Repeated sampling improved the sensitivity of malaria microscopy in children under six years

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Abstract
Objective: Microscopy remains the gold standard for identification of malaria parasites. However, the sensitivity of malaria microscopy is low. This study evaluated the impact of repeated sampling up to 12 h in 177 children < 6 years with suspected malaria.

Results: The median age was 3 years (interquartile range, 2.0–4.0 years). Eighty-nine percent (158/177) presented with hyperthermia together with one or more of the following symptoms: chills, headache, sweating, fatigue, nausea, abdominal pain, vomiting, diarrhea and cough. Baseline microscopy confirmed malaria in 29.9% (53/177) of the suspects. Repeated testing at 6 and 12 h increased the positive detection rates to 35.0% (62/177) and 41.8% (74/177), respectively. Microscopy underestimated malaria diagnosis by 11.9% on single testing. Children showing classical signs of malaria but with initial negative parasitological reports should be retested between 6 to 12 h to confirm or rule out a diagnosis of malaria.

Keywords: Malaria microscopy, Repeated sampling, Baseline parasitaemia, Children up to 5 years, Suspected malaria patients

Introduction
Globally, human malaria is caused by five *Plasmodium* species, namely, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* [1]. These parasites are responsible for over 228 million cases of malaria annually, resulting in about 405,000 deaths [2]. In sub-Saharan Africa, children under 5 years are at risk for malaria, and a significant number succumb to malaria if the diagnosis and treatment are delayed [3]. In malaria endemic countries, the number of malaria cases seems to be increasing, due to a decline in malaria control strategies which result in increasing transmission of parasites [4]. In malaria endemic countries, the number of malaria cases seems to be increasing, due to a decline in malaria control strategies which result in increasing transmission of parasites [4]. Key to malaria control is accurate detection of the parasites and treating with potent anti-malarials. Due to this, the World Health Organization (WHO) recommends parasitological confirmation of all suspected malaria cases before commencement of treatment [5].

Microscopy remains the gold standard in the laboratory identification of malaria [6]. However, the technique is unreliable in detection parasitaemia less than 500 parasites/μL of blood [7–9]. Despite being of low sensitivity, malaria microscopy could speciate *Plasmodium* parasites, and is useful in determining the degree of parasitaemia [10]. In cases of submicroscopic parasitaemia, microscopy is almost useless [11]. All *P. falciparum* infections start sub-microscopically before the detection limit is exceeded. In individuals with incompetent anti-malaria immunity such as children under 5 years, malaria-specific symptoms are usually provoked by low levels of parasitaemia. In this study, a strategy of repeated malaria testing of children up to 5 years presenting with
suspected malaria was adopted, to evaluate whether smears repeated at 6 and 12 h after the initial sample improved the diagnostic yield.

**Materials and methods**

**Study design, study site, and study population**

An observational study was conducted at Ga West Municipal Hospital, Amasaman, in the Greater Accra Region, Ghana from March through December 2018. The study participants were children up to 5 years that were suspected of malaria. Parental written informed consent was obtained for serial finger prick samples at baseline, 6 h and 12 h. During admission, children with negative malaria smears did not receive parenteral fluids nor antimalarial drugs. Once malaria was confirmed, antimalarial chemotherapy was commenced immediately. Children that remained febrile received tepid sponging and antipyretic therapy. Guardians completed a questionnaire to elicit responses on malaria risk assessment.

**Definition of suspected malaria**

Any child with axillary temperature above 37.5 °C or rectal temperature above 38.5 °C of any duration with or without any other clinical complaints was identified as a suspected malaria case. All suspected cases of malaria were identified by pediatricians. Data on all other clinical history and/or presenting complaints were collected from the patients’ folders.

**Parasitological confirmation of malaria**

Detection of *P. falciparum* specific histidine-rich protein 2 (Pfhrp2) was done with CareStart™, following the manufacturer’s instructions. Finger prick blood samples (approximately 6 µL of whole blood) were used to prepare thick blood films which were air dried, stained with 10% Giemsa for 10 min, and examined using light microscopy to quantify parasitaemia according to the WHO protocol [12].

**Data analysis**

The frequencies of demographic parameters, malaria risk assessment, and presenting complaints were presented as percentages. Detection rate for both microscopy and RDT were calculated based on the total of tested for that category. In subsequent testing, all cases previously confirmed as malaria were excluded. The differences in baseline, 6-h and 12-h parasitological assessment were determined by Chi-square. Significant differences were defined as p < 0.05. Statistical analysis was done by SPSS Version 24 (Chicago, IL, USA).

**Results**

Blood samples were taken from 177 children with suspected malaria. The median age was 3 years (interquartile range (IQR), 2.0–4.0 years), and 61.0% (108/177) were male. Most of the guardians (54.8%) were self-employed. Most (61.6%) of the patients lived close to open drains. Although 83.6% households owned long-lasting insecticidal nets (LLIN), these were not regularly used in 61.5% of households. Insecticide repellents were used in 51.4% of households. A large proportion of children (63.8%) were nocturnally active, and 21.4% slept in rooms with eaves (Table 1).

**Table 1 Demographic and malaria risk exposure parameters of the suspected patients**

| Parameters                          | Number of patients | %   |
|-------------------------------------|--------------------|-----|
| Age (years)                         |                    |     |
| 1                                   | 17                 | 9.6 |
| 2                                   | 33                 | 18.6|
| 3                                   | 39                 | 22.0|
| 4                                   | 47                 | 26.5|
| 5                                   | 41                 | 23.2|
| Gender                              |                    |     |
| Male                                | 108                | 61.0|
| Female                              | 69                 | 39.0|
| Category of guardian’s occupation  |                    |     |
| Unemployed                          | 16                 | 9.0 |
| Self-employed                       | 97                 | 54.8|
| Regularized private sector          | 43                 | 24.3|
| Civil servant                       | 21                 | 11.8|
| Living close to open drains         |                    |     |
| Yes                                 | 109                | 61.6|
| No                                  | 68                 | 38.4|
| LLIN availability                   |                    |     |
| Yes                                 | 148                | 83.6|
| No                                  | 29                 | 16.4|
| Regular LLIN usage                  |                    |     |
| Yes                                 | 57                 | 38.5|
| No                                  | 91                 | 61.5|
| Insecticide repellent usage         |                    |     |
| Yes                                 | 91                 | 51.4|
| No                                  | 86                 | 48.6|
| Staying in rooms with eaves         |                    |     |
| Yes                                 | 38                 | 21.4|
| No                                  | 139                | 78.5|
| Nocturnal outdoor activity          |                    |     |
| Yes                                 | 113                | 63.8|
| No                                  | 64                 | 36.2|

**LLIN long-lasting insecticide net**
Table 2 represents the clinical history of the children on direct questioning and examination. Almost all the children were hyperthermic (98.3%). The majority (> 50%) also experienced chills, headache, sweating, fatigue, and nausea while < 50% reported abdominal pain, vomiting, diarrhea, cough or rash. 89.2% (158/177) presented with more than one symptom.

The detection rate of malaria in baseline blood samples was 29.9% (53/177) using microscopy and 42.9% (76/177) using RDT ($x^2 = 5.90; p = 0.015$). At 6 h repeat sampling in children with initially negative results, 9 (5.1%) and 3 (1.7%) additional positive malaria cases were detected using smear and RDT, respectively. At 12 h repeat sampling in children with negative results at the baseline and 6 h testing time points, and additional 12 (6.8%) and 5 (2.8%) positive malaria cases were detected using smear and RDT, respectively. Serial microscopy testing increased the malaria detection rate by 11.9% compared to a single smear at baseline (from 29.9% to 41.8%) which was equivalent to the detection rates from a single RDT (42.9%) (Table 3). Serial RDT testing increased the detection rate by 4.6% (from 42.9% to 47.5%). At baseline, RDT was superior ($p < 0.0001$). However, testing 6 ($p = 0.041$) and 12 ($p = 0.023$) hours respectively after baseline, microscopy became better. Baseline parasitaemia range

**Table 2 Presenting history of suspected malaria patients on arrival and during follow-up**

| Clinical symptoms | On arrival | %  |
|-------------------|------------|----|
| Fever             | 174        | 98.3|
| Chills            | 153        | 86.4|
| Headache          | 148        | 83.6|
| Sweating          | 133        | 75.1|
| Fatigue           | 131        | 74.0|
| Nausea            | 98         | 55.4|
| Abdominal pain    | 77         | 43.5|
| Vomiting          | 68         | 38.4|
| Diarrhea          | 63         | 35.6|
| Cough             | 61         | 34.5|
| Rashes            | 53         | 29.9|

**Table 3 Baseline and follow-up detection of malaria parasites**

| Sampling time                  | Diagnostic methods   | Microscopy | Malaria rapid test | McNemar’s test |
|--------------------------------|----------------------|------------|--------------------|----------------|
|                                |                      | Number of patients | %       | Number of patients | %       |                     |
| Baseline                       |                      | Positive      | 53<sup>ab</sup> | 29.9  | 76<sup>cd</sup> | 42.9  | 21.0 ($p < 0.0001$) |
|                                | Range of parasitaemia (/µL) | 9815–83,452 | Mean axillary temperature | 38.3 °C | Mean axillary temperature | 37.1 °C |
|                                |                      | Negative      | 124                 | 70.1  | 101 | 52.5  |                     |
|                                |                      | Mean axillary temperature | 38.3 °C | Mean axillary temperature | 38.3 °C |                     |
| 6 h re-sampling on baseline negative patients |                      | Positive | 9                  | 7.9   | 3    | 3.0   | 4.1 ($p = 0.041$) |
|                                | Range of parasitaemia (/µL) | 1708–4416 | Mean axillary temperature | 37.7 °C | Mean axillary temperature | 37.7 °C |                     |
|                                |                      | Negative | 105                | 92.1  | 98   | 97.0  |                     |
|                                |                      | Mean axillary temperature | 37.7 °C | Mean axillary temperature | 37.7 °C |                     |
|                                | Cumulative positive patients | 62<sup>a</sup> | 35.0  | 79<sup>c</sup> | 44.6  |                     |
| 12 h sampling on 12-h negative results |                      | Positive | 12                | 11.4  | 5    | 5.1   | 5.1 ($p = 0.023$) |
|                                | Range of parasitaemia (/µL) | 2205–7110 | Mean axillary temperature | 37.8 °C | Mean axillary temperature | 37.6 °C |                     |
|                                |                      | Negative | 93                 | 88.6  | 93   | 94.9  |                     |
|                                |                      | Mean axillary temperature | 37.6 °C | Mean axillary temperature | 37.6 °C |                     |
|                                | Cumulative positive patients | 74<sup>b</sup> | 41.8  | 84<sup>d</sup> | 47.5  |                     |
|                                | Total malaria negative samples | 103      | 58.2  | 93   | 52.5  |                     |

<sup>a</sup> $x^2$ statistic 1.04, $p = 0.307$
<sup>b</sup> $x^2$ statistic 5.42, $p = 0.019$
<sup>c</sup> $x^2$ statistic 0.10, $p = 0.748$
<sup>d</sup> $x^2$ statistic 0.73, $p = 0.392$
was 9815–83,452 parasites/µL of blood. Parasitaemia range drastically reduced at 6-h (1708–4416) and 12-h (2205–7110) retesting (Table 3).

Discussion

Compared to microscopy, the sensitivity of rapid diagnostic test (RDT) kits is significantly higher. This is because RDT kits are manufactured to detect Plasmodium spp antigens in individuals with parasitaemia of 50 to –100 parasites/µL of blood [7]. Even though RDT kits are very useful in malaria diagnosis, they are unable to determine parasite densities as well as treatment success or failure. Additionally, considering increasing prevalence of Pfhrp2/Pfhrp3 mutant P. falciparum in Ghana [13] and elsewhere [14], malaria microscopy becomes extremely useful in their detection. In view of this, microscopy remains essential in malaria management. Several studies have confirmed the lower sensitivity associated with malaria microscopy [7–9]. However, to be able to monitor malaria treatment success, determination of baseline malaria parasitemia prior to treatment is essential.

In this study, malaria microscopy at baseline detected malaria parasites in 30% of children up to 5 years that presented with a clinical suspicion of malaria. Repeat smears, conducted at 6 and 12 h after admission increased the diagnostic yield considerably, with an additional 21 (11.9%) children diagnosed smear positive. Even though tepid sponging and the use of antipyretics were expected to significantly reduce body temperature within an hour [15], axillary temperature of 37.7–37.8 °C was obtained for the additional patients in which malaria parasite were confirmed. Strikingly, the cumulative detection rate of malaria microscopy up to 12 h of initial negative parasitological results was very comparable to the detection rate of baseline RDT ($x^2 = 0.046, p = 0.83$).

In a previous study, it was established that liver stage of merozoites double every 8 h. Hence, each hepatocyte contains thousands of merozoites [16]. Moreover, in early stage infections in non- or partially immune patients, such as the children included in this study, parasite multiplication during intra-erythrocytic stage range from six to tenfold per cycle, and in some cases up to 20-fold has been reported [17, 18]. It takes 48 h for P. falciparum to complete one cycle. Within 3–4 cycles, falciparum parasitaemia increase exponentially from one parasite to $10^8$ (thus 100 million) parasites, an increase of over 520,000 parasites each hour which corresponds to an increase of 50 parasites/µL hourly [19]. This level of parasitaemia is usually associated with the onset of parasite-induced fever and malaria-related symptoms in non-immune individuals [20]. It could therefore be speculated that malaria parasites were released slowly into peripheral blood. The most probable reason underpinning this speculation was that submicroscopic parasitaemia may exist in all the patients that were previously negative by microscopy but later tested positive. This was because malaria antigens were detected in all of them. Rapidly dividing pre-erythrocytic parasites together with multiplying intra-erythrocytic parasites increased parasitaemia in an exponential manner.

After the 12 h microscopy testing procedure, 103 patients with suspected malaria remained smear negative for Plasmodium spp. The mean temperature in these patients was 37.6 °C, and it is possible that subsequent parasitological evaluation may have yielded further positive result. Furthermore, RDT yields improved with serial testing. These findings reveal that a single microscopic or RDT test for malaria reporting underestimates detection by 11.9% and 4.6% respectively.

Not every pyrexia is due to malaria. Viral, bacterial, mycobacterial, fungal, other parasitic infections, systemic inflammatory conditions and malignancies are important alternative diagnoses in children that present with fever. It is recommended that clinical and laboratory evaluation of pyrexia must be done exhaustively to unearth the actual origin of pyrexia.

Conclusion

This study reports that one-time testing for malaria parasites underestimates malaria diagnosis by 12%. We recommend that children showing classical symptoms of malaria, but with initial negative parasitological reports be retested at 6 and 12 h to confirm or exclude the diagnosis of malaria.

Limitation

In order not to unduly keep the pyretic children in the health facility, parasitological assessment was done up to 12 h.

Abbreviations

µL: Microliter; LLIN: Long-lasting insecticide net; RDT: Rapid diagnostic test; WHO: World Health Organization.

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Authors’ contributions

EA conceptualised the study, collected data, analysed data and wrote the manuscript.

Authors’ information

Dr Enoch Aninagyei was the Head of Clinical Laboratory Unit at Ga West Municipal Hospital, Amasaman. Currently, I lecture on Microbiology and Molecular Biology at the University of Health and Allied Sciences, Ho in the Volta Region of Ghana.
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Availability of data and materials
All study data collected in this study are presented in this publication.

Ethics approval and consent to participate
This study was approved by Ghana Health Service Ethical Review Committee (GHS-REC002/03/18). Written consent was obtained from all the parents of children used in this study.

Consent for publication
Not applicable.

Competing interests
None to declare.

Consent for publication
None to declare.

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