Estimation of *Plasmodium falciparum* Transmission Intensity in Lilongwe, Malawi, by Microscopy, Rapid Diagnostic Testing, and Nucleic Acid Detection

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Abstract

Estimates of malaria transmission intensity (MTI) typically rely upon microscopy or rapid diagnostic testing (RDT). However, these methods are less sensitive than nucleic acid amplification techniques and may underestimate parasite prevalence. We compared microscopy, RDT, and polymerase chain reaction (PCR) for the diagnosis of *Plasmodium falciparum* parasitemia as part of an MTI study of 800 children and adults conducted in Lilongwe, Malawi. PCR detected more cases of parasitemia than either microscopy or RDT. Age younger than five years predicted parasitemia detected by PCR alone (adjusted odds ratio 1.61, 95% CI 1.09-2.38, Wald p = 0.02). Additionally, we identified one *P. falciparum* parasite with a false-negative RDT result due to a suspected deletion of the *hrp2* gene and employed a novel, ultra-sensitive PCR assay to detect low-level parasitemia missed by traditional PCR. Molecular methods should be considered for use in future transmission studies as a supplement to RDT or microscopy.

Plasmodium falciparum infects hundreds of millions of individuals and kills 600,000 persons every year.[1] A recently completed phase three trial of the RTS,S/ASO1 malaria vaccine demonstrated moderate and varying efficacy in different transmission settings.[2] Study sites conducted annual malaria transmission intensity (MTI) studies during the trial using microscopy and rapid diagnostic test (RDT) parasite prevalence as a surrogate for transmission intensity.

Both microscopy and RDT are known to produce negative results in Africa when parasitemia levels are beneath their limits of detection, 50 and 200 parasites per microliter of blood, respectively.[3, 4, 5] Infections not detected by microscopy or RDT, often referred to as subpatent infections, can often be detected by nucleic acid detection methods.[6] Though infections detected by microscopy and RDT are likely responsible for the majority of transmission, further studies are needed to assess the impact of subpatent infections on the transmission reservoir.[7, 8] In the context of vaccine trials, these low-level, subpatent parasitemias may confound estimates of transmission intensity used to study vaccine efficacy.

To evaluate the impact of subpatent parasitemias on parasite prevalence estimates, we collected dried blood spots (DBS) from all participants during the final year of the three year MTI study in Lilongwe, Malawi, to allow for nucleic acid detection of parasitemia. Using clinical data and a spatial database to evaluate ecological factors that may impact transmission, we attempted to identify risk factors for subpatent parasitemia. We also investigated the impact of other factors on discordances between PCR and other assays (RDT and microscopy), including: 1) non-falciparum parasitemia, 2) the occurrence of histidine-rich protein 2 (*hrp2*) deletions on false negative RDTs, and 3) low-level parasitemia detectable by ultra-sensitive PCR targeting high copy number genes.[9] This work adds to the growing literature concerning the

relationship between testing characteristics for malaria detection and provides new information about *hrp2* deletions in Malawi and the use of ultra-sensitive, falciparum-specific, high-copy telomeric-associated repetitive element 2 (TARE-2) PCR to address diagnostic discordances.

The MTI study was conducted in Lilongwe, Malawi, from 2011-2013 within the catchment area of the phase three RTS,S/ASO1 trial.[2] 800 participants were included each year from randomly selected households in the area surrounding the Malawi Ministry of Health Area 18 Health Centre in Lilongwe. Each annual cohort of enrollees in the MTI study included 400 subjects ≥ 6 months and <5 years of age, 200 subjects ≥ 5 years of age and < 20 years of age, and 200 subjects ≥ 20 years of age. Participants in the phase three RTS,S/ASO1 trial were excluded from the MTI study. The current study includes only the 2013 cohort, all of whom had a finger prick with blood collected for RDT and microscopy and dried on Whatman 3 filter paper (GE Healthcare, Piscataway, NJ). Microscopy slides were read by two expert microscopists who received training every 4 months, with discordances referred to a third microscopist. RDT testing was performed using SD Bioline HRP-2 Kits (Gyeonggi-do, Republic of Korea). Filter papers were labeled with an identifier and stored individually with dessicant at -20°C. This study was approved by the Malawian National Health Sciences Research Committee and the University of North Carolina (UNC) Institutional Review Board. All participants provided written informed consent.

DNA was extracted from filter paper using the PureLink Pro 96 Genomic DNA Purification Kit (Life Technologies, Grand Island, NY) at UNC. We used a predetermined screening algorithm for testing samples **(Figure 1** and **Supplementary Table 1)**. Initial testing for parasitemia involved a real-time PCR assay to detect the falciparum-specific lactate dehydrogenase gene (*pfldh*).[10] All samples were tested with 1µl of template DNA per reaction in

duplicate, followed by 4µl of template DNA in duplicate for discordant replicates and for microscopy-positive, PCR-negative samples.

The clinical characteristics of subjects were compared using Fisher's exact test for categorical variables, the student's t-test for normally distributed continuous variables, and the Wilcoxon rank-sum test for non-normally distributed continuous variables. Risk factors for subpatent malaria were investigated by constructing a binomial regression model. We used directed acyclic graphs (DAGs) to assess all possible relationships between risk factors of interest and subpatent malaria and to identify potential confounders. We calculated crude and adjusted odds ratios for each risk factor. Kappa statistics were calculated to quantify the agreement between diagnostic testing methods in subjects who underwent testing with all three methods (PCR, microscopy, and RDT), in addition to sensitivity and specificity using PCR as the gold standard. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC).

The majority of malaria infections identified in 2013 were subpatent (**Table 1** and **Figure 2**). This finding was most prominent amongst children younger than five, in whom RDT and microscopy failed to detect two-thirds of all PCR-positive parasitemias. These children were more likely to have subpatent malaria than patent malaria, defined as PCR-positive and either microscopy- or RDT-positive ($p = 0.04$). Subjects with subpatent and patent malaria malaria had similar demographic and clinical characteristics (**Table 1**). Modeling confirmed that age younger than five years predicted subpatent malaria in our cohort (**Supplementary Table 2**; adjusted OR 1.61, 95% CI 1.09-2.38, Wald $p = 0.02$). Neither bed net use nor anemia predicted subpatent malaria in the crude or adjusted models.

PCR was the most sensitive diagnostic test for parasitemia, producing a prevalance 76% higher than microscopy and 16% higher than RDT in 2013. Malaria was detected by *pfldh* PCR

in 79 (10.0%) subjects, by microscopy in 45 (5.7%), and by RDT in 68 (8.6%). There was moderate agreement between all three tests (**Figure 2**). RDT and microscopy had the highest level of agreement (Kappa 0.64, 95% CI 0.54-0.74), PCR and microscopy less agreement (Kappa 0.50, 95% CI 0.38-0.61), and PCR and RDT the lowest level of agreement (Kappa 0.44, 95% CI 0.33-0.54). Using PCR as the gold standard, both microscopy and RDT lacked sensitivity but were specific. For microscopy, the sensitivity and specificity were 41.8% (95% CI 30.9-52.7) and 98.3% (95% CI 97.4-99.3), while RDT had a sensitivity and specificity of 45.6% (95% CI 34.6-56.6) and 95.5% (95% CI 94.0-97.0). Among 12 subjects with indeterminant microscopy results (microscopy-positive but RDT- and *pfldh* PCR-negative), there were no cases of non-falciparum malaria, but one subject with parasitemia beneath the *pfldh* PCR assay's limit of detection was detected using ultra-sensitive TARE-2 PCR **(Figure 1)**. All 26 subjects evaluated for possible RDT false-positive results (RDT-positive but microscopy- and *pfldh* PCR-negative) had negative TARE-2 PCR results. Notably, one parasite detected by both PCR and microscopy but not detected by RDT likely harbored a deletion of the *hrp2* gene, which is responsible for production of the antigen detected by most RDTs. The geographical distribution of positive test results by PCR, microscopy, and RDT was similar.

These results confirm that studies that rely upon RDT and/or microscopy underestimate malaria prevalence. The striking difference in parasitemia prevalence estimates amongst children younger than five is especially concerning, as these children are at highest risk of malaria-related morbidity and mortality.[1] Even if most subpatent infections in these young children are asymptomatic, they may represent a transmission reservoir that requires further study and may need to be addressed by ongoing malaria control efforts.[6]

Age younger than five was a risk factor for subpatent malaria in our cohort, a finding that differs from other published reports. Specifically, submicroscopic malaria has been associated with older age, presumably due to partial immunity.[6, 11, 12] While high levels of bed net usage during the study period suggested that bed nets may be partly responsible for this finding, bed net usage was not a risk factor for subpatent infection in our models, a finding consistent with prior reports.[12] Submicroscopic infections are also thought to be more common in settings of low transmission intensity.[11, 13] Because PCR testing was only performed in the final year of the study, we could not assess changes in subpatent malaria over time.

Using individual-level data, we found a lower level of concordance between microscopy and RDT results than a recent analysis of prevalence studies, which depended upon cluster-level data.[6,10] Although malaria prevalence as determined by PCR and RDT differed by only 16%, there was only moderate agreement between tests (Kappa 0.44), likely due in part to the large number of "false-positive" RDT results **(Figure 1** and **Supplementary Figure 2)**. "False-positive" RDT results have been described in the setting of persistent circulating HRP2 antigen after clearance of *P. falciparum* parasitemia, indicating not a failure of the assay itself but recently resolved malaria.[5, 14, 15] We confirmed the absence of low-level parasitemia in samples with false-positive RDT results using TARE-2 PCR, a novel ultra-sensitive PCR assay that can detect *P. falciparum* parasitemias of 0.03-0.15 parasites/uL blood. We also employed this ultra-sensitive assay to evaluate microscopy-positive but RDT-/PCR-negative results and successfully identified low-level parasitemia in one of 12 subjects.

The *hrp2*-deleted *P. falciparum* parasite identified in this study represents the first suspected case reported in Malawi. There are increasing reports of these stealth parasites, which

evade detection by RDTs that depend upon identification of circulating *P. falciparum* HRP2 antigen, but their public health significance remains unknown.[9, 16]

In conclusion, we employed an epidemiological and molecular approach to evaluate the diagnostic methods employed during a large malaria transmission intensity study conducted as part of the RTS,S/ASO1 vaccine trials. Our findings confirm that RDT and microscopy underestimate the prevalence of *P. falciparum* parasitemia. RDTs may currently lack the sensitivity and specificity for accurate assessment of transmission intensity.[15, 16] Molecular methods should be considered for use in future transmission studies as a supplement to RDT or microscopy.

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Figure 1. Diagnostic testing algorithm and results

Figure 2. Proportional Venn diagram comparing P. falciparum prevalence by diagnostic method.

Number of results and percent overall prevalence by testing method. Diagram generated using eulerAPE, version 3.0.0.[17]

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