AGE-DEPENDENT DISTRIBUTION OF PLASMODIUM FALCIPARUM GAMETOCYTES QUANTIFIED BY PFS25 REAL-TIME QT-NASBA IN A CROSS-SECTIONAL STUDY IN BURKINA FASO

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Abstract. Sexual stages of Plasmodium falciparum play a key role in the transmission of malaria. Studies on gametocytes are generally based on microscopic detection, but more sensitive detection methods for P. falciparum gametocytes frequently detect sub-patent gametocytes. We used Pfs25 mRNA quantitative-nucleic acid sequence-based amplification (QT-NASBA) to quantify gametocytes in 412 samples from a cross-sectional study in Burkina Faso, covering all age groups, to determine age-related patterns in gametocyte carriage and gametocyte density. The more sensitive QT-NASBA technique gave estimates of gametocyte prevalence 3.3-fold higher than microscopy (70.1% versus 21.4%, respectively). Prevalence of gametocytes significantly decreased with age. Our data suggest that asexual parasite densities are primarily responsible for the age-related decrease of gametocyte prevalence, possibly because of developing asexual stage immunity. Gametocyte densities decrease also with age, primarily because of decreasing asexual parasite densities; only a small but significant age effect on gametocyte density may be caused by developing sexual stage-specific immunity.

INTRODUCTION

Malaria is caused by protozoan parasites of the genus Plasmodium. The life cycle of these parasites consists of both asexual and sexual phases occurring in two hosts. Sexual reproduction occurs in the invertebrate vector, Anopheles mosquitoes for human malaria, whereas reproduction in the vertebrate host is solely asexual. However, sexual stage development starts in the vertebrate host where a proportion of the asexual parasites transform into sexual stages, called gametocytes. The gametocytes can infect mosquitoes, reproduce sexually, and are responsible for ongoing transmission of malaria to the next host. Despite the importance of gametocytes for the spread of malaria, relatively little is known about sexual stage development in comparison to the asexual stages that cause disease symptoms. Until recently, studies on gametocytes were based on microscopy, which is rather insensitive and inaccurate in quantification of gametocytes in blood samples (A. L. Ouédraogo and others, unpublished data). Individuals without microscopically detectable gametocytes can infect mosquitoes, and higher gametocyte prevalences are found when larger volumes of blood are used for analysis. More sensitive detection methods for Plasmodium falciparum gametocytes such as the Pfs25 or Pfg377 reverse transcriptase-polymerase chain reaction (RT-PCR) are able to detect sub-patent gametocytes, which can be quantified by Pfs25 mRNA quantitative-nucleic acid sequence-based amplification (QT-NASBA). The Pfs25 QT-NASBA has a detection limit of 20–100 gametocytes/mL of blood, and the high-throughput format allows its use in large epidemiologic studies. A previous study with Pfs25 QT-NASBA showed very high prevalence of gametocytes in symptomatic children in Kenya. The objective of this study was to determine submicroscopic levels of gametocytes in a different epidemiologic setting. We used Pfs25 QT-NASBA to quantify gametocytes in 412 samples from a cross-sectional study in Burkina Faso, covering all age groups, and determined age-related patterns in gametocyte carriage and density.

MATERIALS AND METHODS

Field study. The study took place in six small rural villages 30 km north of Ouagadougou, Burkina Faso. These villages are situated in a Sudanese savannah area with a marked wet season from June to October. The distance between villages varies from 1 to 5 km. Malaria transmission is highest in the wet season and peaks around September. Plasmodium falciparum is responsible for 90% of malaria infections with Anopheles gambiae and A. funestus as major vectors. Health care facilities are equally distributed in the six villages, and residents live by subsistence farming.

A cross-sectional survey was carried out at the end of the transmission season (December 2003), at which time 412 villagers of all ages were enrolled. Adults and children of both sexes and exclusively of the Mossi ethnic were randomly included in the study by order of arrival until a minimum number of 10 individuals per age group with informed consent were included. A finger prick blood sample was taken from all participants. Thick and thin blood films were made, air-dried, and stained with 5% Giemsa. For collection of nucleic acids, 100 μL of blood was mixed with 900 μL of L6 lysis buffer and stored for RNA extraction. Most participants were asymptomatic; only 7% had a body temperature of > 37.5°C and were treated with chloroquine according to the national policy after blood samples for the study had been obtained. The study received ethical approval of the Ministry of Health of Burkina Faso.

Microscopic detection of P. falciparum parasites. Samples were considered negative if no parasites were detected in 100 fields (10 × 100 magnification). Both asexual stage and gametocyte densities were simultaneously assessed by counting against 500 leukocytes in the thick smear. The lower limit of microscopy for gametocyte quantification was therefore evaluated to 16 gametocytes/μL of blood. Parasite counts were converted to numbers of parasites per microliter by assuming a standard count of 8,000 leukocytes/μL of blood.
Each sample was read independently by two microscopists. A third reader was involved when the difference between the readers exceeded 30%, and in such a case, the median reading was used (CNRFP Ouagadougou).

**Real-time Pfs25 QT-NASBA and nucleic acid extraction.** Nucleic acids were extracted from blood samples using the guanidiumisothiocyanate (GuSCN)/silica procedure and 18S rRNA real-time QT-NASBA and Pfs25 real-time mRNA QT-NASBA were performed as described elsewhere. Briefly, real-time QT-NASBA for Pfs25 mRNA (Genbank accession no. AF193769.1) was performed on a Nuclisens EasyQ analyser (bioMérieux sa, Lyon, France) using the Nuclisens BasicKit for amplification according to the manufacturer’s instructions at a KCl concentration of 80 mmol/L. Reactions were performed in a total reaction volume of 10 μL per reaction (forward primer: 5'-gactgtaataacatggagaaggattaccgc-3'; reverse primer: 5'-aattctaatgacgaagagacctttaccgt-taccacaagtt-3'; Pfs25 molecular beacon: 5'-TexasRed-egatgctcggattcagttgcc-DABSYL-3'). For quantification, time to positivity was calculated (i.e., the time-point during amplification at which the fluorescence detecting target amplicons becomes higher than the mean fluorescence of three negative controls + 20 SD). The use of a standard gametocytes stage 5 dilution series allowed exact calculation of the number of gametocytes present in unknown samples.

**Data analysis.** Statistical analyses were performed in SPSS (SPSS Inc., Chicago, IL) 12.0.1. Because parasitologic parameters, including total parasite and gametocyte densities, were not different between the six villages (data not shown), samples were pooled for analyses. Spearman correlation was used to determine correlation between results of microscopy and QT-NASBA. A \(10\log(x + 1)\) transformation was applied to both asexual parasite and gametocyte counts to allow negative samples in the analysis. Geometric mean of gametocyte density was calculated for gametocyte positive samples and for all samples, including the negatives. To determine the relation to age group and asexual parasite density, logistic regression was used for gametocyte prevalence and linear regression for gametocyte density. The age range was very large, and therefore, we analyzed age group as a categorical variable in comparison with the oldest age group of 25+ years (adults). With such analyses, a decrease of the correlation coefficient with increasing age indicates a negative relation between age group and the parameter under study.

**RESULTS**

**Comparison of QT-NASBA and microscopy for gametocyte detection.** Blood samples for QT-NASBA analysis were collected randomly from 412 individuals with a mean age of 17.5 years (range, 2–83 years) after informed consent. All participants were assigned to age groups: < 5 (N = 79), 5–9 (N = 97), 10–14 (N = 78), 15–24 (N = 71), and ≥ 25 years (N = 87).

Pfs25 mRNA QT-NASBA confirmed that 92% (81/88) of samples were gametocyte positive by microscopy, with a geometric mean QT-NASBA gametocyte density of \(3.90 \times 10^3\)/mL blood (IQR, \(5.11 \times 10^2\-2.72 \times 10^3\)/mL blood). In general, gametocyte densities detected by microscopy were close to the microscopical detection limit with 1 gametocyte/500 leukocytes counted in 51.1% of these samples. The more sensitive QT-NASBA detected gametocytes in an additional 208 samples, with a geometric mean density below the detection limit of microscopy (1.98 \(\times 10^3\)/mL blood; IQR, 3.95 \(\times 10^2\-9.60 \times 10^3\)/mL blood).

**Parasite prevalence.** Detection of gametocytes by Pfs25 real-time QT-NASBA considerably increased gametocyte prevalence from 21.4% (microscopy) to 70.1%. Total parasite and gametocyte prevalence as detected by the two methods are shown for all age groups in Figure 1. The detection of total parasite prevalence and gametocyte prevalence were higher by QT-NASBA compared with standard microscopy. A decrease of \(P. falciparum\) prevalence with age was found by microscopy but not by QT-NASBA, indicating that parasite densities in adults were merely reduced to submicroscopic levels. Gametocyte prevalence was negatively associated with age and 3.2- to 3.8-fold higher in the three youngest age groups (0–15 years) compared with that in adults (25+ years) by QT-NASBA analyses (Table 1). For microscopy, this was 1.7- to 1.9-fold higher (data not shown).

Asexual parasite densities detected by microscopy decrease significantly with age (A. L. Ouédraogo and others, unpublished data). Because gametocytes are derived from their asexual progenitors, it is important to separately detect the
Calculations of mean gametocyte densities are influenced by gametocyte prevalence if gametocyte-negative samples are included. Therefore, any factor that influences gametocyte prevalence may have been included in the analysis, without necessarily having a direct relation to gametocyte density. To avoid the risk of incorporating such indirect effects, the analyses were repeated in only QT-NASBA gametocyte-positive samples. Asexual density still tended to decrease with age, although the relation was not significant ($P = 0.378$). However, QT-NASBA gametocyte densities still decreased slightly with age. Table 1 shows that the two youngest age groups ($< 10$ years) carry significantly higher gametocyte densities compared with adults. This effect of age is independent of asexual density. Such an age effect was not significant for microscopy, with or without asexual parasite density as a covariate.

**DISCUSSION**

Pfs25 real-time QT-NASBA showed an overall gametocyte prevalence of 70.1% during a cross-sectional study at the end of the rainy season in Burkina Faso, which is higher than in studies based on microscopy.\textsuperscript{15,16} Although the additionally detected gametocyte carriers in general have very low gametocyte densities, the potential contribution of this group to the infectious reservoir should not be ignored (P. Schneider and others, unpublished data).\textsuperscript{11} Therefore, transmission studies based on mosquito feeds with random population samples\textsuperscript{16–18} will give a more adequate estimation of population-wide transmission potential than studies based on microscopy.\textsuperscript{15,19–21}

Age-related decreases in both asexual parasites and gametocytes have been shown before.\textsuperscript{15,16,18,22–24} Such relations may be the result of developing immunity to asexual and sexual stages of *P. falciparum* over time. In this study, the age-dependent decrease of asexual parasites suggests the development of asexual stage immunity. Gametocyte prevalence and densities also decreased with age. As these stages are formed from their asexual progenitors, this may be the result of a lower availability of asexual progenitors (sexual stage immunity), of cross-stage immunity,\textsuperscript{25} or of sexual stage-specific immunity. Our results show that the relation be-

### Table 1

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>N</th>
<th>OR* (95% CI)</th>
<th>P value</th>
<th>OR† (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5</td>
<td>79</td>
<td>3.16 (1.61–6.20)</td>
<td>0.001</td>
<td>1.53 (0.73–3.24)</td>
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<tr>
<td>5–9</td>
<td>97</td>
<td>3.83 (2.00–7.37)</td>
<td>0.000</td>
<td>1.86 (0.90–3.83)</td>
<td>0.094</td>
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<tr>
<td>10–14</td>
<td>78</td>
<td>3.35 (1.69–6.63)</td>
<td>0.001</td>
<td>1.85 (0.89–3.88)</td>
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</tr>
<tr>
<td>15–24</td>
<td>71</td>
<td>1.52 (0.80–2.88)</td>
<td>0.197</td>
<td>1.05 (0.53–2.08)</td>
<td>0.884</td>
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<tr>
<td>≥ 25</td>
<td>87</td>
<td>1.0‡</td>
<td></td>
<td>1.0‡</td>
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</tr>
</tbody>
</table>

**Gametocyte density¶**

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>N</th>
<th>β* [SE (β)]</th>
<th>P value</th>
<th>β† [SE (β)]</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5</td>
<td>79</td>
<td>1.11 (0.26)</td>
<td>0.000</td>
<td>0.50 (0.28)</td>
<td>0.071</td>
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<tr>
<td>5–9</td>
<td>97</td>
<td>1.19 (0.25)</td>
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<td>0.59 (0.27)</td>
<td>0.027</td>
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<tr>
<td>10–14</td>
<td>78</td>
<td>1.02 (0.27)</td>
<td>0.000</td>
<td>0.53 (0.27)</td>
<td>0.053</td>
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<tr>
<td>15–24</td>
<td>71</td>
<td>0.45 (0.27)</td>
<td>0.098</td>
<td>0.16 (0.27)</td>
<td>0.556</td>
</tr>
<tr>
<td>≥ 25</td>
<td>87</td>
<td>0.0‡</td>
<td></td>
<td>0.0‡</td>
<td></td>
</tr>
</tbody>
</table>

**Gametocyte density¶**

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>N</th>
<th>β* [SE (β)]</th>
<th>P value</th>
<th>β† [SE (β)]</th>
<th>P value</th>
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<tbody>
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<tr>
<td>5–9</td>
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<td>10–14</td>
<td>61</td>
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<tr>
<td>15–24</td>
<td>44</td>
<td>0.213 (0.21)</td>
<td>0.309</td>
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<td></td>
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<tr>
<td>≥ 25</td>
<td>45</td>
<td>0.0‡</td>
<td></td>
<td>0.0‡</td>
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</tbody>
</table>

*Crude values of odds ratio (OR) and β.
† OR and β adjusted for **log asexual parasite density. Asexual density OR = 1.27 (CI = 1.16–1.38; $P < 0.001$) for gametocyte prevalence and β = 0.18 [SE (β) = 0.03; $P < 0.001$] for gametocyte density.
‡ Age group ≥ 25 years was used as reference group.
¶ All samples.
$^*$ Samples from Pfs25 QT-NASBA-positive gametocyte carriers.
** Adjustment for asexual density was not significant with β = 0.023; SE (β) = 0.026; and $P = 0.378$.**

*Parasite density.* Figure 2 shows that gametocyte densities and the asexual parasite densities decrease with age. Linear regression analyses showed a significant negative effect of age on gametocyte density detected by either QT-NASBA (Table1) or microscopy (data not shown), was found with increasing age. A significant positive association was shown between asexual parasite density and gametocyte prevalence (OR = 1.27; 95% CI = 1.16–1.38; $P < 0.001$) for QT-NASBA (Table1); OR = 1.12; 95% CI = 1.01–1.25; $P = 0.040$ for microscopy). These data suggest that the age-related decrease of gametocyte prevalence is probably the result of decreasing asexual parasite densities.

**Figure 2.** Mean parasite densities for different age groups, calculated for all samples. Solid bars represent asexual parasite density measured by microscopy. Striped bars represent gametocyte density measured by QT-NASBA. Numbers of individuals per group are presented on top of bars. Error bars indicate SD.
between age and both gametocyte prevalence and density depends primarily on asexual parasite density, suggesting that asexual or cross-stage immunity may be an important determinant. This effect may obscure direct influences of age (i.e., anti-gametocyte immunity). We adjusted for this effect in two different ways. 1) We adjusted for asexual parasite density. Although a trend was seen, age-related decreases in gametocyte density were non-significant after this adjustment. However, as a result of adjustment with highly variable asexual parasite densities, larger samples sizes may be needed to obtain significant relationships. 2) We selected only Pf525 QT-NASBA–positive samples. The influence of asexual parasite density was reduced to insignificant levels in this selection, whereas an age-related decrease in gametocyte density was still seen. This decrease of gametocyte densities with age, unaffected by asexual parasite densities, may be the result of anti-gametocyte immunity. However, the age-related decrease of gametocytes is likely determined by immunity that results in primarily a decreased asexual parasite density rather than direct effect on gametocytes.

The influence of asexual parasite density on gametocytes, described above, was based on analyses including microscopically counted asexual parasites. Although microscopic detection of asexual parasites is more robust than that of gametocytes, more sensitive methods like QT-NASBA may also detect sub-patient asexual parasites. Ideally, we would have included asexual parasite density determined by QT-NASBA as a covariate. QT-NASBA is currently available for quantification of the total parasite load or for gametocytes only, but not yet for specific quantification of asexual parasites as is the case for other molecular biology methods such as PCR.^{7,14}

Our results were obtained with samples collected at the end of the wet season. With a marked seasonal transmission in Burkina Faso, we cannot generalize these results to all seasons. It is possible that gametocyte prevalence and mean gametocyte densities, as well as the relations with age, vary over time. However, in a preceding study over various seasons, of which the present samples are a small part, we came to similar conclusions with microscopic parasite detection (A. L. Ouédraogo and others, unpublished data). QT-NASBA analysis of samples of cross-sectional surveys in other seasons will be performed.

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