

# Malaria prevalence defined by microscopy, antigen detection, DNA amplification and total nucleic acid amplification in a malaria-endemic region during the peak malaria transmission season

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## Summary

**OBJECTIVES** To determine the malaria prevalence by microscopy, antigen detection and nucleic acid detection in a defined subpopulation in a *Plasmodium falciparum*-endemic region during the peak transmission season.

**METHODS** Blood specimens were collected in a cross-sectional study involving children aged 5–10 years ( $n = 195$ ) presenting with acute fever to two clinics in Western Kenya. All specimens underwent microscopy, HRP2 and aldolase antigen detection by enzyme immunoassay (EIA), parasite-specific DNA and total nucleic acid (RNA and DNA) by real-time PCR (qPCR) and reverse-transcriptase PCR (qRT-PCR).

**RESULTS** Microscopy detected 65/195 cases of malaria infection [95% confidence interval (CI) 52–78]. HRP2 and aldolase EIA had similar sensitivity levels detecting antigen in 65/195 (95% CI, 52–78) and 57/195 (95% CI, 45–70) cases. Discordants in antigen detection *vs.* microscopy occurred at <470 parasites/ $\mu$ l and <4900 parasites/ $\mu$ l for HRP2 and aldolase, respectively. Detection of total nucleic acid allowed a 3 log lower limit of detection than just DNA detection by real-time PCR *in vitro*. In clinical specimens, 114/195 (95% CI, 100–127) were qPCR positive (DNA), and 187/195 (95% CI, 179–191) were qRT-PCR positive (DNA plus RNA).

**CONCLUSIONS** The prevalence of submicroscopic malaria infection was significantly higher when detecting total nucleic acid than just DNA in this outpatient population during the high transmission season. Defining standards for submicroscopic infection will be important for control programmes, diagnostics development efforts and molecular epidemiology studies.

**keywords** malaria, prevalence, molecular diagnostics

## Introduction

Parasite detection by microscopy, antigen detection by enzyme immunoassay (EIA) or rapid diagnostic tests (RDTs) and nucleic acid amplification tests (NAATs) are the most common methodologies used to determine malaria infection in a patient. The prevalence of malaria as determined by each method is a function of many variables, including parasite density, assay limit of detection (LOD), as well as population exposure to malaria, age and specific *Plasmodium* genotype.

Microscopy the most widely used test has a relatively high LOD of approximately 50–500 parasites/ $\mu$ l. This can

result in under estimation of infection rates particularly in regions of low malaria transmission or in cases of imported malaria (Menge *et al.* 2008). *Plasmodium*-specific antigen detection is also widely used to support malaria diagnosis, with approximately equivalent performance to microscopy, depending, again, on the endemicity and *Plasmodium* subspecies (Noedl *et al.* 2006; Kifude *et al.* 2008; Msellem *et al.* 2009).

Several NAATs have been adopted for detection and speciation of *Plasmodium* in clinical specimens (Barker *et al.* 1992; Kain *et al.* 1993; Snounou *et al.* 1993b; Hermsen *et al.* 2001; McNamara *et al.* 2004; Mens *et al.* 2006; Menge *et al.* 2008; Steenkeste *et al.* 2009). Most of

these technologies focus on the detection of DNA, while RNA has been a target for the quantification of *P. vivax* and *P. falciparum* gametocytes in clinical specimens (Babiker *et al.* 1999; Schneider *et al.* 2004; Mlambo *et al.* 2008; Beurskens *et al.* 2009). Most NAATs are significantly more sensitive than microscopy leading to the detection of submicroscopic parasitemia. A meta-analysis of the sensitivity of microscopy compared to NAAT techniques suggests significant underestimation of malaria prevalence by microscopy, which is in part dependent on transmission rates (Okell *et al.* 2009).

Identifying markers for the definition of submicroscopic malaria prevalence in a given population is increasingly necessary both for control programmes and for the performance evaluation of new more sensitive malaria diagnostic platforms. Here we present data comparing malaria prevalence as determined by microscopy, antigen detection, and both DNA and total nucleic acid in febrile children aged 5–10 years presenting to outpatient clinics in a malaria holoendemic region during an intense malaria transmission season.

## Methods

### Patients and samples

Patients aged 5–10 years presenting with acute fever (onset within the previous 4 days) and malaria symptoms were recruited at two outpatient clinics: Kombewa Subdistrict Hospital and Kondele Children's Hospital, in western Kenya as part of a cross-sectional study evaluating aetiologies of fever (Waitumbi *et al.* 2010). Malaria epidemiology in this region is holoendemic. The study was conducted over the months of April to July 2007 corresponding to the period of intense malaria transmission. Whole blood count (CBC) and malaria blood smear results performed at a nearby Walter Reed Program laboratory were promptly provided to the healthcare provider for patient management. All other tests were performed retrospectively. The study protocol was reviewed and approved by the following institutional review boards: Kenya Ethical Review Committee ERC #1117, Walter Reed Army Institute of Research (WRAIR) HURC #1315 and PATH Research Ethics Committee HS #358.

### Microscopy

Thin and thick malaria blood films (MBFs) were prepared and read by two blinded, experienced microscopists according to published methodology (Ohr *et al.* 2007). For quantification of malaria parasites in the thick film, a total of 200 white blood cells (WBC) were examined while simultaneously counting the malaria parasites. If the

parasite/WBC ratio exceeded 2 in the thick MBF, the parasite density was evaluated from the thin MBF. The number of infected RBCs per 2000 total RBCs was counted.

### Antigen detection testing

Enzyme immunoassay detection of pfHRP2 antigen: All tests were performed with plasma. Murine anti-pfHRP2 (MPFM-45A; Immunology Consultants Laboratories, Inc. [ICL], USA) was diluted to 1 µg/ml in phosphate-buffered saline (PBS) and used to coat Immulon II HighBind plates (CoStar, #3590). The plates were washed with PBS, 0.05% Tween-20 (PBS-T), and blocked for 2 h at 25 °C with 2% bovine serum albumin (BSA) in PBS. Following a wash step, the wells were incubated with 25 µl of plasma for 1 h at 25 °C. The plates were washed four times with PBS-T and incubated with 25 µl/well of 0.05 µg/ml anti-pfHRP2-HRP (ICL, #MPFG-45P) diluted in 2% BSA with 1% Tween-20 for 1 h at 25 °C and then washed four times with PBS-T. The plates were developed with 25 µl/well of TMB chromogen (Zymed Labs, Inc., USA) and incubated for 10 min at room temperature in the dark. The reaction was stopped with 25 µl of 1 M sulphuric acid, and the absorbance at 450 nm was read. LOD was determined using recombinant antigen (ICL, #AGPF-55). Samples were positive if absorbance values were greater than or equal to three times the average absorbance values from the negative buffer control.

Enzyme immunoassay detection of aldolase antigen: Immulon II HighBind plates (CoStar, #3590) were coated overnight at 4 °C with 50 µl/well of murine monoclonal IgG anti-*plasmodium* aldolase (ICL, #MPVA-55A) diluted to 1 µg/ml in PBS. The assay was then performed as described above for HRP2 except using 25 ng/ml HRP-conjugated rabbit anti-*plasmodium* aldolase (ICL Consultants, #RPVA-55A) diluted in 2% BSA as the reporter molecule. LOD was determined using recombinant antigen (#A3001; CTK Biotech, San Diego, CA).

### Nucleic acid extraction

For the assay validation, a highly synchronised ring stage (~98%) culture of *P. falciparum* (3D7) was established and allowed to reach a parasitemia level of 3%. A parasitemia dilution series was prepared in normal whole blood. In an evaluation for DNA, RNA and total nucleic acid using blood pellets spiked with infected red blood cells using both QIAGEN QIAmp RNA and DNA blood mini kits, the QIAmp DNA blood mini kit consistently provided lower Cq values for both qPCR and qRT-PCR and thus was used for the study. For all extractions, 200 µl of

specimen was used, and nucleic acid was eluted in 200  $\mu$ l of elution buffer. Clinical specimens were stored immediately at  $-70^{\circ}\text{C}$ . Frozen blood pellets were re-suspended and homogenised immediately into lysis buffer to minimise RNA degradation.

The NIBSC international standard for *P. falciparum* DNA (NIBSC#: 04/176) procured from the Health Protection Agency (UK) was re-suspended in 500  $\mu$ l of water of which 200  $\mu$ l was used for extraction with the QIAmp DNA blood mini kit and eluted in 200  $\mu$ l of elution buffer. For clinical specimens, total nucleic acid was extracted from 200  $\mu$ l whole blood cell pellets and processed as described for the NIBSC standard. The same volume (2  $\mu$ l) of elution buffer was used for both a one-step qRT-PCR and the qPCR.

#### qPCR primers and reaction conditions

Biplexed real-time PCR reagents targeting the *Plasmodium spp* 18S ribosomal gene and the PHPRT1 human housekeeping gene as endogenous control were developed (Table 1). The assays use hybridisation Pleiades probe chemistry (Lukhtanov *et al.* 2007) with minor groove binder (MGB) attached to the 5'-end of the probe allowing for an end-of-PCR melt analysis. Twelve-mer AT-rich non-complementary 'flaps' were added to each primer to increase fluorescent signal (Afonina *et al.* 2007). Modified bases in the sequence were introduced to obtain the desired oligonucleotide stability. *Plasmodium spp*, forward primer, reverse primer and probe were used at 100, 2000 and 200 nM concentrations, respectively. PHPRT1 human housekeeping gene forward primer, reverse primer and probe were used at 40, 2000 and 500 nM concentrations, respectively.

Samples were amplified with QuantiTect Probe PCR Kit (catalogue #204343; Qiagen) using the following cycling parameters: 2 min at  $50^{\circ}\text{C}$ , 15 min at  $95^{\circ}\text{C}$ , followed by 50 cycles of 15 s at  $95^{\circ}\text{C}$ , 1 min at  $56^{\circ}\text{C}$ , 5 min at  $76^{\circ}\text{C}$ , final extension hold and dissociation stage of  $95^{\circ}\text{C}$

denaturation for 15 s, beginning melt of  $35^{\circ}\text{C}$  for 15 s, ramping (5% ramp rate), to an ending melt of  $95^{\circ}\text{C}$  for 15 s. The same primers and probes were used for qRT-PCR with the QuantiTect Probe qPCR Kit (catalogue #204443; Qiagen) with the same cycling parameters except for the addition of an initial step of 30 min at  $50^{\circ}\text{C}$ .

#### Statistical methods

Correlations between different analytical outputs were determined by Pearson correlation fits.

#### Results

##### Clinical profiles and malaria infection status by microscopy

All patients were between 5 and 10 years of age (median 7 years old) presenting to out patient clinics within 4 days of having fever (median 3 days). Of the 195 patients recruited, 65 (33%) were positive for malaria infection as determined by microscopy. All infections were *P. falciparum*, one of which was co-infected with *P. ovale* and another with *P. malariae*. The arithmetic mean parasite density was 89 358 parasites/ $\mu$ l (range: 46–491,640 parasites/ $\mu$ l).

##### Antigen detection

The presence of *P. falciparum*-specific HRP2 and pan-*Plasmodium spp*. aldolase antigen was determined in all specimens by EIA using plasma. A total of 65 specimens were positive for HRP2, 60 of which were microscopy positive and an additional 5 specimens were negative by microscopy. All specimens positive for HRP2 were positive by PCR. The LOD as determined with recombinant antigen for this test is 50 ng/ml in plasma (5 ng/ml in PBS). A total of 57 specimens were positive for aldolase, 53 of these were positive by microscopy and an additional 4 specimens were negative by microscopy (Table 2). All aldolase-positive specimens were also positive by PCR.

#### Primer sequences

##### *Plasmodium spp*. 18S ribosomal gene

Forward primer	5'- <u>AATAAAATCATAA</u> AGTATTTCAGATGTCAGAGGTG -3'
Reverse primer	5'- <u>AATAAAATCATAA</u> GRCAAATGCTTTCGAGTTG -3'
Probe	5'- MGB-FAM- TTCTGGAGACG* <u>A</u> * <u>G</u> *CAA*CT -Quencher -3'

##### PHPRT1 human housekeeping gene

Forward primer	5'-CCGCAGCCCTGGCGTCGTGATTAGTGA -3'
Reverse primer	5'- CCCTTCCAAATCCTCAGCATAATGATTAGGT -3'
Probe	5'- MGB-Yellow Dye- G*ATTTATTTTGCA*TACCTQ -3'

Underlined in primer sequences are twelve-mer AT-rich non-complementary 'flaps'. Modified bases are indicated by \* in the sequence.

**Table 1** Primers and probes for *Plasmodium spp* real-time PCR assays targeting the 18S ribosomal gene sequence. The assay is biplexed with a PHPRT1 human housekeeping gene assay as endogenous control

**Table 2** Performance of tests on clinical specimens. *Plasmodium falciparum* HRP2 and pan-malaria aldolase were detected by sandwich enzyme immunoassay. DNA and DNA + RNA were detected by real-time PCR and real-time reverse-transcriptase PCR. Positive and negatives are compared to the microscopy results

LOD/performance	Microscopy		HRP2		Aldolase		DNA		DNA + RNA	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
	46* parasites/μl	50 ng/ml†, 470‡ parasites/μl	50 ng/ml†, 4900‡ parasites/μl	32 IU/reaction§	0.32 IU/reaction§					
Microscopy Positive	65	0	60	5	53	12	65	0	65	0
Microscopy Negative	0	130	5	125	4	126	49	81	122	8
Total number of positive specimens	65	65	65	57	57	114	114	114	187	187
Prevalence	33%	33%	29%	29%	29%	58%	58%	58%	96%	96%

\*Lowest parasitemia counted.

†Limit of detection (LOD) determined with recombinant antigen in plasma.

‡Lowest parasitemia above which 100% of specimens were positive for both microscopy and antigen detection.

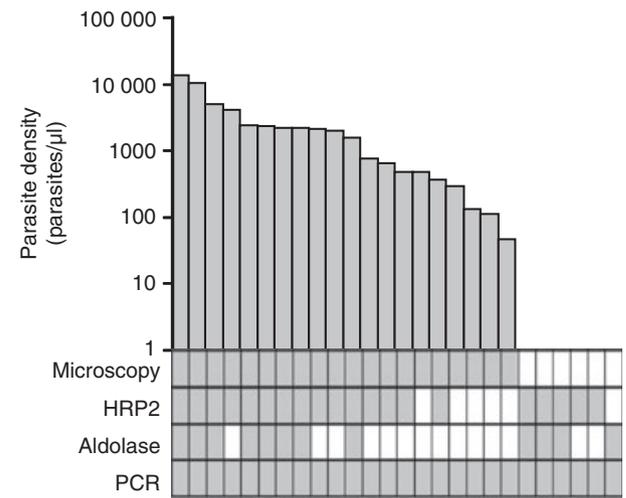
§Limit of detection determined with the WHO malaria molecular diagnostic standard.

The LOD for the aldolase EIA test as determined using recombinant antigen was 37 ng/ml in plasma (5 ng/ml in PBS). The microscopy-positive/antigen-negative specimens had a parasite density of <470 parasites/μL for the HRP2 EIA and <4900 parasites/μL for the aldolase EIA (Figure 1). The Pearson correlation coefficients between the EIA signal (absorbance) and the log parasite densities for HRP2 were  $r = 0.45$  ( $P < 0.001$ ) and for aldolase,  $r = 0.62$  ( $P < 0.001$ ) (Figure 2a,b).

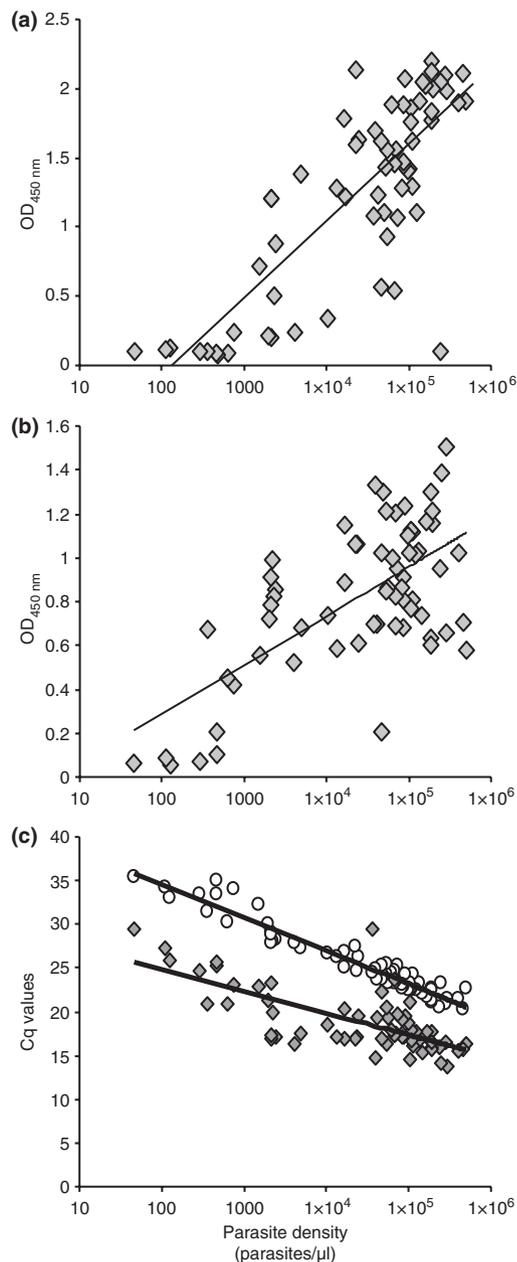
*Design and performance of universal Plasmodium spp. qPCR reagents*

Pleiades probe-based qPCR and qRT-PCR tests were developed targeting the 18S ribosomal RNA gene for *Plasmodium* spp. All assays were performed bplexed with PCR reagents targeting the *Plasmodium* spp 18S sRNA gene and PHPRT1 human housekeeping gene as an endogenous control as described in the materials and methods. In the PCRs, 2 μl of template nucleic acid was used.

The Pleiades probe-based reagents were tested for specificity against a negative pooled human genomic DNA



**Figure 1** Comparison of microscopy, *Plasmodium falciparum*-specific HRP2 antigen detection and *Plasmodium* spp. aldolase antigen detection. Results are shown for specimens with parasite density under 15 000 parasites/μl that were positive for one of the three tests. All specimens with parasite densities >4900 parasites/μl were positive by all assays performed in this study. Positive results are shaded gray. Each column represents one specimen, and the corresponding microscopy, HRP2, and aldolase results are shown. Parasite density as determined by microscopy is shown on top in the bar chart on a logarithmic scale. All specimens represented on this chart contained *Plasmodium* spp. nucleic acid as determined by qRT-PCR.



**Figure 2** Correlations between parasite density and nucleic acid and antigen signal. (a) Relationship between parasite density and HRP2 enzyme immunoassay (EIA) optical density values for the clinical specimens. (b) Relationship between parasite density and aldolase EIA optical density values for the clinical specimens. (c) Relationship between parasite density and real-time PCR Cq values for the clinical specimens. The data set indicated with shaded rhombus corresponds to total nucleic acid amplification by qRT-PCR. The data set indicated with empty circles corresponds to DNA amplification by qPCR. The graphs show data points for which both parasite density and the other marker were positive.

that was obtained from Coriell Institute (DNA source: 25 African-American, five Chinese, and three Japanese individuals). Additionally, a blinded panel comprising 35 human genomic DNA specimens spiked with *P. falciparum* DNA and 35 *P. falciparum*-negative human genomic DNA specimens was tested for specificity by qRT-PCR and qPCR. The 35 human genome specimens were composed of eight US Caucasians, eight North Africans, nine sub-Saharan Africans and 10 Southeast Asians (procured from the Coriell Institute). No false positives or false negatives were observed. The reagents also did not show any cross-reactivity in PCRs with  $1 \times 10^6$  copies of each of *Trypanosoma brucei*, *T. gondii*, *Leishmania infantum/donovani* spp., *Salmonella typhi*, *S. paratyphi*, *Rickettsia* spp., dengue virus, measles virus, and influenza A and B.

Under the conditions described previously, the PCR assay showed an LOD of <50 copies of plasmid target DNA. The qPCR reagents were also tested against the WHO international standard for *Plasmodium falciparum* DNA nucleic acid amplification techniques (NIBSC#: 04/176) (Padley *et al.* 2008). The LOD determination was performed as recommended, yielding an LOD of 32 International Units (IU)/reaction.

To determine the relative contributions of DNA and RNA to total nucleic acid signal, ring-stage-enriched infected red blood cells (*P. falciparum* 3D7) were used in combination with qPCR and qRT-PCR. The Cq values for total nucleic acid amplification and RNA-only amplification (DNase-treated template) were lower than for DNA amplification with a mean  $\Delta$ Cq of 11.3 and 10.8, respectively (Table 3). Amplification of total nucleic acid had a 3 log lower LOD than amplification of DNA alone.

#### Performance characteristics of qPCR and qRT-PCR on clinical specimens

Total nucleic acid was extracted from whole blood specimens using the QIAmp DNA mini kits. Two microlitres of whole blood equivalents was used per PCR; 114/195 and 187/195 specimens were positive by qPCR and qRT-PCR, respectively. All microscopy-positive specimens were positive by PCR (Table 2), and all qPCR samples were positive by qRT-PCR. The sum of target 18S ribosomal RNA and DNA was significantly more than just 18S ribosomal DNA as confirmed by the relative mean Cq values for qPCR and q-RT-PCR (Table 4). As these specimens were not specifically preserved for RNA extraction, it is likely that specimen degradation had occurred prior to and during extraction. RNA extraction on fresh specimens yielded an average difference in Cq

**Table 3** Relative real-time PCR (qPCR) signals for nucleic acid extracted from blood specimens spiked with cultured *Plasmodium falciparum*. A 2.1% ring-stage-enriched *Plasmodium falciparum* 3D7 stock specimen serially diluted tenfold nine times yielding 10 dilutions total. Signals from amplification of DNA (qPCR), total nucleic acid (qRT-PCR) and RNA (DNase-treated qRT-PCR)

Dilution (log)	DNA qPCR Cq		RNA qRT-PCR Cq DNase treated		DNA + RNA qRT-PCR Cq	
	Cq	SD	Cq	SD	Cq	SD
0	23.36	0.03	15.06	0.01	15.02	0.01
1	28.65	0.03	18.24	0.02	16.87	0.00
2	30.89	0.06	19.64	0.00	19.47	0.01
3	34.03	0.08	21.88	0.01	21.81	0.05
4	35.18	0.22	23.69	0.01	22.73	0.03
5	37.20	0.32	29.16	0.04	27.87	0.15
6	39.30	0.51	27.26	0.01	27.07	0.00
7	43.46	2.10	30.72	0.02	30.56	0.16
8	Undetermined	NA	33.47	0.02	33.46	0.08
9	Undetermined	NA	41.63	2.07	38.17	0.83
10	Undetermined	NA	46.83	0.00	46.79	2.15
11	Undetermined	NA	Undetermined	NA	Undetermined	NA

Standard deviations are derived from triplicate PCRs.  
NA, not applicable.

**Table 4** Comparison of real-time PCR data for amplification of total nucleic acid (DNA + RNA) by RT-PCR *vs.* DNA only by PCR

DNA (qPCR) mean Cq	33
Total nucleic acid (qRT-PCR), mean Cq	27
Mean $\Delta$ Cq (DNA Cq–Total nucleic acid Cq)	6*, 8†

\*All  $\Delta$  Cq are included.

†Only  $\Delta$  Cq > 0 are included.

between total nucleic acid and DNA amplification corresponding to a three log difference in template amounts similar to that of cultured *P. falciparum* (data not shown). For both the qPCR and qRT-PCR, the Cq values were negatively correlated to the log of the parasite density with Pearson correlation coefficients of  $r = 0.70$  ( $P < 0.001$ ) for the qPCR and  $r = 0.71$  ( $P < 0.001$ ) for the qRT-PCR (Figure 2c). The Pearson correlation coefficients for the Cq values for qPCR and qRT-PCR is  $-0.75$  ( $P < 0.001$ ).

## Discussion

We compared the use of microscopy, antigen detection and qPCR to detect the presence of *Plasmodium* infection in 5- to 10-year-old children living in the malaria holoendemic Lake Victoria basin, Western Kenya, who presented to outpatient clinics with acute fever during the intense malaria transmission period. All individuals in this population are parasitemic multiple times over a lifetime. A

total of 195 children were recruited, 65 of whom were confirmed positive for malaria infection by microscopy.

*Plasmodium falciparum*-specific HRP2 antigen detection and pan-malarial aldolase by sandwich EIA were performed on plasma from the same sample set. The HRP2 EIA was more sensitive than the aldolase EIA and identified clinical specimens with lower parasite density than the aldolase EIA. This corroborates previous RDT results that showed greater sensitivity for HRP2-detection tests than the aldolase (Iqbal *et al.* 2002; Murray *et al.* 2008). We also found that HRP2 levels did not correspond to parasitemia as shown previously (Kifude *et al.* 2008; Martin *et al.* 2009). It has been suggested that plasma levels of HRP2 reflect total *P. falciparum* biomass rather than just circulating parasitemia (Dondorp *et al.* 2005; Martin *et al.* 2009), but our study was not designed to determine this. As would be expected for a short-lived antigen in circulation, the correlation between the aldolase EIA signal and parasite density was better, but not as good as previously reported for *Plasmodium* lactate dehydrogenase (Martin *et al.* 2009). In this population, patients that were positive for HRP2 positives and negative for microscopy all had submicroscopic levels of parasitemia.

The data in this study show that 96% of the specimens in this population have *Plasmodium* infection as demonstrated by qRT-PCR (DNA plus RNA) compared to 58% by qPCR (DNA only) and 33% by microscopy. Most likely in this population (children of ages between 5 and 10),

many of the cases of submicroscopic parasitemia described here are asymptomatic (Waitumbi *et al.* 2010). We attribute the difference in malaria prevalence to the greater sensitivity of qRT-PCR over qPCR: LOD for qRT-PCR was 3 log lower over the detection of DNA only (qPCR), as shown with axenic parasite samples (Tables 2 and 3). All specimens that were malaria positive by microscopy were also positive by the qPCR. Poorer sensitivities for NAAT assays are often observed at low parasite densities possibly because of the use of dry filter spots (DBS) and, as shown in this study and others, because of exclusion of the RNA contribution (Coleman *et al.* 2006; Menge *et al.* 2008; Nicastrì *et al.* 2009; Steenkeste *et al.* 2009). In contrast, for gametocyte detection, RNA is routinely targeted (Babiker *et al.* 1999; Schneider *et al.* 2004; Beurskens *et al.* 2009) indicating clearly that it is possible to take advantage of RNA in field samples. A recent report demonstrates sufficient RNA stability in DBS to allow gametocyte determination by qPCR (Mlambo *et al.* 2008). It will be valuable to evaluate the relative performances of the qRT-PCR assay with the qPCR assay on DBS, given the significant convenience of collecting DBS. This may allow determining submicroscopic prevalence in a manner consistent with gametocyte carrier prevalence determination.

### Conclusions

Microscopy and antigen detection identified similar numbers of malaria infections in 5- to 10-year-old children in a high transmission area in Western Kenya, although the correlation between antigen levels and parasite densities was poor, whereas qPCR and qRT-PCR correlated well with parasite density. For HRP2, we attribute the lack of correlation with patent parasitemia as determined by microscopy and PCR to cumulative HRP2 levels from previous parasitemia. Detection of total nucleic acid (qRT-PCR) identified the highest levels of submicroscopic malaria infection in this cohort. These differences need to be taken into consideration as new, more sensitive diagnostic platforms are developed for malaria diagnosis, surveillance and coinfection research applications. More sensitive assays such as the qRT-PCR while not suitable for clinical diagnosis may be critical for accurate determination of the asymptomatic reservoir in a given population. Careful consideration will have to be taken in setting standards for submicroscopic malaria prevalence determination.

### Acknowledgements

Support for this project was provided through funding from the Bill & Melinda Gates Foundation's Grand

Challenges in Global Health Initiative awarded to the University of Washington and collaborators at Micronics, Inc., Epoch Biosciences, PATH and the Stayton Group. The views expressed by the authors do not necessarily reflect the views of the funding agency. This work is published with the permission of the director of KEMRI.

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