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Infectiousness of the Human Population to *Anopheles arabiensis* by Direct Skin Feeding in an Area Hypoendemic for Malaria in Senegal

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Abstract. Direct skin feeding experiments are sensitive assays to determine human infectiousness to mosquitoes but are rarely used in malaria epidemiological surveys. We determined the infectiousness of inhabitants of a malaria hypoendemic area in Senegal. Gametocyte prevalence by microscopy was 13.5% (26 of 192). Of all individuals who were gametocyte positive, 44.4% (11 of 25) infected ≥ 1 *Anopheles arabiensis* mosquito and 10.8% (54 of 500) of mosquitoes became infected. Of all individuals who were gametocyte negative by microscopy, 4.3% (7 of 162) infected ≥ 1 mosquito and 0.4% (12 of 3240) of mosquitoes became infected. The 18.2% (12 of 66) of all mosquito infections was a result of submicroscopic gametocyte carriage and two individuals without asexual parasites or gametocytes by microscopy were infectious to mosquitoes. When infectivity and local demography was taken into account, children 5–14 years of age contributed 50.8% of the human infectious reservoir for malaria. Adults and submicroscopic gametocyte carriers may contribute considerably to onward malaria transmission in our setting.

INTRODUCTION

Malaria is one of the most deadly infections in the world with 600,000 deaths annually, predominantly in young children in sub-Saharan Africa.1,2 In Senegal, malaria transmission has declined recently,3 although *Plasmodium falciparum* remains endemic throughout the country.4 Malaria transmission occurs from mosquito to man and from man to mosquito. Transmission from man to mosquitoes is poorly quantified5 and a focus often lies on entomological parameters like aggressiveness of anophelines to humans, the entomological inoculation rate, vectorial capacity,6,7 and indicators of parasite prevalence in the human population.8 However, these parameters fail to directly assess human infectiousity that is most relevant in describing malaria transmission from mosquito to man.10 Only a handful of surveys on the natural process of transmission of *Plasmodium* from man to mosquito have been taken. Some indices have been defined, including the proportion of humans with malaria transmission stages, gametocytes in their peripheral blood, the proportion of infectious individuals, and the proportion of mosquitoes that have become infected after feeding.11–15 Often, these studies were restricted to microscopically detected gametocyte carriers, even though it has been repeatedly shown that submicroscopic gametocyte densities may result in mosquito infection.16,17 Although microscopically detectable gametocyte densities are more likely to result in mosquito infections compared with submicroscopic gametocyte densities, the contribution of submicroscopic gametocyte carriers to the total infectious reservoir may be considerable18 and can only be determined in xenodiagnostic surveys where individuals are recruited for feeding experiments, regardless of their parasitemic status. A recent meta-analysis further suggests that direct skin feeding assays may be the most sensitive approach to determine human infectiousness to mosquitoes.16

In this study, we determine infectivity of the human population to mosquitoes in a hypoendemic area of Senegal using direct skin feeding mosquito assays and observe a considerable contribution of adults and submicroscopic gametocyte carriers to malaria transmission.

MATERIALS AND METHODS

This study was carried out in 2010 in the Thies region 90 Km north of Dakar. Malaria transmission was described by Robert and others9 in the bordering zone of Niakhar. *Anopheles arabiensis* is the main vector. Malaria transmission occurs mainly during the rainy season from July to October with about 400–500 mm of annual rainfall and a long dry season from November to June. The level of malaria transmission in this area is hypo-endemic; older estimates of the sporozoite rate in field-caught mosquitoes were 1.6% to 1.8%.19 The study procedures were previously described by Bonnet and others.20 Families were invited to participate in the study during community sensitization meetings. From all participating families, family members ≥ 5 years of age were invited for mosquito feeding assays. Children < 5 years of age were not included because of ethical concerns.

Ethical approval was granted by the National Ethical committees of Senegal for this study. The study protocol and objectives were carefully explained to the local administrative, community leaders, and the assembled village population. Participants were offered free treatment of malaria, intestinal helminthes infections and anemia, and received during the project an insecticide impregnated bed net. Individual written informed consent was obtained from all participants and all caretakers of participants < 18 years of age.

Measurements. Thick blood smears were prepared from each individual and slides were stained with 10% giemsa for 30 minutes and microscopically examined. Two-hundred fields were read for asexual parasites and gametocytes. Assuming
TABLE 1
Characteristics of the study population

<table>
<thead>
<tr>
<th>Ages groups</th>
<th>5–9</th>
<th>10–14</th>
<th>15–24</th>
<th>25–44</th>
<th>≥45</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>45</td>
<td>44</td>
<td>31</td>
<td>38</td>
<td>34</td>
</tr>
<tr>
<td>Asexual parasite prevalence, % (n/N)*</td>
<td>53.3 (24/45)</td>
<td>63.6 (28/44)</td>
<td>45.2 (14/31)</td>
<td>34.2 (13/38)</td>
<td>44.1 (15/34)</td>
</tr>
<tr>
<td>Asexual parasite density, GM (IQR)</td>
<td>418.4 (90.5–2192.5)</td>
<td>500.3 (94.5–5052.0)</td>
<td>176.7 (76.0–198.0)</td>
<td>83.8 (65.0–119.0)</td>
<td>129.2 (56.0–195.0)</td>
</tr>
<tr>
<td>Gametocyte prevalence, % (<strong>n/N</strong>)</td>
<td>28.9 (13/45)</td>
<td>11.4 (5/44)</td>
<td>9.7 (3/31)</td>
<td>5.3 (2/38)</td>
<td>8.8 (3/34)</td>
</tr>
</tbody>
</table>

N = proportion of each age group in the sample population.
* n = number of individuals with asexual parasite.
** n = number of gametocyte carriers in each age group.
IQR = interquartile range; GM = Geometric mean.

10–20 white blood cells per high power field and 8,000 white blood cells per µL, this gives an estimated sensitivity of microscopy of 2–4 parasites/µL. Sulfadoxine-pyrimethamine (Fansidar, Laboratoire Roche, France) was provided for individuals who were infected with > 1,000 parasites/µL.

Mosquito feeding assays. Study participants > 5 years of age were included in an evaluation of infectiousness by feeding laboratory-bred mosquitoes directly on their skin.11,16 A local strain of *An. arabiensis* that has been reared in IRD (Institut de Recherche pour le Developpement) insectary in Dakar was used. Batches containing 25 3-day-old mosquitoes, female anopheles, were starved for 12 hours before being applied to the participant arm. Each individual receives one batch of mosquitoes. Mosquitoes were allowed to feed 10–15 minutes and after feeding, disinfected with alcohol and anti-histamine ointment was put on the skin. Participants were instructed to avoid movement and tolerated the blood feeding without any side effects. After feeding, mosquitoes were transferred from the cups to the cages 12 × 12 × 12 cm and labeled (number of the gametocyte carrier, date of infection). With a mouth aspirator, unfed mosquitoes were removed. Sugar solution was provided for 7 days during which mosquitoes were kept at 25°C, 70–80% humidity. Only cages with at least 20 mosquitoes surviving until Day 7 were included in the analysis. The midguts of surviving females were dissected in a drop of physiological saline (PBS) with 1% mercurchrome to facilitate observation of oocysts detection at the microscope. The infectivity of each patient participant was assessed by determining the percentage of mosquito midguts infected with at least one oocyst.

Statistical analysis. Data were analyzed in Epi Info version 7 (Centers for Disease Control and Prevention, Atlanta, GA) and Stata version 12 (StataCorp, College Station, TX). Proportions were compared by the χ² test or Fisher exact; mean densities by the Mann-Whitney test or the Kruskall Wallis test. The contribution of different age groups to malaria transmission (Ci) was estimated using the proportion (Pi) of the total population in each age group and the age group-specific probability of mosquito infection (Ti), which corresponds to the proportion of mosquitoes infected in feeding assays involving individuals from each age group, regardless of their parasite status:

\[ C_i = \left( \frac{T_i P_i}{\sum_{5-9 \text{ years}}^{>45 \text{ years}} (T_i P_i)} \right) \]

RESULTS

We enrolled a total of 192 individuals 5–65 years of age. Parasite prevalence was 49.0% (94 of 192) and negatively associated with age as a categorical variable (Table 1, P = 0.05). Gametocyte prevalence was 13.5% (26/192) and negatively associated with age as a categorical variable (Table 1, P = 0.005). Geometric mean gametocyte density was 53.7 gametocytes/µL (interquartile range [IQR] 4–226) and there were too few microscopy positive gametocyte observations to allow a robust statistical analysis of gametocyte density in relation to age.

Mosquito feeding experiments. Direct skin feeding experiments were successful for 187 individuals (97.4%, 187 of 192); four experiments failed because fewer than 20 mosquitoes survived until Day 7 post feeding (5, 7, 9, and 11 mosquito observations) and one subject was found to be *Plasmodium malariae* infected; this individual did not infect mosquitoes and was excluded from the analyses. Exactly 20 mosquitoes were dissected and examined for the remaining 187 experiments, giving 3,740 mosquito observations. Eighteen experiments (9.4%, 18 of 187) resulted in at least one infected mosquito; 66 mosquitoes (1.8%, 66 of 3740) became infected. The results of the feeding experiment for each age group and

TABLE 2
Infectiousness in relation to age and parasite status

<table>
<thead>
<tr>
<th>Gametocyte status</th>
<th>Number of experiments</th>
<th>Proportion of individuals infecting ≥ 1 mosquito, % (n/N)</th>
<th>Proportion of infected mosquitoes, % (n/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–9</td>
<td>Positive</td>
<td>12</td>
<td>25.0 (3/12)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>31</td>
<td>6.5 (2/31)</td>
</tr>
<tr>
<td>10–14</td>
<td>Positive</td>
<td>5</td>
<td>80.0 (4/5)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>39</td>
<td>5.1 (2/39)</td>
</tr>
<tr>
<td>15–24</td>
<td>Positive</td>
<td>3</td>
<td>66.7 (2/3)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>28</td>
<td>0.0 (0/28)</td>
</tr>
<tr>
<td>25–44</td>
<td>Positive</td>
<td>2</td>
<td>50.0 (1/2)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>35</td>
<td>0.0 (0/35)</td>
</tr>
<tr>
<td>≥45</td>
<td>Positive</td>
<td>3</td>
<td>33.3 (1/3)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>29</td>
<td>10.3 (3/29)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>187</td>
<td>9.6 (18/187)</td>
</tr>
</tbody>
</table>
gametocyte-positive and -negative individuals are reported in Table 2 and shows that gametocyte-positive individuals were considerably more likely to infect mosquitoes ($P < 0.001$) and infected more mosquitoes ($P < 0.001$). Of all individuals who were gametocyte positive by microscopy, 44.4% (11 of 25) infected at least one mosquito and 10.8% (54 of 500) of mosquitoes became infected. Of all individuals who were gametocyte negative by microscopy, 4.3% (7 of 162) infected at least one mosquito and 0.4% (12 of 3,240) of mosquitoes became infected. In children and adults some individuals who were gametocyte negative by microscopy infected mosquitoes. In our study setting 18.2% (12 of 66) of all mosquito infections was a result of submicroscopic gametocyte carriage. Among the seven individuals who infected mosquitoes despite being gametocyte negative, two were completely slide negative (asexual and sexual parasite slide negative); the remaining five had asexual parasites detected by microscopy but no gametocytes. Although there was a positive correlation between gametocyte density and mosquito infection rates (Figure 1; $r = 0.47$, $P < 0.0001$), the association appeared non-linear and there were no individuals with high gametocyte densities that did not infect any mosquitoes.

The human infectious reservoir for malaria. The contribution of different age groups to the human infectious reservoir for malaria was estimated by including demographic data from a recent demographic survey in the study area (Table 3).

The validity of the estimation of malaria transmission from man to mosquito depends on the representativeness of our sample to the total population. We compared the proportions of each age group between this sample and the national census in the study area. There was a significant difference between the demographic profile of the population on which mosquitoes fed and the demographic profile of the total population in this area. The under-representation of 5–14 age groups and the over 44 age-groups was a result of the absence of some schoolboys far from the village, and some adults working outside the study area and thereby not available for the surveys. When we adjust our estimates of infectiousness to mosquitoes for the contribution of different age groups to the total population, we found that 51.5% of all mosquito infections that resulted from blood sampling on individuals ≥ 5 years of age was a result of children 5–14 years of age. Young adults, 15–24 years of age comprised 17.3% of the village population according to census data and contributed to 33.3% of all mosquito infections. The remaining infected mosquitoes 15.15% (10 of 66) were contributed by those over 24 years of age.

### DISCUSSION

In this study, we determined the human infectious reservoir in an area of hypoendemic malaria transmission in Senegal. We observed that 44% of the microscopically detectable gametocyte carriers were capable of infecting *An. arabiensis* mosquitoes. Although we did not directly determine gametocyte carriage by molecular methods, we found evidence for subpatent gametocyte carriage: 4.3% of gametocyte-negative individuals were capable of infecting mosquitoes, although with low mosquito infection rates. Adults comprised a considerable proportion of the human infectious reservoir, more than half of all mosquito infections occurred from individuals > 15 years of age.

We performed a xenodiagnostic evaluation of transmission from man to mosquito selecting individuals irrespective of gametocytaemia or parasitaemia. Direct feeding of laboratory-bred *An. arabiensis* was performed by using a convenience sampling approach in which families were invited to participate during community meetings and all family members 5 years of age and above were invited to participate in the study. Although there is variation in the manner in which skin feeding is performed, it appears to be a more efficient tool to measure *Plasmodium falciparum* transmission from man to mosquito than the more widely used membrane feeding assays. We chose a minimum number of fed mosquitoes per batch to maximize the informativeness of individual feeding experiments. Our findings confirm previous findings on a higher likelihood of mosquito infection from microscopically detectable gametocyte densities and a relevant contribution of submicroscopic gametocyte carriers to onward malaria transmission. Because of the methodology we used, direct skin feeding, we could not directly determine gametocyte density in the mosquito blood meal. There are indications that

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Proportion in the current study (n)</th>
<th>Proportion of population in a census (%)</th>
<th>Proportion of individuals with ≥ 1 mosquitos % (n/N)</th>
<th>Proportion of infected mosquitoes, % (n/N)</th>
<th>Relative contribution to the infectious reservoir of individuals ≥ 5 years*</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 5 years</td>
<td>ND</td>
<td>19.3</td>
<td>ND</td>
<td>1.6 (14/860)</td>
<td>19.7%</td>
</tr>
<tr>
<td>5–9</td>
<td>23.0 (43)</td>
<td>17.0</td>
<td>11.6 (5/43)</td>
<td>2.3 (20/880)</td>
<td>19.9%</td>
</tr>
<tr>
<td>10–14</td>
<td>23.5 (44)</td>
<td>12.3</td>
<td>13.6 (6/44)</td>
<td>3.5 (22/620)</td>
<td>43.7%</td>
</tr>
<tr>
<td>15–24</td>
<td>16.6 (31)</td>
<td>17.3</td>
<td>6.5 (2/31)</td>
<td>0.4 (3/740)</td>
<td>5.9%</td>
</tr>
<tr>
<td>25–44</td>
<td>19.8 (37)</td>
<td>20.3</td>
<td>2.7 (1/36)</td>
<td>1.1 (7/640)</td>
<td>10.8%</td>
</tr>
<tr>
<td>≥ 45</td>
<td>17.1 (32)</td>
<td>13.8</td>
<td>12.5 (4/32)</td>
<td>1.8 (66/3740)</td>
<td>100%</td>
</tr>
<tr>
<td>Total</td>
<td>(187)</td>
<td>100.0</td>
<td>9.6 (18/187)</td>
<td>1.8 (66/3740)</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

ND = non-determined; contribution to the infectious reservoir estimated by the formula.
gametocyte densities may be higher in the subdermal vasculature compared with venous blood and potentially that there are higher gametocyte densities in the subdermal vasculature compared with finger prick blood samples. One may therefore hypothesize that mosquitoes sample gametocytes more efficiently from the subdermal vasculature than microscopy can detect gametocytes in finger prick blood samples that are taken from slightly deeper capillaries. The phenomenon of relatively efficient mosquito transmission from low gametocyte densities has also been reported for membrane feeding assays where gametocytes were detected in the venous blood sample that was offered to mosquitoes and suggests that clustering of gametocytes in the mosquito blood meal may contribute to the efficient transmission of malaria at gametocyte densities below the threshold density, which allows gametocyte detection by microscopy. Specific studies must be carried out to elucidate this long-standing question on gametocyte densities and infectiousness in different blood compartments that may have fundamental consequences for the interpretation of malarialmetric indices from venous blood or finger prick blood samples.

Shortcomings of our study include the fact that we did not perform a formal random selection of individuals but approached families and recruited all family members who were willing to participate. The most important shortcoming may be that we excluded children < 5 years of age for ethical reasons. In our study setting, it was considered unacceptable to include this young age group. Membrane feeding assays may be more acceptable but have a lower sensitivity and form a less natural assessment of infectivity where loss of gametocyte infectiousness caused by handling of venous blood samples cannot be excluded. Our choice for direct skin feeding precluded any statements on the infectiousness of the youngest age group who may have the highest gametocyte densities and may be particularly infectious to mosquitoes and form an important part of the human infectious reservoir. Despite this important limitation, our findings illustrate that adults, in particular young adults 15–24 years of age, contribute considerably to onward malaria transmission. This age group is currently not directly targeted in malaria control interventions in Senegal, and this may limit the impact of current interventions on malaria transmission intensity because a considerable proportion of the human infectious reservoir is neglected. Another shortcoming is our dependence on microscopy. We did not use molecular methods to detect gametocytes that are known to substantially increase the proportion of gametocyte carriers that are detected in a population. By reading 200 microscopic fields, double the number of fields routinely examined, we have attempted to maximize the sensitivity of microscopy detection. Despite this extensive slide reading, not all infectious individuals were detected. Many authors report that individuals with submicroscopic gametocytes can successfully infect mosquitoes, and we show that reading 200 microscopic fields does not overcome the limited sensitivity of microscopy for gametocyte detection. Although the limited sensitivity of microscopy to detect infectious individuals is widely accepted, this is not the same as evidence for a contribution of submicroscopic infections to malaria transmission. In our setting, the majority of individuals who were infectious to mosquitoes but gametocyte negative by microscopy had asexual parasites detected by microscopy and were therefore not truly subpatent infections. This is important to put our findings in perspective because these submicroscopic gametocyte carriers would have been detected by conventional diagnostics that detect (asexual) malaria parasites. We observed that two individuals without asexual parasites or gametocytes by microscopy were infectious to mosquitoes, comprising 11% of the total number of infectious individuals. One of these two individuals infected 20% (4 of 20) of the feeding mosquitoes, the other 5% (1 of 20). These individuals will have remained undetected by conventional diagnostics. If such subpatent infections are also found to be infectious in low endemic areas, where subpatent infections are particularly prevalent, they may form a stumbling block for successful elimination initiatives and may need to be included in malaria interventions to maximize their effect.

In conclusion, our study showed that 10% of all individuals ≥ 5 years of age were infectious to An. arabiensis mosquitoes in the peak transmission season in an area of intense malaria transmission. Infectiousness to mosquitoes was not restricted to any age group or to individuals with gametocytes detected by microscopy. We observed successful mosquito infections from individuals without microscopically detectable asexual parasites or gametocytes. If the infectiousness of such subpatent infections is confirmed in other settings, notably low endemic settings that aim for malaria elimination, they may fulfill an important role in maintaining malaria transmission and may require inclusion in malaria control and elimination efforts.

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