

Molecular methods for tracking residual *P. falciparum* transmission in a close-to-elimination setting in Zanzibar

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Research

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Abstract

Background Molecular detection of low-density *Plasmodium falciparum* infections is essential for surveillance studies conducted to inform malaria control strategies in close-to-elimination settings. Molecular monitoring of residual malaria infections usually requires a large study size, therefore sampling and diagnostic processes need to be economical and optimized for high-throughput. In a method comparison, we aimed at identifying the most efficient diagnostic procedure for processing large collections of community samples with optimal test sensitivity, simplicity, and minimal costs.

Methods In a reactive case detection study conducted on Zanzibar, parasitemia of 4590 individuals of all ages was investigated by a highly sensitive quantitative (q) PCR that targets multiple var gene copies per parasite genome. To reduce cost, a first round of positivity screening was performed on pools of dried blood spots from five individuals. Ten cycles of a pre-PCR were performed directly on the filter paper punches, followed by qPCR. In a second round, samples of positive pools were individually analyzed by pre-PCR and qPCR.

Results Prevalence in household members and neighbors of index cases was 1.7% (78/4590) with a geometric mean parasite density of 58 parasites/ μ l blood. Using qPCR as gold standard, diagnostic sensitivity of rapid diagnostic tests (RDTs) was 37% (29/78). Infections positive by qPCR but negative by RDT had mean densities of 15 parasites/ μ l blood.

Conclusion The approach of pre-screening reactive case detection samples in pools of five was ideal for a low prevalence setting such as in Zanzibar. Performing direct PCR on filter paper punches saves substantial time and justifies the higher cost for a polymerase suitable for amplifying DNA directly from whole blood. Molecular monitoring in community samples provided a more accurate picture of infection prevalence compared to RDT results alone, by identifying a reservoir of infection which is largely missed by RDT. qPCR as a research and surveillance tool can great benefits the evaluation of strategies or programs to eliminate malaria.

Background

Surveillance is a key component of malaria control and elimination programmes (Malaria Surveillance, Monitoring & Evaluation: A Reference Manual. 2018). Surveillance and response approaches require specific adaptation to the epidemiological and operational setting in which they are implemented. Depending on the exact objective of the surveillance activities, suitable data collection strategies and diagnostic tools may vary. A key interest is to understand population prevalence as a proxy for the transmission reservoir and transmission potential in a certain area. A number of publications have presented mathematical models and gametocyte data from endemic communities (Gonçalves et al. 2017; Bradley et al. 2018; Gruenberg et al. 2019; A. Björkman et al. 2019; Churcher et al. 2013), all concurring in that, in addition to symptomatic malaria cases, also individuals with asymptomatic as well as submicroscopic infections contribute to the infectious reservoir. This silent reservoir is currently under

investigation in many malaria endemic countries, and malaria elimination programs aim to uncover and potentially track this source of transmission. To understand the relative importance of submicroscopic infections, their prevalence and densities are investigated in community samples of low parasite density. This is feasible by using molecular diagnostic methods. These methods are employed with the expectation that such data from the community may help to investigate residual transmission in close-to-elimination settings.

At low endemicity malaria is known to cluster geographically and the exposure of individuals to malaria infection may vary substantially within a village and over time (Bousema et al. 2012; N. E. Hofmann et al. 2017; Bejon et al. 2014). To capture such heterogeneity in case distribution, asymptomatic malaria cases are identified in a reactive case detection (RCD) approach (Stresman et al. 2010; 2015). RCD is triggered by patients reporting to a health facility and diagnosed with malaria, based on laboratory confirmation. These index cases prompt a visit to the household of the patient (and sometimes to neighboring households) to identify additional malaria infections, most of which are expected to be asymptomatic (Yukich et al. 2017). The RCD strategy generally entails a targeted response, such as treating individuals of identified transmission foci. Thus, implementation of RCD aids in the containment of local epidemics of malaria and may help to control onward transmission from imported infections. Modeling indicated that RCD seems to be a promising approach to control residual malaria by complementing non-targeted interventions with targeting additional interventions or to support elimination in areas where the transmission potential is very low (Chitnis et al. 2019).

In pre-elimination settings, such as our study site in Zanzibar, passive case surveillance and elimination strategies further struggle with the fact that symptomatic cases are rare. On the other hand, active and reactive case detection include asymptomatic individuals, but parasite densities tend to be very low and difficult to detect with routinely applied diagnostic tools such as RDTs or light microscopy (LM). Meta-analyses comparing prevalence rates determined by PCR versus LM, have demonstrated that the proportion of submicroscopic *P. falciparum* infections in community samples substantially increases with declining malaria transmission intensity (Okell et al. 2009; 2012). This trend was confirmed in the past few years by numerous molecular-epidemiological studies (Morris et al. 2015; Niang et al. 2017; Nguyen et al. 2018; Zemene et al. 2018; Jiram et al. 2019; Okell et al. 2012; Shekalaghe et al. 2007). Also earlier RCD pilot projects have shown that molecular diagnostic contributes a more comprehensive picture of the infectious reservoir than would be possible by malaria RDT or LM (Stresman et al. 2015).

An extensive subpatent reservoir of malaria infections has major consequences for malaria surveillance activities, in particular in pre-elimination settings, where the aim is to interrupt local transmission. Recent data from a pre-elimination setting in Zambia showed that almost half of all infections remained undetected by RDT (Kobayashi et al. 2019). About a quarter of these infections were subpatent by RDT but carried gametocytes. To measure the magnitude of the infection reservoir missed in household surveys by employing RDTs, the current diagnostic tool for RCD, we undertook a molecular-epidemiological study in a close-to-elimination setting on Zanzibar. This study was nested into a larger project (Reactive Case Detection in Zanzibar: System Effectiveness and Cost, RADZEC) to evaluate the

effectiveness of RCD in Zanzibar. Study details and epidemiological results were presented elsewhere (Stuck et al. submitted, van der Horst et al. submitted). Here we focus on the development and evaluation of an efficient diagnostic strategy for large numbers of samples collected during RCD in a pre-elimination setting. As samples collected from asymptomatic carriers in such settings mostly harbor low parasite densities, high diagnostic sensitivity and high throughput was the priority. In a low transmission setting with only few infected individuals in the community, molecular-epidemiological studies requires screening of a large number of samples with high diagnostic sensitivity to identify the remaining infected individuals. A reduction of work load may be achieved by a sample pooling strategy prior to molecular diagnostics (Taylor et al. 2010; Hsiang et al. 2010). Applying a multi-target molecular test is a precondition for both, pooling of several samples without losing sensitivity, and detection of asymptomatic, low-density infections. A quantitative PCR (qPCR) that targets the *P. falciparum* var gene family (varATS us-qPCR) was previously developed to permit pooling without losing sensitivity (N. Hofmann et al. 2015). The aims of this study therefore were to identify a time-efficient strategy for pooling multiple DBS samples, to simplify DNA extraction, and to develop a diagnostic method with high sensitivity and robust quantitation in detecting submicroscopic parasitemia.

Methods

Study design

Since 2008, the Zanzibar Malaria Elimination Program (ZAMEP) has been implementing a RCD system (Ashton et al. 2019; van der Horst et al. submitted). The RADZEC project represents a rolling cross-sectional survey, whereby field data collectors accompanied district malaria surveillance officers (DMSOs) during follow-up visits to the households of malaria index cases who were detected at health facilities and flagged up through an electronic malaria case notification system (index cases). After the DMSO finished with their investigation of the index household and departed, study staff continued surveying the four nearest neighbors and 5 households along a 200 meter transect drawn in a random direction away from the index case household. Details on the study design were described previously elsewhere (Stuck et al. submitted). The samples used in this molecular analysis were collected between June 1, 2017 and August 13, 2018.

The molecular epidemiological study consisted of a subsample of 156 clusters from the full rolling cross sectional study, each representing a follow-up investigation of an index case in two selected study districts on Pemba and Unguja islands of the Zanzibar archipelago, Tanzania, in 2017–2018. From a large sample collection, clusters were selected for molecular analyses which contained samples from at least seven households. Each cluster included members of the index case household, of the four nearest neighboring households and five transect households. A total of 664 residents of index case households were included, as well as 1'955 members of neighboring and 1'971 of transect households. The index cases themselves were not included as they already had received treatment at the health facility 1 to 2 days prior to the follow-up household visit.

Sample collection, storage and transport

During the follow-up household visits, usually within 5 days after the reporting of an index case, capillary blood was collected by finger prick from all consenting household members and neighbors older than 3 months. A malaria RDT (SD BIOLINE Malaria Ag Pf/Pan (Abbott)) was performed for on-the-spot malaria diagnosis. The DMSO treated all RDT-positive individuals within the index household according to the national guidelines. Individuals testing positive in non-index households, in which the DMSO was not present, were referred to the nearest health facility. A 100–150 μl whole blood sample was directly added to a pre-folded Whatman 3MM filter paper. Blood spots were dried in the field and then packed in individual plastic bags with desiccant and humidity indicator papers. Dried blood spots (DBS) were unpacked and re-dried after transport to the study office, re-packed with desiccant and humidity indicator paper, sealed in a zip-lock plastic bag and thereafter stored at room temperature.

Preparation of dried blood spots with known parasite densities

For assay development and optimization, *P. falciparum* strain 3D7 was cultured in vitro and parasitemia was determined by microscopy. To mimic an infected blood sample 3D7 culture was diluted in malaria-negative whole blood to parasite densities ranging from 0.05 parasites/ μl to 10^4 parasites/ μl . Negative controls consisted of whole blood from an uninfected volunteer. To simulate the conditions of DBS collected in the RADZEC study, blood of this volunteer was spotted in 50 μl aliquots onto Whatmann 3MM filter paper, air dried overnight and stored at room temperature in plastic bags with desiccants.

Comparison of DNA extraction by chelex, glassmilk, boil-and- spin to direct pre-PCR

For DNA extraction, five 3 mm discs were punched from the DBS using a hand-held paper craft punch. varATS qPCR (details described below) was used to compare four different DNA extraction methods for DBS: (i) chelex extraction was performed according to Plowe and coworkers, with minor modifications (Plowe et al. 1995). (ii) For boil-and-spin extraction, punches were incubated over night at 4 °C in 0.5% Saponin/phosphate buffered saline (PBS). Saponin was removed after incubation and punches were washed twice by adding 1 ml of PBS, inverting tubes several times, spinning down briefly and removing PBS. Punches were transferred into a clean 1.5 ml Eppendorf tube and centrifuged briefly to collect and remove any remaining liquid. 50 μl ddH₂O was added and samples were boiled at 95 °C for 30 min. Tubes were centrifuged for 5 min at 14'000 rpm. 25 μl of DNA solution was transferred into a new tube. (iii) Boil and spin plus glassmilk purification: After boil-and-spin extraction, DNA was purified using glassmilk (MP Biomedicals) according to the supplier's instructions. (iv) Direct amplification of *P. falciparum* DNA in pre-PCR (details of final protocol described below) did not require any processing of punches from DBS. Four alternative polymerases for direct pre-PCR were tested during assay optimization: Phusion Blood Polymerase (Thermo Fisher Scientific), 2X KAPA HiFi HotStart (KAPA

Biosystem), Hemo KlenTaq Polymerase (NEB), MyTaq DNA Polymerase (Bioline). Protocols and test results are provided in Supplementary material: Tables S1-S10.

Molecular diagnosis in community samples

For detection of *P. falciparum* infections, samples collected from RDT-negative household members were screened by pre-PCR/qPCR using a two-step process. First, pools of five samples were screened using one 3 mm punch per sample (one punch is equivalent to 3–4 µl of whole blood). In a second step, samples from positive pools were screened individually using five 3 mm punches per sample (equal to 15 µl of whole blood). Samples from high- and low-positive pools (above/below 10 parasites/µl) were processed separately. Samples from RDT-positive household members and from index cases were not included in the sample pools, but directly screened individually in pre-PCR/qPCR using five 3 mm punches.

Direct on-DBS pre-PCR: *P. falciparum* DNA was amplified directly from DBS punches using Phusion Blood Direct PCR Kit (Thermo Fisher Scientific). Primers targeted the conserved C-terminal region of the multi-copy var gene family (N. Hofmann et al. 2015). A 55 µl reaction contained 1x Phusion Blood Buffer supplemented with 150 µM nucleotides and 450 nM forward and reverse primers (final concentration), 1 µl Phusion Blood Polymerase and five 3-mm punches. Cycling conditions were 98 °C for 5 min, followed by 10 cycles of 98 °C for 15 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds. Pre-amplified PCR products were diluted 1:50 and used as template in varATS qPCR.

Quantitative PCR: varATS qPCR was performed using 4 µl of diluted pre-amplification product or DNA extracted by various approaches during comparison of extraction methods. The 12 µl reaction contained 1x GoTaq Probe Mastermix (Promega), 833 nM forward and reverse primer, and 416 nM probe (final concentrations). Cycling conditions in an ABI StepOne System were 95 °C for 2 min, 45 cycles of 95 °C for 15 seconds and 55 °C for 1 min. Parasite density was determined using a 10-fold dilution row of the WHO 1st international standard for *P. falciparum* DNA Nucleic Amplification Techniques (NIBSC), ranging from 4880 to 0.048 parasites/ul (the two lowest concentrations were run in duplicate). For direct on-DBS pre-PCR followed by qPCR, this DNA standard was included in qPCR analysis starting from the pre-amplification step. In order to ensure equal amplification conditions of standard DNA and DBS samples, one 3-mm punch from a malaria-negative DBS was added to the pre-amplification reactions containing the standard DNA dilution. Two types of negative controls were used for all analyses: (i) parasite-negative DBS processed alongside samples through all extraction and amplification procedures, and (ii), 4 µl ddH₂O added as blank to qPCR mix.

Reproducibility, limit of detection (LOD), introduction of cut-off of parasite quantification by pre-PCR/qPCR

Reproducibility

Intra- and inter-assay variance, assessed by calculating the coefficient of variation for Ct values (CV, expressed in %) were determined by testing replicate DNA dilution rows of the WHO 1st international standard for *P. falciparum* DNA Nucleic Amplification Techniques (NIBSC), supplemented with negative DBS punches as described above. Intra-assay variation was determined using 5 replicates of eight serially diluted samples (corresponding to 48800, 4880, 488, 48.8, 4.88, 2.44, 0.488, and 0.244 parasites/ μ l), supplemented with 5 negative DBS punches, within a single pre-PCR/qPCR run. Inter-assay variation was determined using six serially diluted samples (corresponding to 4880, 488, 48.8, 4.88, 0.488, and 0.048 parasites/ μ l), supplemented with one negative DBS punch, in 28 separate pre-PCR/qPCR runs. Limit of Detection (LOD): The LOD was determined separately for the two PCR rounds: (i) screening of sample pools (one 3 mm DBS punch per sample in the pool) and (ii) testing of individual samples (using five 3 mm DBS punches). For this purpose, serial dilutions of the WHO DNA standard (NIBSC) were made and combined with punches from DBS impregnated with uninfected human whole blood. Pre-PCR and qPCR were performed in quintuple on 12 dilutions (corresponding to 48800, 4880, 488, 48.8, 4.88, 2.44, 0.488, 0.244, 0.122, 0.048, 0.024, and 0.0048 parasites/ μ l). The LOD was determined using 3 μ l and 15 μ l of DNA, supplemented with 1 and 5 negative DBS, respectively, which represents the LODs using 1 and 5 punches from field sample DBS. A probit model was used to produce a regression line based on experimental replicates of the dilution series. The LOD was 1.12 parasites/ μ l for 3 μ l of DNA (CI95 [0.39–27.81]) and 0.13 parasites/ μ l (CI95 [0.07–2.32]) for 15 μ l DNA (Fig. 1). Hence, using five-fold more template material increased sensitivity 8.6-fold.

Cut-off setting

Since qPCR positivity for a sample of low parasitemia strongly depends on a stochastic distribution of scarce parasites on the DBS, a positivity cut-off based on the LOD was introduced. The selected LOD of 0.13 parasites/ μ l permits detection of a parasite infection with 95% sensitivity when pools of 5 punches per DBS are analyzed. This cut-off value for positivity was applied for all diagnostic assays performed on community samples.

Results

Comparison of extraction/amplification methods for DBS

In search of the best suitable protocol for processing pooled DBS from large community surveys, the most sensitive and time-efficient combination of extraction and amplification was identified. To this end, dilutions of cultured 3D7 parasites in whole blood, ranging from 10^4 parasites/ μ l to 0.05 parasites/ μ l, were spotted onto filter papers. These test samples were analyzed by chelex extraction, boil-and-spin extraction, boil-and-spin extraction followed by glassmilk purification, and pre-PCR performed directly on DBS punches (direct pre-PCR) (Figure 2) followed by varATS qPCR. Direct pre-PCR was the most sensitive method, giving positive results in 3/4 replicates of very low parasite densities (0.1 parasites/ μ l – 0.05 parasites/ μ l) with least variation in measured densities between replicates. The direct pre-PCR also represents the least laborious method, as it requires no processing of DBS before PCR analysis. The

second- and third- best methods were chelex extraction and boil-and-spin extraction, respectively. The least sensitive method was boil-and-spin followed by glassmilk purification. Because direct pre-PCR proved the fastest and most sensitive of all tested methods, this approach was chosen as standard procedure for the RADZEC RCD samples. In a well-controlled laboratory set-up, where the dangers of contamination, arising from performing a pre-PCR, are customarily monitored tightly, the gain in sensitivity and ease overrides the controllable and low risk of false positive results.

Validation of parasite quantification

Prior to analyzing DBS from study participants, we validated parasite quantification when performing direct-on-DBS PCR. A 10-fold dilution row of 3D7 parasites in whole blood spotted onto filter paper was analyzed in parallel to a 10-fold dilution row of the WHO 1st international standard for *P. falciparum* DNA Nucleic Amplification Techniques (NIBSC). To ensure that dilutions of WHO DNA standard and of 3D7 parasites were amplified under the same conditions, we mimicked DBS by supplementing the WHO standard dilutions with DBS impregnated with uninfected blood for the pre-PCR step. This was necessary, as pilot experiments had shown that the Phusion mastermix amplified purified DNA more efficiently when a negative DBS was added to the mastermix than purified DNA alone (**Supplementary material: Figure S1 and Figure S2**). This may be due to an optimization of Phusion direct blood kit specifically for DNA amplification in the presence of blood. Our experiments showed a qPCR efficiency of only 83% when the WHO standard DNA alone was pre-amplified in Phusion mastermix. In contrast, efficiency was 94% for DBS punches carrying the 3D7 culture dilution. Addition of a *P. falciparum*-negative DBS punch to pre-PCRs with WHO standard DNA restored qPCR efficiency to 100% (**Figure S1**).

To assess the reproducibility of quantification, intra- und inter-assay coefficients of variation (CV) were determined by serial dilutions of the WHO standard DNA (**Table 1A and 1B**). Intra-assay CV of replicates ranged between 0.39-5.65% for different dilutions. These experiments used serially diluted DNA samples (in quintuplicate, supplemented with negative DBS) using 3µl of DNA (equal to one punch per DBS). When 15µl of DNA (equal to five punches per DBS) were used, intra-assay CV ranged from 0.64-2.49%. Results were equally reproducible between runs, with inter-assay CV using 3µl of DNA in 28 replicates (equal to one punch per DBS) ranging between 0.93-3.49% for the different dilutions.

Table 1: Reproducibility of *P. falciparum* quantification by qPCR. A. Intra-assay variation of varATS standard curve determined via Ct-values of eight serially diluted DNA samples (in quintuplicate, supplemented with negative DBS) using either 3µl of DNA (equal to one punch per DBS) or 15µl of DNA

(equal to five punches per DBS). B. Inter-assay variation of varATS standard curve determined via Ct-values of six serially diluted DNA samples (supplemented by negative DBS) using 3µl of DNA (equal to one punch per DBS) in 28 replicates.

A

Parasites/µl	Mean (Ct)		SD		%CV	
	3 µl	15 µl	3 µl	15 µl	3 µl	15 µl
48800	19.19	18.05	0.15	0.45	0.81	2.49
4880	20.63	19.41	0.06	0.25	0.28	1.26
488	24.34	22.68	0.12	0.24	0.50	1.06
48.8	27.58	25.95	0.12	0.17	0.45	0.64
4.88	31.12	29.76	0.12	0.25	0.39	0.83
2.44	31.91	30.40	0.63	0.49	1.99	1.62
0.48	35.61	32.83	2.01	0.46	5.65	1.39
0.24	34.98	33.78	1.12	0.98	3.20	2.89

B

Parasites/µl	Mean (Ct)	SD	%CV
4880	20.39	0.22	1.07
488	23.72	0.26	1.09
48.8	27.17	0.25	0.93
4.88	30.47	0.38	1.25
0.488	33.35	0.9	2.68
0.048	34.76	1.21	3.49

Sensitivity after applying a robust density cut-off

Because many study participants carried very low parasite densities, we tested the reproducibility of parasite detection specifically in low-density field samples by repeating molecular diagnosis in independent replicates. Starting from the original DBS and using 5 DBS punches per sample, triplicates were analyzed for 10 field samples with less than 0.1 parasites/ μl , thus all representing samples that were positive but with densities below the cut-off set at 0.13 parasites/ μl . We confirmed positivity for all 10 samples in at least one of the triplicates. For 6/10 samples at least 2 replicates were positive. These results confirm that such very low-density infections detected by our method are true positives and emphasize how much the detection of such infections by qPCR is determined by stochastic distribution of template in the starting material. Despite the repeated detection of low-positives with densities below 0.1 parasites/ μl , we opted to introduce a cut off for qPCR-positivity at 0.13 parasites/ μl , i.e. the LOD at which samples are detected with 95% probability. This cut-off may exclude some very low positive samples.

The stochastic distribution of parasite in samples with very low densities concentrations also affected our sample screening strategy: samples were first screened in pools of 5 samples with 1 DBS punch per sample, while the follow-up screens were performed on individual samples using 5 DBS punches, the latter aimed at reducing the stochastic effects. To understand the consequences of the stochastic parasite distribution when pooling 5 field samples for initial screening, we assessed the proportion of samples missed by this pooling strategy. For this purpose, we randomly selected 48 pools, corresponding to 240 individual samples, which all had been qPCR-negative in the first screening round. These 240 originally negative samples were screened individually using five punches per DBS. Before applying the cut-off for positivity, 18 low-positive individual samples (7.5%; 18/240) we found with densities of 0.01 parasites/ μl – 2.03 parasites/ μl . When applying the chosen cut-off of 0.13 parasites/ μl , 10 low-positive individuals (4.2%; 10/240) with densities of 0.13–2.03 parasites/ μl remained positive and 8 individuals were below the cut-off of 0.13 parasites/ μl and to be considered negative. Thus, in this small subset of samples, we showed empirically a loss in positivity by both, the cut-off for density and the stochastic distribution of low parasite densities on the DBS. Nevertheless, to maintain consistency in the database, all 18 positive samples newly identified in this additional experiment performed with in 48 originally negative pools were recorded negative in the final database as per protocol for community screening and analysis.

Quality assurance for processing DBS from areas of very low prevalence and density

In order to validate the risk of cross-contamination on pre-PCR/qPCR plates, we repeated: (i) 22 samples with densities below 8 parasites/ μl derived from RDT-positive household members of symptomatic index cases. These were repeated (in triplicate) because all samples from RDT-positive individuals, some of which with potentially very high parasite densities representing a risk for cross-contamination, were screened on a common PCR plate. All 22 low-positive samples were confirmed in independent repeat

analyses. (ii) We also repeated molecular diagnostics on 7 RDT-negative field samples that had densities below 2 parasites/ μl and had been identified in pools that contained a high-positive sample. Positivity was confirmed for all these samples. No further replicates were performed because of limited sample material.

Prevalence of infection in community members from 156 clusters

Direct on-DBS pre-PCR was performed on samples from 4'590 individuals derived from 156 clusters, each triggered by an index case. 664 individuals belonged to the households of index cases, the remainder belonged to neighboring and transect houses.

By RDT, 0.7% of all individuals were *P. falciparum*-positive. Positivity by qPCR was 1.7% (Table 1). Of 33 RDT-positive individuals, 29 were confirmed positive by qPCR. RDT detected 37% (29/78) of qPCR-positive samples.

Table 1: Number of study participants that were *P. falciparum* positive or -negative by RDT and qPCR.

	RDT result	qPCR result
Negative	99.3% (4'557/4'590)	98.3% (4'512/4'590)
Positive	0.7% (33/4'590)	1.7% (78/4'590)

The sensitivity of RDT compared to qPCR as gold standard was 37%, while the specificity was 100% (Table 2). Most individuals with a *P. falciparum* density below 100 parasites/ μl were RDT-negative, whereas the majority of those with a density of 100 parasites/ μl or higher were RDT-positive (Figure 3).

Table 2: Comparison of RDT results with qPCR results.

		RDT negative	RDT positive	Total
qPCR negative	Frequency	4'508	4	4'512
	<i>Percentage</i>	<i>99.9</i>	<i>0.09</i>	<i>100</i>
qPCR positive	Frequency	49	29	78
	<i>Percentage</i>	<i>62.8</i>	<i>37.2</i>	<i>100</i>
Total	Frequency	4'557	33	4'590

Discussion

Sensitive detection and accurate quantification of low-density *P. falciparum* infections from DBS has become increasingly important in the context of describing residual malaria transmission in close-to-elimination settings. In areas of highly heterogeneous transmission it is of interest to identify risk factors of residual infections and to understand the infectious reservoir in the population. In low transmission settings most DBS will yield a negative qPCR result; therefore, a pooling strategy may reduce costs substantially (Hsiang et al. 2010, 2012, N. Hofmann et al. 2015). To provide optimal protocols for large-scale molecular-epidemiological studies in a pre-elimination setting, we developed a simple but sensitive method combining pooling, extraction and amplification. The optimal strategy consisted of direct pre-PCR of pooled 3-mm punches from DBS, followed by varATS qPCR.

Despite using a single DBS punch in a pool of 5 samples, we were able to reliably identify 1 parasite/ μ l blood in a dilution series where DBS were reconstituted with WHO parasite density standard mixed with uninfected blood. The ultra-sensitive qPCR assay applied in this study targets multiple copies of *P. falciparum* var genes. A higher number of templates per parasite contributes to robust results in low-density infections and pools of these (N. Hofmann et al. 2015). Nonetheless, the uneven distribution of the parasites in blood spotted on DBS poses an additional limiting factor. Hence, the sensitivity of our method might be slightly overestimated, as the WHO density standard was added as a DNA solution directly into the PCR mastermix, thus more even distribution of templates compared to spotting parasites. This discrepancy cannot be bypassed, because the use of an international reference standard is indispensable for inter-laboratory comparisons. The level of sensitivity of our method complies with the malERA consultative group-recommended detection limit for malaria pre-elimination settings of 1 parasite/ μ l blood (malERA Consultative Group on Diagnoses and Diagnostics 2011). Another previously published pooling approach used chelex extraction followed by cytochrome b nested PCR and reported a 100% sensitivity to detect a single positive sample with a density of 100 parasites/ μ l blood in a pool of 5 samples, and a 80% sensitivity to detect an infection of 10 parasites/ μ l in pooled analysis (Hsiang et al. 2010). Compared to this previous study, our limit of detection was substantially more sensitive with 95% sensitivity to detect 1.12 parasites/ μ l blood on a single positive DBS punch screened in pools of five punches, i.e. together with 4 negative DBS. As both studies use multi-target qPCRs, this difference seems

to be due to the chelex extraction used in this previous study. The chelex protocol has the advantage of being low-cost, but its disadvantage consists in a dilution of DNA in a larger volume than the original volume of blood, thus, sensitivity is compromised. It should be emphasized that for standardization sake, we measured both, LOD and CV, using the WHO standard DNA mixed with negative blood on DBS. The difference between introducing DNA or parasites into a reaction is that DNA in solution is more evenly distributed than when all target copies were contained in parasites. Owing to multi-copy genomic targets of our varATS qPCR assay, the values for LOD and CV could prove slightly less sensitive, if whole parasites were analyzed.

The advantage of direct on DBS pre-PCR is that the time-consuming extraction and purification of DNA is omitted, such as an overnight incubation in saponin as required in the chelex extraction. Introduction of pre-PCR permitted to introduce the highest possible concentration of parasite DNA into the amplification reaction. All alternative methods tested would have introduced less template into the qPCR mix, as pre-PCR overcomes loss of DNA during the extraction process. The major disadvantage of performing pre-PCR directly on filter paper was its high potential for contamination. Ten cycles of pre-amplification directly on DBS harbors dangers, mainly because it requires transfer of amplified product from pre-PCR into a second reaction tube or plate for qPCR. Such an open tube system requires utmost care because amplicons can potentially be transferred via aerosols or spills to neighboring wells leading to false positive results. The risk of contamination increases with increasing parasite density on the DBS. This risk requires an upscale in safety measures and controls as well as an adequate laboratory set up. In this study, we have minimized the risk for contamination by dedicated rooms for master mix preparation, punching of samples, handling of post amplification product, and final qPCR reaction setup. Importantly, surfaces were decontaminated by exposure to UV light and bleach prior to and after completion of pipetting. Several negative controls were included in pre-PCR as well as qPCR to monitor any contamination. One way to further minimize cross-contamination, also employed in this study, was to analyze pools with high parasite concentrations (including all cases of symptomatic malaria) separately from low-density samples.

A relevant consideration in a molecular-epidemiological study is that pooling of samples from several individuals trades off test-sensitivity against the potential for high-throughput processing. This is because pooling of DBS reduces sensitivity of parasite detection because of a smaller blood volume processed. Due to space limits in reaction tubes, only five punches of 3 mm diameter corresponding to 3 μ l blood each could be processed using our method. When samples were analyzed individually, all five punches derived from one DBS, whereas for analysis of sample pools, only one punch per DBS could be processed.

When we evaluated the difference in LOD between the analysis of one sample (5 punches per DBS) versus a pool of 5 samples (one punch each), 8.6-fold loss in sensitivity was observed for pooling compared to individual screening. The thus reduced sensitivity by processing pools is in the sensitivity range of current molecular diagnostic assays used for screening of community samples. Using the varATS us-qPCR compensated partly for the loss of sensitivity through pooling.

For processing a large set of samples from low-transmission settings as in this study, performing an initial screen on sample pools was necessary. When evaluating the potential to miss samples through pooling, we found that 7.5% (18/240) low-positive samples would be gained by individual analysis, 10/240 (4.2%) would be positive above the cut-off. These numbers highlight the limitations in large studies and reporting the potential for false negatives is relevant. However, in the context of investigating the extent of the asymptomatic parasitemia in the community, there is no need to identify the full depth of the subclinical reservoir, as such very low-density infections are unlikely transmitted (N. E. Hofmann et al. 2018).

This equally applies to definition of a density cut-off. We have introduced a cut-off for qPCR-positivity of 0.13 parasites/ μ l blood to compensate for the variance caused by stochastic distribution of scarce parasites. Using the cut-off of 0.13 parasites/ μ l leads to omission of all low positive samples that would not be detected with certainty of less than 95%. Although we could show that samples below this cut-off were detected in some independent replicates, we opted to create a very robust data set with records of positive samples that would be reproduced if repeatedly analyzed.

Earlier Mass Screening and Treatment (MSAT) campaigns relying on RDT-based or LM-based diagnosis have not produced convincing results: A study in Burkina Faso found no sustained effect on incidence of anti-malarial treatment of asymptomatic *P. falciparum* carriers after three screening and treatment campaigns (Tiono et al. 2013). A population-wide malaria testing and treatment with RDTs and artemether-lumefantrine in southern Zambia, an area with heterogeneous transmission, showed an overall modest impact on decreasing the malaria infection burden (Larsen et al. 2015). A recent study in Indonesia reported similar results; after two or three rounds of MSAT using microscopy, little or no impact on malaria incidence was found (Sutanto et al. 2018). Such little effect on incidence and prevalence is likely due to the large proportion of missed low-density infections, which will sustain transmission despite treatment of RDT-positive infections. A recent study in Zambia, performed in a close-to-elimination setting, showed that almost half of all PCR-diagnosed infections remained undetected by RDT, and about a quarter of these RDT-negative infections carried gametocytes and therefore may be infectious to mosquitoes (Kobayashi et al. 2019).

Our results gained in Zanzibar are in line with the previous observations of additionally detected *P. falciparum* infections by PCR (Anders Björkman et al. 2017; Morris et al. 2015; Shakely et al. 2013). In our study, the absolute numbers of infections gained by performing us-qPCR were small, with 45 infections detected additionally to RDT-positive individuals in a total of 4590 blood samples screened. RDT detected only 29 of the 78 qPCR-positive individuals and had a diagnostic sensitivity of 37%. Thus, to inform targeted response interventions, such as focal testing and treatment, RDT alone might not be sufficiently sensitive. However, it remains to be shown by additional epidemiological analyses of our data whether both diagnostics reveal the same epidemiological patterns and risk factor for infection in the various household types. Performing molecular diagnostics in the framework of elimination research represents a relevant expansion into a not yet well characterized, potential reservoir of infection.

In recent years, increasingly sensitive diagnostic methods uncovered a large proportion of low-density infections. The relevance of infections of very low densities to malaria transmission is under investigation in many endemic areas (Bousema et al. 2014). An unexpected large reservoir of asymptomatic parasite infections was identified by analyzing large blood volumes (Imwong et al. 2014) combined with us-qPCR (N. E. Hofmann et al. 2018). Malaria transmission modeling suggested that asymptomatic infections including submicroscopic infections may contribute to ongoing malaria transmission (Churcher et al. 2013). The reason for a significant relevance of asymptomatic infections is a much higher proportion of low-density infections compared to that of high-density clinical episodes. The data on infectivity available so far collectively suggest that sensitivity of LM and RDT does not suffice to detect the asymptomatic reservoir (Tadesse et al. 2018; Gonçalves et al. 2017). Several authors suggested that diagnostic methods applied in the field should be adjusted to more sensitive detection of low-density infections (Britton, Cheng, and McCarthy 2016; Cook et al. 2016; Mahende et al. 2016).

In routine surveillance, only cheap and simple-to-use methods, such as LM or RDT, are generally available. Although our new approach simplifies malaria diagnosis from DBS and supports high throughput screening, molecular diagnostic for programmatic use and routine implementation does not seem realistic currently, mainly due to lack of funding, capacity and appropriate laboratory set-up. Molecular parasite detection, on the other hand, is very useful as a research tool for gaining knowledge on foci of residual malaria or the silent reservoir of transmission, as well as for informing mathematical modeling.

In conclusion, we developed and applied a qPCR-based pooling approach that allows high-throughput and ultra-sensitive detection of *P. falciparum* DNA from DBS. This diagnostic approach applies pre-PCR amplification, which circumvents DNA extraction and facilitates pooling of five samples, but at the same time increases the risk of contamination. Thus, a laboratory set-up dedicated to PCR work is essential. This approach is suitable to quantify low-density *P. falciparum* infections and to generate accurate prevalence data to monitor intervention success or guide targeted interventions. While the presented diagnostics is primarily an efficient research tool, it may also be of use for fast parasite characterization in focal transmission or surveillance-response activities.

Abbreviations

DBS
dried blood spot
LM
light microscopy
MCP
malaria control programme
MEEDS
Malaria Epidemic Early Detection System
PCR
polymerase chain reaction

Pf-varATS

P falciparum var gene acidic terminal sequence

qPCR

quantitative PCR

RADZEC

Reactive Case Detection in Zanzibar: Effectiveness and Cost

RCD

reactive case detection

RDT

rapid diagnostic test

ZAMEP

Zanzibar Malaria Elimination Programme

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the institutional review boards of Tulane University and IHI, from the Zanzibar Medical Research Advisory Committee and from the Ethics Committee of Northwest and Central Switzerland (EKNZ). Written informed consent was obtained from all study participants or their caregivers prior to data and sample collection.

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Consent for publication

All authors declare their consent to publish the manuscript and agree to the proposed authorship order.

Availability of data and material

All data generated or analyzed during this study are included in this published article or accompanying supplementary files. The full data set can be made available upon request to M. Hetzel.

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Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

B.S.F. and A.A. performed and supervised field work

B.G., A.H., K.A.O., N.H. and I.F. performed laboratory work and wrote the manuscript

M.W.H., J.Y. and L.S. contributed to conception, study design, supervision of field work and helped to edit the manuscript.

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References

Ashton, Ruth A., Adam Bennett, Abdul-Wahid Al-Mafazy, Ali K. Abass, Mwinyi I. Msellem, Peter McElroy, S. Patrick Kachur, et al. 2019. "Use of Routine Health Information System Data to Evaluate Impact of Malaria Control Interventions in Zanzibar, Tanzania from 2000 to 2015." *EClinicalMedicine* 12 (July): 11–19. <https://doi.org/10.1016/j.eclinm.2019.05.011>.

Bejon, Philip, Thomas N Williams, Christopher Nyundo, Simon I Hay, David Benz, Peter W Gething, Mark Otiende, et al. 2014. "A Micro-Epidemiological Analysis of Febrile Malaria in Coastal Kenya Showing Hotspots within Hotspots." Edited by Mercedes Pascual. *ELife* 3 (April): e02130. <https://doi.org/10.7554/eLife.02130>.

Björkman, A., D. Shakely, A. S. Ali, U. Morris, H. Mkali, A. K. Abbas, A-W Al-Mafazy, et al. 2019. "From High to Low Malaria Transmission in Zanzibar—Challenges and Opportunities to Achieve Elimination." *BMC Medicine* 17 (1): 14. <https://doi.org/10.1186/s12916-018-1243-z>.

Björkman, Anders, Jackie Cook, Hugh Sturrock, Mwinyi Msellem, Abdullah Ali, Weiping Xu, Fabrizio Molteni, Roly Gosling, Chris Drakeley, and Andreas Mårtensson. 2017. "Spatial Distribution of Falciparum Malaria Infections in Zanzibar: Implications for Focal Drug Administration Strategies Targeting Asymptomatic Parasite Carriers." *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 64 (9): 1236–43. <https://doi.org/10.1093/cid/cix136>.

Bousema, Teun, Jamie T. Griffin, Robert W. Sauerwein, David L. Smith, Thomas S. Churcher, Willem Takken, Azra Ghani, Chris Drakeley, and Roly Gosling. 2012. "Hitting Hotspots: Spatial Targeting of Malaria for Control and Elimination." *PLoS Medicine* 9 (1). <https://doi.org/10.1371/journal.pmed.1001165>.

Bousema, Teun, Lucy Okell, Ingrid Felger, and Chris Drakeley. 2014. "Asymptomatic Malaria Infections: Detectability, Transmissibility and Public Health Relevance." *Nature Reviews. Microbiology* 12 (12): 833–40. <https://doi.org/10.1038/nrmicro3364>.

Bradley, John, Will Stone, Dari F. Da, Isabelle Morlais, Alassane Dicko, Anna Cohuet, Wamdaogo M. Guelbeogo, et al. 2018. "Predicting the Likelihood and Intensity of Mosquito Infection from Sex Specific Plasmodium Falciparum Gametocyte Density." *ELife* 7. <https://doi.org/10.7554/eLife.34463>.

Britton, Sumudu, Qin Cheng, and James S. McCarthy. 2016. "Novel Molecular Diagnostic Tools for Malaria Elimination: A Review of Options from the Point of View of High-Throughput and Applicability in Resource Limited Settings." *Malaria Journal* 15 (February). <https://doi.org/10.1186/s12936-016-1158-0>.

Chitnis, Nakul, Peter Pemberton-Ross, Josh Yukich, Busiku Hamainza, John Miller, Theresa Reiker, Thomas P. Eisele, and Thomas A. Smith. 2019. "Theory of Reactive Interventions in the Elimination and Control of Malaria." *Malaria Journal* 18 (1): 266. <https://doi.org/10.1186/s12936-019-2882-z>.

Churcher, Thomas S, Teun Bousema, Martin Walker, Chris Drakeley, Petra Schneider, André Lin Ouédraogo, and María-Gloria Basáñez. 2013. "Predicting Mosquito Infection from Plasmodium Falciparum Gametocyte Density and Estimating the Reservoir of Infection." *ELife* 2 (May). <https://doi.org/10.7554/eLife.00626>.

Cook, Jackie, Lynn Grignard, Samira Al-Eryani, Mustafa Al-Selwei, Abraham Mnzava, Hafed Al-Yarie, Alison Rand, Immo Kleinschmidt, and Chris Drakeley. 2016. "High Heterogeneity of Malaria Transmission and a Large Sub-Patent and Diverse Reservoir of Infection in Wusab As Safil District, Republic of Yemen." *Malaria Journal* 15 (April). <https://doi.org/10.1186/s12936-016-1249-y>.

Gonçalves, Bronner P., Melissa C. Kapulu, Patrick Sawa, Wamdaogo M. Guelbéogo, Alfred B. Tiono, Lynn Grignard, Will Stone, et al. 2017. "Examining the Human Infectious Reservoir for Plasmodium Falciparum

Malaria in Areas of Differing Transmission Intensity.” *Nature Communications* 8 (1): 1133. <https://doi.org/10.1038/s41467-017-01270-4>.

Gruenberg, Maria, Natalie E. Hofmann, Elma Nate, Stephan Karl, Leanne J. Robinson, Kjerstin Lanke, Thomas A. Smith, Teun Bousema, and Ingrid Felger. 2019. “Molecular and Immunofluorescence-Based Quantification of Male and Female Gametocytes in Low-Density *P. Falciparum* Infections and Their Relevance for Transmission.” *The Journal of Infectious Diseases*, August. <https://doi.org/10.1093/infdis/jiz420>.

Hofmann, Natalie E., Maria Gruenberg, Elma Nate, Alice Ura, Daniela Rodriguez-Rodriguez, Mary Salib, Ivo Mueller, et al. 2018. “Assessment of Ultra-Sensitive Malaria Diagnosis versus Standard Molecular Diagnostics for Malaria Elimination: An in-Depth Molecular Community Cross-Sectional Study.” *The Lancet. Infectious Diseases* 18 (10): 1108–16. [https://doi.org/10.1016/S1473-3099\(18\)30411-0](https://doi.org/10.1016/S1473-3099(18)30411-0).

Hofmann, Natalie E., Stephan Karl, Rahel Wampfler, Benson Kiniboro, Albina Teliki, Jonah Iga, Andreea Waltmann, et al. 2017. “The Complex Relationship of Exposure to New Plasmodium Infections and Incidence of Clinical Malaria in Papua New Guinea.” *ELife* 6. <https://doi.org/10.7554/eLife.23708>.

Hofmann, Natalie, Felista Mwingira, Seif Shekalaghe, Leanne J. Robinson, Ivo Mueller, and Ingrid Felger. 2015. “Ultra-Sensitive Detection of Plasmodium Falciparum by Amplification of Multi-Copy Subtelomeric Targets.” *PLoS Medicine* 12 (3): e1001788. <https://doi.org/10.1371/journal.pmed.1001788>.

Hsiang, Michelle S., Jimmie Hwang, Simon Kunene, Chris Drakeley, Deepika Kandula, Joseph Novotny, Justin Parizo, et al. 2012. “Surveillance for Malaria Elimination in Swaziland: A National Cross-Sectional Study Using Pooled PCR and Serology.” *PloS One* 7 (1): e29550. <https://doi.org/10.1371/journal.pone.0029550>.

Hsiang, Michelle S., Michael Lin, Christian Dokomajilar, Jordan Kemere, Christopher D. Pilcher, Grant Dorsey, and Bryan Greenhouse. 2010. “PCR-Based Pooling of Dried Blood Spots for Detection of Malaria Parasites: Optimization and Application to a Cohort of Ugandan Children.” *Journal of Clinical Microbiology* 48 (10): 3539–43. <https://doi.org/10.1128/JCM.00522-10>.

Imwong, Mallika, Sarun Hanchana, Benoit Malleret, Laurent Rénia, Nicholas P. J. Day, Arjen Dondorp, Francois Nosten, Georges Snounou, and Nicholas J. White. 2014. “High-Throughput Ultrasensitive Molecular Techniques for Quantifying Low-Density Malaria Parasitemias.” *Journal of Clinical Microbiology* 52 (9): 3303–9. <https://doi.org/10.1128/JCM.01057-14>.

Jiram, Adela Ida, Choo Huck Ooi, José Miguel Rubio, Shamilah Hisam, Govindarajoo Karnan, Nurnadiah Mohd Sukor, Mohd Mafie Artic, Nor Parina Ismail, and Nor Wahida Alias. 2019. “Evidence of Asymptomatic Submicroscopic Malaria in Low Transmission Areas in Belaga District, Kapit Division, Sarawak, Malaysia.” *Malaria Journal* 18 (1): 156. <https://doi.org/10.1186/s12936-019-2786-y>.

Kobayashi, Tamaki, Mufaro Kanyangarara, Natasha M. Laban, Masiliso Phiri, Harry Hamapumbu, Kelly M. Searle, Jennifer C. Stevenson, Philip E. Thuma, William J. Moss, and for the Southern Africa International Centers of Excellence for Malaria Research. 2019. "Characteristics of Subpatent Malaria in a Pre-Elimination Setting in Southern Zambia." *The American Journal of Tropical Medicine and Hygiene* 100 (2): 280–86. <https://doi.org/10.4269/ajtmh.18-0399>.

Larsen, David A., Adam Bennett, Kafula Silumbe, Busiku Hamainza, Joshua O. Yukich, Joseph Keating, Megan Littrell, John M. Miller, Richard W. Steketee, and Thomas P. Eisele. 2015. "Population-Wide Malaria Testing and Treatment with Rapid Diagnostic Tests and Artemether-Lumefantrine in Southern Zambia: A Community Randomized Step-Wedge Control Trial Design." *The American Journal of Tropical Medicine and Hygiene* 92 (5): 913–21. <https://doi.org/10.4269/ajtmh.14-0347>.

Mahende, Coline, Billy Ngasala, John Lusingu, Tai-Soon Yong, Paminus Lushino, Martha Lemnge, Bruno Mmbando, and Zul Premji. 2016. "Performance of Rapid Diagnostic Test, Blood-Film Microscopy and PCR for the Diagnosis of Malaria Infection among Febrile Children from Korogwe District, Tanzania." *Malaria Journal* 15 (July). <https://doi.org/10.1186/s12936-016-1450-z>.

Malaria Surveillance, Monitoring & Evaluation: A Reference Manual. 2018. Genève: World Health Organization.

malERA Consultative Group on Diagnoses and Diagnostics. 2011. "A Research Agenda for Malaria Eradication: Diagnoses and Diagnostics." *PLoS Medicine* 8 (1): e1000396. <https://doi.org/10.1371/journal.pmed.1000396>.

Morris, Ulrika, Weiping Xu, Mwinyi I. Msellem, Alanna Schwartz, Ali Abass, Delér Shakely, Jackie Cook, et al. 2015. "Characterising Temporal Trends in Asymptomatic Plasmodium Infections and Transporter Polymorphisms during Transition from High to Low Transmission in Zanzibar, 2005-2013." *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* 33 (July): 110–17. <https://doi.org/10.1016/j.meegid.2015.04.018>.

Nguyen, Thuy-Nhien, Lorenz von Seidlein, Tuong-Vy Nguyen, Phuc-Nhi Truong, Son Do Hung, Huong-Thu Pham, Tam-Uyen Nguyen, et al. 2018. "The Persistence and Oscillations of Submicroscopic Plasmodium Falciparum and Plasmodium Vivax Infections over Time in Vietnam: An Open Cohort Study." *The Lancet. Infectious Diseases* 18 (5): 565–72. [https://doi.org/10.1016/S1473-3099\(18\)30046-X](https://doi.org/10.1016/S1473-3099(18)30046-X).

Niang, Makhtar, Laty Gaye Thiam, Rokhaya Sane, Nafissatou Diagne, Cheikh Talla, Souleymane Doucoure, Joseph Faye, et al. 2017. "Substantial Asymptomatic Submicroscopic Plasmodium Carriage during Dry Season in Low Transmission Areas in Senegal: Implications for Malaria Control and Elimination." *PloS One* 12 (8): e0182189. <https://doi.org/10.1371/journal.pone.0182189>.

Okell, Lucy C., Teun Bousema, Jamie T. Griffin, André Lin Ouédraogo, Azra C. Ghani, and Chris J. Drakeley. 2012. "Factors Determining the Occurrence of Submicroscopic Malaria Infections and Their Relevance for Control." *Nature Communications* 3: 1237. <https://doi.org/10.1038/ncomms2241>.

- Okell, Lucy C., Azra C. Ghani, Emily Lyons, and Chris J. Drakeley. 2009. "Submicroscopic Infection in Plasmodium Falciparum-Endemic Populations: A Systematic Review and Meta-Analysis." *The Journal of Infectious Diseases* 200 (10): 1509–17. <https://doi.org/10.1086/644781>.
- Plowe, C. V., A. Djimde, M. Bouare, O. Doumbo, and T. E. Wellems. 1995. "Pyrimethamine and Proguanil Resistance-Confering Mutations in Plasmodium Falciparum Dihydrofolate Reductase: Polymerase Chain Reaction Methods for Surveillance in Africa." *The American Journal of Tropical Medicine and Hygiene* 52 (6): 565–68.
- Shakely, Delér, Kristina Elfving, Berit Aydin-Schmidt, Mwinyi I. Msellem, Ulrika Morris, Rahila Omar, Xu Weiping, et al. 2013. "The Usefulness of Rapid Diagnostic Tests in the New Context of Low Malaria Transmission in Zanzibar." *PLOS ONE* 8 (9): e72912. <https://doi.org/10.1371/journal.pone.0072912>.
- Shekalaghe, Seif A., J. Teun Bousema, Karaine K. Kunei, Paminus Lushino, Alutu Masokoto, Liselotte R. Wolters, Steve Mwakalinga, Frank W. Mosha, Robert W. Sauerwein, and Chris J. Drakeley. 2007. "Submicroscopic Plasmodium Falciparum Gametocyte Carriage Is Common in an Area of Low and Seasonal Transmission in Tanzania." *Tropical Medicine & International Health: TM & IH* 12 (4): 547–53. <https://doi.org/10.1111/j.1365-3156.2007.01821.x>.
- Stresman, Gillian H., Amrish Y. Baidjoe, Jennifer Stevenson, Lynn Grignard, Wycliffe Odongo, Chrispin Owaga, Victor Osofi, et al. 2015. "Focal Screening to Identify the Subpatent Parasite Reservoir in an Area of Low and Heterogeneous Transmission in the Kenya Highlands." *The Journal of Infectious Diseases* 212 (11): 1768–77. <https://doi.org/10.1093/infdis/jiv302>.
- Stresman, Gillian H., Aniset Kamanga, Petros Moono, Harry Hamapumbu, Sungano Mharakurwa, Tamaki Kobayashi, William J. Moss, and Clive Shiff. 2010. "A Method of Active Case Detection to Target Reservoirs of Asymptomatic Malaria and Gametocyte Carriers in a Rural Area in Southern Province, Zambia." *Malaria Journal* 9 (October): 265. <https://doi.org/10.1186/1475-2875-9-265>.
- Sutanto, Inge, Ayleen Kosasih, Iqbal R. F. Elyazar, Deddy R. Simanjuntak, Tri A. Larasati, M. Sopiudin Dahlan, Isra Wahid, et al. 2018. "Negligible Impact of Mass Screening and Treatment on Mesoendemic Malaria Transmission at West Timor in Eastern Indonesia: A Cluster-Randomized Trial." *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 67 (9): 1364–72. <https://doi.org/10.1093/cid/ciy231>.
- Tadesse, Fitsum G., Hannah C. Slater, Wakweya Chali, Karina Teelen, Kjerstin Lanke, Muluaem Belachew, Temesgen Menberu, et al. 2018. "The Relative Contribution of Symptomatic and Asymptomatic Plasmodium Vivax and Plasmodium Falciparum Infections to the Infectious Reservoir in a Low-Endemic Setting in Ethiopia." *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 66 (12): 1883–91. <https://doi.org/10.1093/cid/cix1123>.
- Taylor, Steve M., Jonathan J. Juliano, Paul A. Trottman, Jennifer B. Griffin, Sarah H. Landis, Paluku Kitsa, Antoinette K. Tshefu, and Steven R. Meshnick. 2010. "High-Throughput Pooling and Real-Time PCR-

Based Strategy for Malaria Detection.” *Journal of Clinical Microbiology* 48 (2): 512–19.
<https://doi.org/10.1128/JCM.01800-09>.

Tiono, Alfred B., Alphonse Ouédraogo, Bernhards Ogutu, Amidou Diarra, Sam Coulibaly, Adama Gansané, Sodiomon B. Sirima, Gregory O’Neil, Amitava Mukhopadhyay, and Kamal Hamed. 2013. “A Controlled, Parallel, Cluster-Randomized Trial of Community-Wide Screening and Treatment of Asymptomatic Carriers of *Plasmodium Falciparum* in Burkina Faso.” *Malaria Journal* 12 (February): 79.
<https://doi.org/10.1186/1475-2875-12-79>.

Yukich, Joshua, Adam Bennett, Rudy Yukich, Logan Stuck, Busiku Hamainza, Kafula Silumbe, Tom Smith, et al. 2017. “Estimation of Malaria Parasite Reservoir Coverage Using Reactive Case Detection and Active Community Fever Screening from Census Data with Rapid Diagnostic Tests in Southern Zambia: A Re-Sampling Approach.” *Malaria Journal* 16 (1): 317. <https://doi.org/10.1186/s12936-017-1962-1>.

Zemene, Endalew, Cristian Koepfli, Abebaw Tiruneh, Asnakew K. Yeshiwondim, Dinberu Seyoum, Ming-Chieh Lee, Guiyun Yan, and Delenasaw Yewhalaw. 2018. “Detection of Foci of Residual Malaria Transmission through Reactive Case Detection in Ethiopia.” *Malaria Journal* 17 (1): 390.
<https://doi.org/10.1186/s12936-018-2537-5>.

Figures

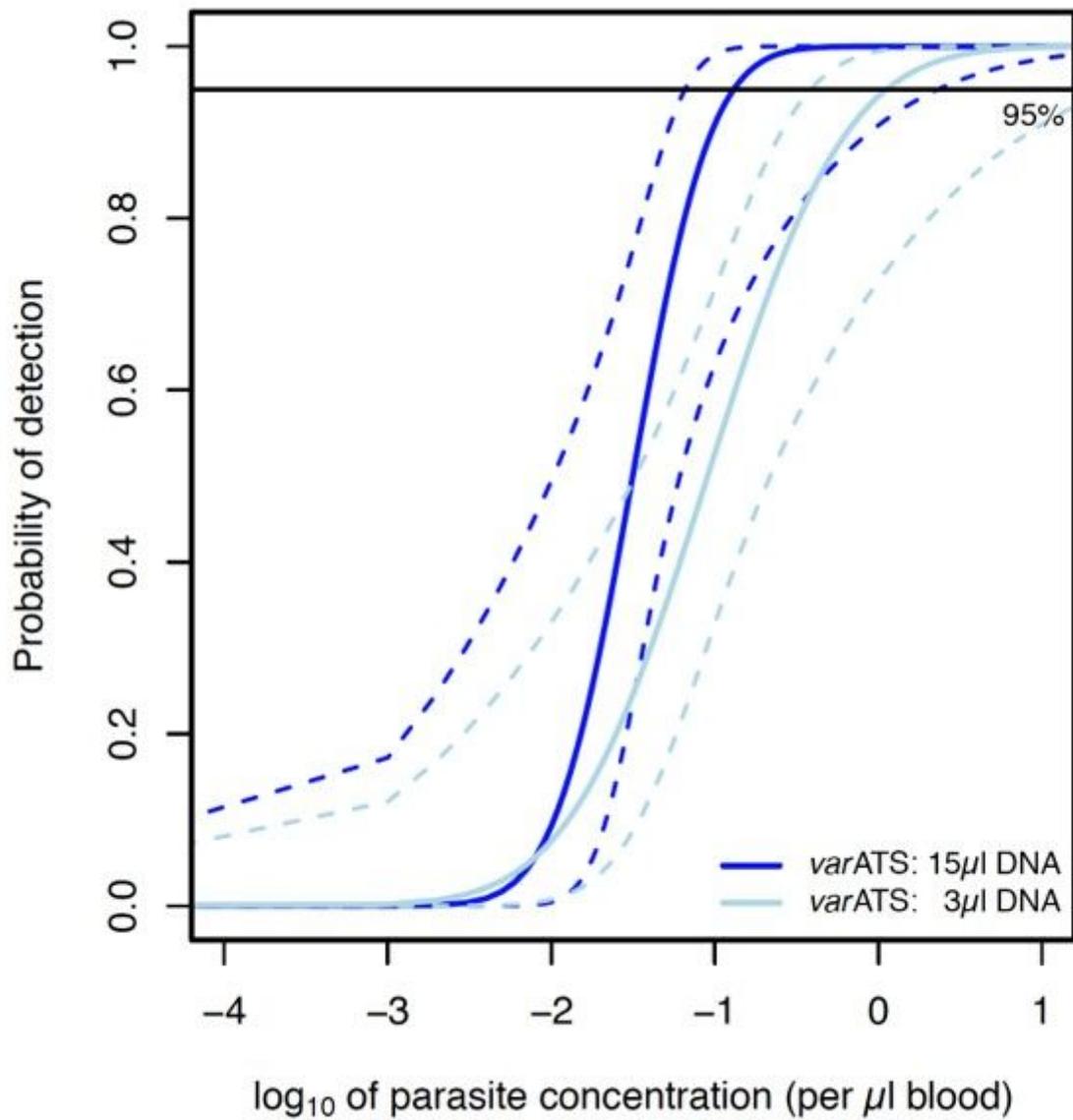


Figure 1

Limit of detection of varATS qPCR determined by probit analysis. Based on a serial dilution of WHO standard material using either 3 μl of DNA supplemented by one negative DBS punch (representing LOD using 1 punch per DBS sample, light blue) or 15 μl of DNA supplemented with five negative DBS punches (representing LOD using 5 punches per DBS sample, dark blue).

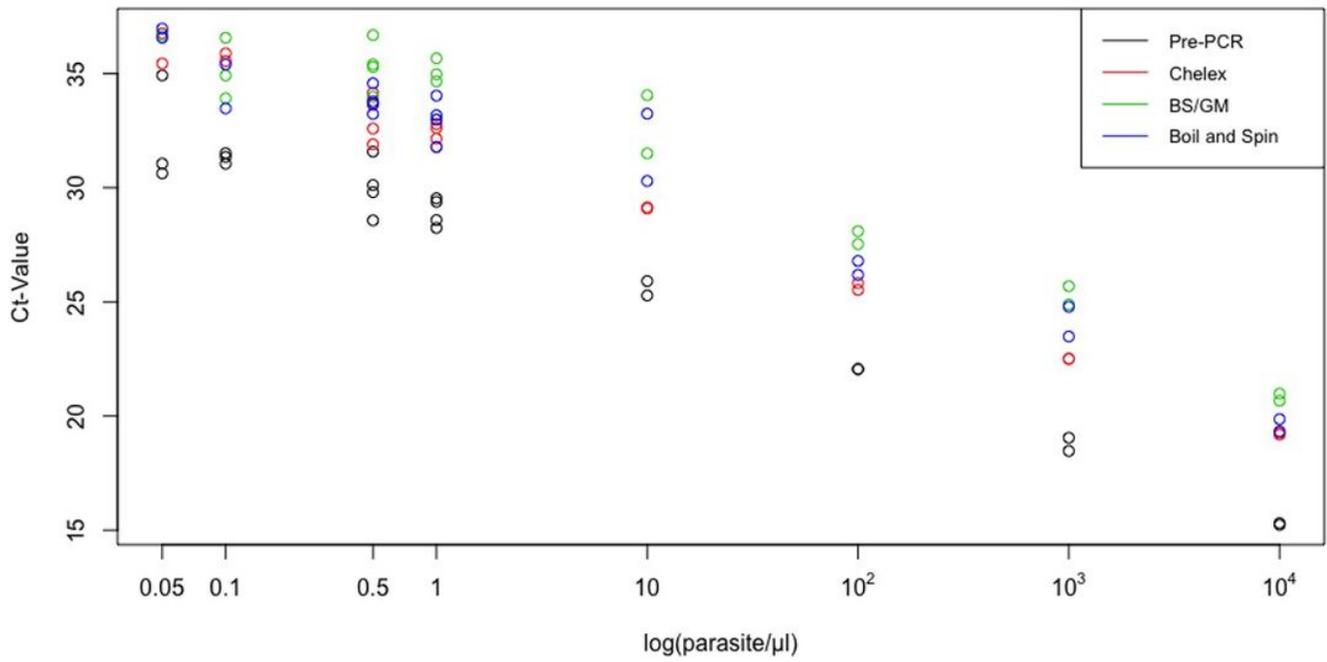


Figure 2

Comparison of Ct-values obtained by 4 DNA extraction methods in a *P. falciparum* dilution row spotted on DBS. Direct pre-PCR (black), chelex extraction (red circles), boil and spin extraction followed by glassmilk purification (green) and boil and spin extraction (blue).

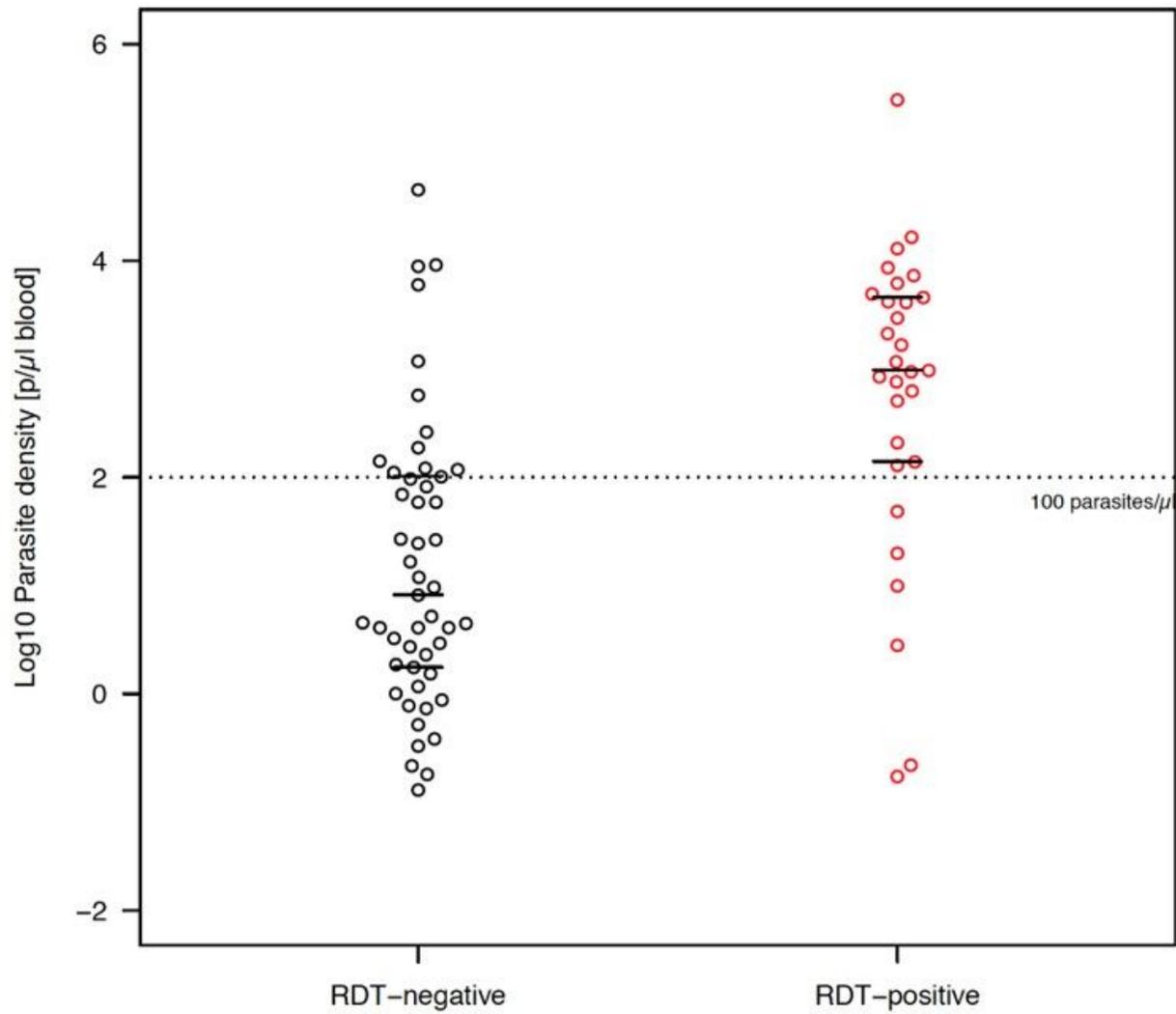


Figure 3

Proportion of *P. falciparum* density in RDT negative and RDT positive individuals detected by qPCR. Black lines indicate the median, respectively the IQR. Dotted line indicates parasite concentration 100parasites/ μ l blood.