplification sensitivity when using long amplicon primers. In addition, the acidity of the buccal acidity may also promote degradation of parasite DNA, leading to poor amplicon yields. However, mere primer annealing properties may

also lead to such differences, regardless of amplicon size. We further spun the saliva to obtain pellet and soluble fractions. We sought to understand which of the two fractions of saliva would provide better quality of amplifiable parasite DNA. Based on the nested PCR findings, DNA extracted from the pellet fraction was a more reliable template for the PCR test. It is possible that *P. falciparum* DNA is introduced into human saliva through ruptured RBCs or DNA from parasites trapped in macrophages (Kaufman and Lamster, 2002). The DNA amplified in the supernatant portion of saliva may be due to trace amounts of pellet material taken up with supernatant during sample preparation and extraction. However, further study is still required to clarify how malarial DNA is transported to saliva of malaria infected patients. Through optimising the nested PCR recipe on saliva DNA extracts, this study was able to achieve high amplicon yields than previously reported (Buppan et al., 2010), relative to DNA purified from blood samples. In this study, DNA derived from human saliva samples using the Qiagen extraction kit displayed 94% sensitivity and 97% specificity, using regular PCR on blood as the gold standard. Buppan et al. (2010), suggests that saliva samples preserved in ethanol yielded superior positive PCR results when compared to samples kept on ice. However, the absence of ethanol preservation in this study does not appear to have negatively affected PCR amplification. A very good agreement ($\kappa = 0.907$) was observed for DNA derived from saliva using the Qiagen extraction kit relative to DNA purified from blood samples. This study confirms the reliability of saliva as an alternative source for malaria infection diagnosis which may be adopted for large scale malaria screenings (Bremam et al., 2004). In this study, only 15.9% of the volunteers had a history of malaria-related symptoms. Thus, under normal circumstances the 84.1% asymptomatic patients would not have gone for malaria testing, since they did not experience any need to seek medical services.

To progress towards the goal of effective malaria control and eventual elimination, the accurate identification of asymptomatic parasite carriers who are often sources of perpetual disease transmission is vital. The use of non-invasive saliva-based screening affords an accurate and fundamentally more pragmatic approach for maximizing community participation, especially in detecting foci of asymptomatic reservoir infections. Obviating the use of sharps or needles, blood drawing and associated community taboos, healthy infection carriers and vulnerable groups alike are far more likely to cooperate in surveys for operational research and control programmes.

Conclusion

Our study shows an accurate and reliable method for the PCR detection of malaria using saliva samples as an alternative to blood samples. This non-invasive approach affords fundamental practical advantages on safety,

community participation and minimizing bias in large community surveys with or without need for repeated testing such as drug or vaccine efficacy trials. Future studies could examine the use of loop-mediated isothermal amplification (LAMP) and exploring possibilities of migrating from the bench-top to a point-of-care device such as a biosensor chip. This would introduce sensitive tools for surveillance and targeting of asymptomatic reservoirs of infection in malaria control and elimination programmes.

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