Short Report: Prevalence of PCR Detectable Malaria Infection among Febrile Patients with a Negative *Plasmodium falciparum* Specific Rapid Diagnostic Test in Zanzibar

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Abstract. We screened for malaria in 594 blood samples from febrile patients who tested negative by a *Plasmodium falciparum*-specific histidine-rich protein-2-based rapid diagnostic test at 12 health facilities in Zanzibar districts North A and Micheweni, from May to August 2010. Screening was with microscopy, polymerase chain reaction (PCR) targeting the *cytochrome b* gene (*cytb*PCR) of the four major human malaria species, and quantitative PCR (qPCR). The prevalence of *cytb*PCR-detectable malaria infection was 2% (12 of 594), including 8 *P. falciparum*, 3 *Plasmodium malariae*, and 1 *Plasmodium vivax* infections. Microscopy identified 4 of 8 *P. falciparum* infections. Parasite density as estimated by microscopy or qPCR was > 4,000 parasites/μL in 5 of 8 *cytb*PCR-detectable *P. falciparum* infections. The infections that were missed by the rapid diagnostic test represent a particular challenge in malaria elimination settings and highlight the need for more sensitive point-of-care diagnostic tools to improve case detection of all human malaria species in febrile patients.

Zanzibar has been the site of a substantial recent effort to reduce the overall burden of malaria. With these efforts, the prevalence of parasitologically confirmed malaria infection among febrile children presenting at primary health care facilities in Zanzibar has decreased from ~25% in 2003[1] to 2% in 2010 (Shakely and others, unpublished data). Historically, *Plasmodium falciparum* has played a dominant role in malarial illness in Zanzibar, and elsewhere in sub-Saharan Africa, causing well over 90% of episodes of the disease[2,3]; the remaining reported malaria infections in Zanzibar in recent years have been caused by *Plasmodium malariae*.[2]

Malaria rapid diagnostic tests (RDTs) that detect parasite antigens have improved the availability of parasite-based diagnosis for rural clinics in Africa.[4] However, the RDT currently used in most of sub-Saharan Africa and in Zanzibar at the time of this study detects histidine-rich protein-2 (HRP2), which is specific to *P. falciparum*, and does not detect other species of malaria parasites.[5]

The availability of highly sensitive molecular techniques provides an opportunity to better characterize the species of Plasmodia causing malaria in regions of sub-Saharan Africa experiencing decreasing *P. falciparum* transmission. The aim of this study was to assess the prevalence of polymerase chain reaction (PCR)-detectable malaria infection among febrile patients with a negative *P. falciparum*-specific RDT in Zanzibar.

Samples for this study were from an RDT effectiveness trial (Shakely and others, unpublished data) performed in 12 Zanzibar primary health care facilities, six each in North A and Micheweni districts over a 12-week period during the high transmission season from May to August 2010. Inclusion criteria in this study were: age ≥ 2 months, a measured axillary temperature ≥ 37.5°C or history of fever in the preceding 24 hours, and absence of any danger signs of severe disease.[6]

After obtaining informed written consent, dried blood spots were collected from febrile patients who tested negative for malaria by an HRP2-based RDT (Paracheck Pf) and a short questionnaire was administered to gather demographic information and travel histories. Travel was defined as having spent at least one night away from the home in the past 30 days, either within Zanzibar or abroad. For children < 15 years of age, consent and questionnaire were administered to the accompanying adult. Five hundred ninety-four participants who tested negative by RDT at enrollment were randomly selected for additional blood sampling by microscopy and PCR and are included in this analysis.

Trained health facility personnel performed specimen collection and interpreted RDT results. In 10 out of 12 study sites, RDT use is part of the routine management of febrile illness, whereas in the remaining two sites, microscopy is the standard diagnostic method. Following confirmation of a negative RDT result and informed consent, blood from a finger prick was collected onto a blood slide and a filter paper (Whatman 3MM, Florham Park, NJ), which were labeled with unique identifiers. Filter paper samples were dried overnight and stored in sealed bags at ambient temperature with desiccant. Before the start of the study, district supervisors and clinic health care personnel were trained in Integrated Management of Childhood Illness (IMCI),[6] standardized blood sample collection, sample storage, and questionnaire data collection.

A two-stage PCR-based pooling strategy, as previously described,[7] was used to characterize samples. Briefly, DNA was extracted from sets of 10 pooled samples, and subsequently from all individual samples in pools testing positive. At each stage, a nested PCR reaction using primers common to the cytochrome *b* genes of the four major human malaria species,[8] followed by an *AluI* restriction digest to distinguish species, was performed (*cytb*PCR).[9] Duplex quantitative PCR targeting the human β *tubulin* gene and the plasmodial methionine transfer

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RNA (tRNA) gene (*pgmet*) was performed to quantify parasite density relative to human DNA, as previously described.[10] To obtain estimates of parasite density, a standard curve was derived from filter paper controls created using known densities of cultured parasites serially diluted in whole blood from three donors. We have found this method to reliably detect and quantify concentrations of 25 parasites/μL or higher from filter paper samples (Hsiang and others, unpublished data). Microscopic investigations of Giemsa-stained thick blood smears were performed according to standard practice for malaria diagnosis[11]; asexual parasite densities were calculated against 200 white blood cells, assuming 8,000 white blood cells per microliter of blood. Blood slides were recorded as negative if no parasites were found after examining 100 high power microscopy fields. All blood slides were examined by two qualified laboratory technicians in Zanzibar. Differences in species identification or parasite density of > 50% were subjected to a third decisive examination by an independent expert microscopist.

All data were entered in Excel (Microsoft Corp., Redmond, WA), converted to comma-separated text and imported into SAS Version 9.2 (SAS Institute Inc., Cary, NC). Data were cleaned in SAS to check for duplications and identify any missing or discrepant data. Fisher's exact test was used to compare categorical demographic variables and PCR results. This study was approved by the Zanzibar Medical Research Ethical Committee (ZAMREC), the University of California San Francisco Committee for Human Research (CHR), and the Regional Ethics Committee Stockholm, Sweden.

A total of 594 RDT negative subjects were enrolled: 270 from North A district and 324 from Micheweni district. Demographic data and travel history of the study participants are presented in Table 1. Overall, 12 of 594 (2%) RDT-negative samples were positive by *cytb*PCR, including 8 *P. falciparum*, 3 *P. malariae*, and 1 *Plasmodium vivax* infections (Table 2). No multi-species infections were identified by *cytb*PCR. Microscopy was positive for 4 of 8 *P. falciparum* infections, with parasite densities ranging from 4,309 to 43,886 parasites/μL, but for only 1 of 4 of the *cytb*PCR-detectable non-falciparum infections. The latter infection, which was identified as *P. malariae* by *cytb*PCR, had a very low density, i.e., insufficient to allow species identification, by microscopy. Similarly,

TABLE 1

Selected characteristics among enrolled patients with a negative malaria rapid diagnostic test, Zanzibar districts North A and Micheweni (N = 594)

Variable	n (%)	*cytb*PCR positive n (%)	*cytb*PCR negative n (%)	P value
Sex				
Male	245 (41)	6 (2)	239 (98)	0.57
Female	343 (58)	6 (2)	337 (98)	
Missing data	6 (1)			
Total	594			
Age				
≤ 18 years	360 (61)	4 (1)	356 (99)	0.07
> 18 years	228 (38)	8 (4)	220 (96)	
Missing data	6 (1)			
Total	594			
Travel*				
Yes	72 (12)	1 (1)	71 (99)	1.000
No	522 (88)	11 (2)	511 (98)	
Total	594			

*Having spent at least one night away from home in the past 30 days, either within Zanzibar or abroad.

TABLE 2

Features of infections with a negative malaria rapid diagnostic test and positive microscopy or *cytb*PCR result (N = 12)

*cytb*PCR results	Age (years)	Sex	Travel	Microscopy (parasites/μL)	qPCR (parasites/μL)
P. falciparum	33	male	no	4,309	4,714
P. falciparum	20	male	no	14,420	3,057
P. falciparum	5	male	yes	21,991	report unavailable
P. falciparum	8	female	no	43,886	8,018
P. falciparum	24	female	no	negative	negative
P. falciparum	35	male	no	negative	negative
P. falciparum	1	female	no	negative	negative
P. falciparum	24	female	no	negative	13,516
P. malariae	29	male	no	10*	1.48
P. malariae	30	female	no	negative	negative
P. malariae	25	male	no	negative	negative
P. vivax	4	female	no	negative	negative

*This low parasite density did not allow for species identification.

qPCR verified parasitemia in 4 of 8 of the *ctyb*PCR-detectable *P. falciparum* infections, with parasite densities estimated from 3,057 to 13,516 parasites/μL. However, none of the non-falciparum infections identified by *cytb*PCR had detectable parasitemia by qPCR. There were no statistically significant differences in sex, age, or travel history between *cytb*PCR negative and positive subjects. Of note, none of the *cytb*PCR-detectable non-falciparum infections occurred in subjects that reported travel, suggesting the infections were acquired locally (Table 2).

The prevalence of PCR-detectable malaria infection among febrile patients with a negative *P. falciparum*-specific RDT in Zanzibar was low. A majority of these infections were caused by *P. falciparum*. Of importance, only half of the *cytb*PCR-detectable *P. falciparum* infections, and only one of the non-falciparum infections, were identified by microscopy. The presence of PCR-detectable non-falciparum and *P. falciparum* infections that were missed by the HRP-2-based RDT identifies a particular challenge in a malaria pre-elimination setting like Zanzibar, because such infections are unlikely to be detected by the presently available point-of-care malaria diagnostic tools.[12] Some false-negative RDT results might be explained by infection with *P. falciparum* with altered to absent HRP2, although this possibility requires additional study involving extensive sequencing and consideration of multiple PCR and sequencing primers to account for known sequence variability.[13–16] Additionally, although our health facility personnel were trained in the collection of specimens for RDTs, it is possible that human error in test performance may explain some of the false-negative RDT results. In any event, our results suggest the need in malaria elimination settings for development of more sensitive point-of-care diagnostic tools to ensure improved case detection of all major human malaria species in febrile patients.

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