QUANTIFYING MOSQUITO BITING PATTERNS ON HUMANS BY DNA FINGERPRINTING OF BLOODMEALS


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Abstract. A major debate in infectious disease epidemiology concerns the relative importance of exposure and host factors, such as sex and acquired immunity, in determining observed age patterns of parasitic infection in endemic communities. Nonhomogeneous contact between hosts and vectors is also expected to increase the reproductive rate, and hence transmission, of mosquito-borne infections. Resolution of these questions for human parasitic diseases has been frustrated by the lack of a quantitative tool for quantifying the exposure rate of people in communities. Here, we show that the polymerase chain reaction (PCR) technique for amplifying and fingerprinting human DNA from mosquito bloodmeals can address this problem for mosquito-borne diseases. Analysis of parallel human and mosquito (resting *Culex quinquefasciatus*) samples from the same households in an urban endemic focus for bancroftian filariasis in South India demonstrates that a 9-locus radioactive short-tandem repeat system is able to identify the source of human DNA within the bloodmeals of nearly 80% of mosquitoes. The results show that a person’s exposure rate, and hence the age and sex patterns of exposure to bites in an endemic community, can be successfully quantified by this method. Out of 276 bloodmeal PCR fingerprints, we also found that on average, 27% of the mosquitoes caught resting within individual households had fed on people outside the household. Additionally, 13% of mosquitoes biting within households contained blood from at least 2 people, with the rate of multiple feeding depending on the density of humans in the household. These complex vector feeding behaviors may partly account for the discrepancies in estimates of the infection rates of mosquito-borne diseases calculated parasitologically and entomologically, and they underline the potential of this tool for investigating the transmission dynamics of infection.

INTRODUCTION

Progress in resolving the long-standing epidemiological debate regarding the relative importance of exposure, acquired immunity, and other factors, such as hormonal effects, in determining observed age and sex patterns of parasitic infection or disease depends on the ability to reliably quantify the exposure rate of people. Recent theoretical work has also shown that nonhomogeneous mixing (nonrandom contact) between vectors and hosts has important implications for the persistence, prevalence, and hence control of mosquito-borne diseases. In particular, when vectors concentrate on certain hosts, the basic reproductive rate (R) of the disease (a measure of persistence) and the vectorial capacity both have the potential to be greater than under conditions of homogeneous mixing, thus making disease control more difficult.

Despite these findings and the long-held popular perception by both casual visitors to the tropics and students of mosquito biting behavior that individual humans differ markedly in their attractiveness to mosquito bites, there have been few systematic attempts to investigate the patterns of human biting by hematophagous insects under natural field conditions. A major drawback has been the lack of a sensitive tool for quantifying biting rates on people at an epidemiologically relevant scale in the field. Previous methods of assessing field-biting rates on individual humans by mosquitoes have depended on either making landing catches on exposed people or crudely identifying the human source of mosquito bloodmeals by typing for human blood groups or other polymorphic blood. The former, a laborious and ethically dubious technique, is constrained by biases between catchers; the latter is limited by the degree of polymorphism of the blood markers used. This has led to conflicting results regarding the rate of biting on people by mosquitoes in the field. Thus, although some studies have indicated feeding to be random, others have found adults to be more attractive than children to mosquitoes.

In contrast, recent developments in molecular ecology centered on the polymerase chain reaction (PCR)–based fingerprinting of mosquito bloodmeals have suggested that this approach may provide a sensitive method for quantifying the mosquito biting rate of people. Previous applications of this method have highlighted its potential for addressing epidemiologically important questions regarding malaria transmission. Gokool and others applied the tool to small field samples in malaria bed-net trials and demonstrated the usefulness of the technique for assessing protection provided by impregnated nets. More recently, Koella and others have successfully used the method to investigate the ability of *Plasmodium falciparum* to increase multiple feeding on people by mosquitoes.

Here, we evaluate for the first time the usefulness of this method as an epidemiological tool for quantifying the biting rate on humans in the field by the mosquito species, *Culex quinquefasciatus*, an important vector of bancroftian filariasis in many parts of the world. This investigation forms an integral part of a larger study designed to quantify the impact and sources of exposure heterogeneity on the immunepidemiology of filariasis in South India.

MATERIAL AND METHODS

Study area and samples. Field samples for this study were collected from 19 randomly selected houses in Muthialpet, a moderately dense neighborhood of Pondicherry, South India. All household investigations were made with the consent of the residents, or in the case of children, their
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guardians. Ethical approval for the study was obtained from the institutional ethical committee of the Vector Control Research Center (Indian Council of Medical Research). For all residents in each house, details of age, sex, and familial relationships were recorded during an initial survey before the collection of up to 1 mL of blood in heparinized Vacutainers. All blood samples were frozen within an hour of collection. Female *Cx. quinquefasciatus* were collected inside these houses between 7:30 AM and 9:30 AM. During this time, mosquitoes are believed to rest on the walls and ceilings after feeding during the night. Mosquitoes were caught by means of battery-operated aspirators, each collector spent ~10 minutes per house. Mosquitoes from each house were preserved individually within an hour in 100% isopropanol in 500 μL Eppendorf tubes. Samples were stored at room temperature until used.

**Mosquito bloodmeal dissections.** Mosquito body parts and abdomen membranes were removed from the bloodmeal in order to remove potential PCR inhibitors. Each bloodmeal was sized according to one of the following size categories: one-quarter full, half full, or full. DNA extraction. Aliquots (200 μL) whole blood and each mosquito bloodmeal were digested in 200 μL 50 mM Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate–proteinase K. The bloodmeals were crushed in the digest with micropestles to aid the digestion of clotted blood. All samples were incubated at 37°C overnight. The digested products were purified by treating with equal volumes of phenol-chloroform and chloroform-isooamyl alcohol, followed by the addition of an equal volume of LiCl; DNAs were precipitated by the ethanol precipitation method. DNA amplification reactions consisted of an initial denaturation step of 95°C and consisted of an initial denaturation step of 95°C for 1 min at 95°C; then, 2 μL was loaded on a 6% acrylamide gel. After electrophoresis, the gel was placed onto Whatman 3MM paper, dried, and exposed to Fuji X-ray film by means of intensifying screens at –70°C. We defined a meal as single if all the identified alleles came from one person. A meal was defined as multiple if at least one locus had ≥2 alleles or if the alleles at any of the loci could only have been derived from biting different people in a single household.

**Statistical analyses.** We used logistic and multiple logistic regressions to test for the effects of covariates on binary or proportional response data. For testing the effects of age and sex on the probability that a mosquito took a quarter-sized meal, we specified a logistic regression for clustered data with each individual person forming the cluster to account for the lack of independence for the data recorded per individual person. Generalized additive models with binomial errors were used to examine nonlinearity in these data. Count data (numbers of mosquitoes and biting density per person) were analyzed by either quasi-likelihood Poisson generalized linear models or quasi-likelihood Poisson generalized additive models (to uncover nonlinear structure). Quasi-likelihood estimation was used to account for the significant overdispersion occurring in such data, which may partly reflect the likely clustering of responses within individual households. Note that we simply account for such responses here via the quasi-likelihood estimation. Pearson’s chi-square test was used to assess the goodness of fit of the negative binomial and Poisson probability distributions to the observed frequency distribution of estimated bites per person.

**RESULTS**

The PCR method uses human microsatellite (STR) primers to genotype the bloodmeals of mosquitoes caught while resting in households and compares the resulting DNA fingerprints with those of the humans residing within the same household. We developed and optimized a 9-locus multiplex STR system for this. Figure 1 illustrates an autoradiogram obtained by this system in a pilot study comparing the DNA profiles of 4 people and 16 *Cx. quinquefasciatus* vectors sampled from the same household in Pondicherry, South India. This pilot investigation shows that the band pattern of Individual E9 matches those obtained from bloodmeals of Mosquitoes 5, 8, and 31. Similarly, the pattern obtained for Individual E11 matches that of Mosquitoes 17 and 22; E12 matches Mosquitoes 9 and 29; and Individual E15 had been bitten by Mosquito 11. By contrast, the fingerprint obtained from Mosquito 16 contains bands or alleles from both Individuals E11 and E12, providing evidence for mixed feeding by this mosquito on these people. The profiles of only 9 out of the 15 mosquitoes that were amplified (Figure 1) appear to match those obtained from people living in this household, suggesting that the rest of the mosquitoes may have fed elsewhere or on visitors.

We used this tool to quantify how biting rate varies with host age and sex in human communities by collecting par-
Figure 1. Autoradiogram derived from applying the 9-locus multiplex single-tandem repeat system to human blood samples and bloodmeals of the corresponding mosquitoes sampled from a pilot study household in Pondicherry, South India. The DNA banding patterns depicted in the figure clearly show that polymerase chain reaction fingerprinting of *Culex quinquefasciatus* bloodmeals provides a reliable tool for identifying and hence estimating the biting rate by these mosquitoes on exposed people. Sample labels are arbitrary and were assigned randomly to each human and mosquito blood sample. Lane 1 (left) represents results from the negative control (distilled water) used in this experiment. The bloodmeal of Mosquito 14 failed to amplify.

More than 75% of the bloodmeal samples from the study households provided unambiguous PCR results (Table 1). The earlier study by Koella and others\(^9\) that used this tool had a higher failure rate of 33%. However, in contrast with that study, our results indicated that the lack of success of the PCR was a direct function of bloodmeal size. Of the 3 bloodmeal categories (fully fed, half fed, and one-quarter fed) that we assigned to individual mosquitoes, the propor-
tation of unclear results increased from 16.9% (31 of 183) to 21.8% (26 of 119) to 49.1% (28 of 57) as the bloodmeal size decreased. This inverse relationship was significant for the overall data (logistic regression of PCR success on bloodmeal category; chi-square = 20.05, degree of freedom [df] = 1, P < 0.0001), but did not differ significantly between the categories of half and fully fed (chi-square = 1.88, df = 2, P = 0.188) or people per household (quasi-likelihood linear Poisson regression, F = 0.645).

The results indicate that on average, 27.5% of the mosquitoes caught within a household did not contain alleles of the inhabitants of that household (Table 1). Interestingly, the data in Table 1 suggest that the proportion of such mosquitoes may be high when both the densities of vectors caught and the number of humans per household are either very low or very high. The apparent proportional increase in such mosquitoes at the highest household densities of both variables could, however, represent an anomaly because one house (H43) contained the highest number of both vector and mosquitoes (Table 1). When data from this house were omitted, the number of mosquitoes found biting outside houses did not differ significantly between the study households in relation to either the density of vectors (quasi-likelihood linear Poisson regression, F = 1.88, df = 1, P = 0.188) or people per house (quasi-likelihood linear Poisson regression, F = 0.22, df = 1, P = 0.645).

On the other hand, as shown in Figure 2, the number of mosquitoes biting inhabitants within a house increased significantly and perhaps unsurprisingly with both these variables (quasi-likelihood linear multiple Poisson regression, F = 68.68, df = 1, P < 0.0001, and F = 7.58, df = 2, P = 0.014, respectively, for density of mosquitoes and people per household). This implies that the apparent variation in the proportion of resting mosquitoes found biting people outside households (Table 1) was mainly the result of between-house differences in the numbers of mosquitoes biting people inside households.

A second notable feature regarding the biting behavior of Cx. quinquefasciatus concerns the number and proportion of vectors taking multiple feeds (Table 1). Overall, 13.0% of the mosquitoes biting within households had taken multiple meals in our study sample (Table 1). This compares remarkably well with the findings of Koella and others,19 who found 14.0% of Anopheles gambiae taking multiple meals in their African study site. Interestingly, our data show that this pro-
portion may be negatively associated with the number of inhabitants but not the number of mosquitoes caught per house (Figure 3). This suggests that interrupted feeding of Cx. quinquefasciatus may be host density dependent.

Figure 4 depicts the frequency distribution of the number of bites estimated for each person in this study. The total number of estimated bites and the mean number of bites received per person per night in the study population were 233 and 3.1, respectively. The numbers of bites on people were highly overdispersed (variance to mean ratio = 5.5) and were adequately described by the negative binomial probability distribution (Figure 4). Indeed, > 55% of the total bites were received by < 20% of the sampled people.

Figure 5 examines the variation in the density of mosquito bites per night among people in relation to age and sex. There was no significant difference in the overall number of bites received by males and females (quasi-likelihood Poisson generalized linear model, $F = 0.97$, df = 1.74, $P = 0.327$), but the age-specific biting rate differed markedly between the sexes (Figure 5). In male inhabitants, biting density showed some tendency to increase in inhabitants aged between 10–30 years, but overall, the data showed little age dependency, probably as a result of its high variability (Figure 5a). In the female population, by contrast, there was a clear nonlinear pattern (Figure 5b): biting density increased in girls up to the age of ~15 years before decreasing significantly among women.

Although the numbers of boys and men and girls and women residing within the study households varied (Table 1), such sex ratio differences were not statistically significant for either the overall study population (people of all ages) (chi-square = 11.92, df = 18, $P = 0.85$) or for people aged < 25 years (chi-square = 14.27, df = 17, $P = 0.65$). This suggests that disproportionate mosquito densities at the household level are unlikely to bias the observed age-specific biting patterns depicted in Figure 5 for male and female inhabitants.

**DISCUSSION**

Our results confirm that DNA fingerprinting of blood-meals can provide a sensitive method for identifying which
individual humans are bitten by which hematophagous arthropod under natural field conditions. They also demonstrate for the first time the usefulness of this technique as an epidemiological tool for quantifying patterns of potential human exposure to infection. Our analysis of this topic indicates that both variations in mosquito biting and resting biology at the household level and intrinsic host factors, such as age and sex, may underlie the natural biting rate of people in urban endemic localities.

It is clear that the power of this method would be enhanced if the technical failure of the PCR for a proportion of bloodmeals can be overcome. Our study has shown that this problem may be connected to the low DNA content of partially completed bloodmeals, although the previous study by Koella and others did not find such a relationship despite the greater failure rate (33%) associated with their locus STR system compared with ours (23.5%). Perhaps this is not only a reflection of the greater sensitivity of our 9 locus STR system to DNA quantity but also differences due to the vector species (An. gambiae versus Culex quinquefasciatus) studied, such as variations in nutritional and foraging strategies that may affect human-biting propensity (anthropophagism); frequency of feeding; bloodmeal size; and digestion of intact DNA. We are currently investigating the use of nested PCR protocols as a means to deal with the low or even possibly degraded DNA content of incomplete bloodmeals.

Nonetheless, this study provides the first clear demonstration to date that mosquitoes biting people under field conditions is not random and may be dependent on the person’s age and sex. This contrasts with the mixed results of studies conducted on selective feeding in the past. In part, the outcome of past studies reflects the biases of previous methods used for assessing an individual host’s biting rate and in part the fact that previous investigations (largely due to constraints imposed by previous tools) have tended to study relatively small groups of people. The high sensitivity of the multiplex STR system for identifying bitten people, coupled with its capacity for dealing relatively speedily with large samples (which is required to overcome the high variability of biting data; Figures 4 and 5) means that this newly developed molecular tool may overcome the drawbacks of past studies and for the first time allow realistic investigations of the 2 major unresolved questions connected with mosquito-transmitted diseases, namely the factors that underlie differential biting on humans by mosquitoes and the impact of exposure on the immunogenicity of such infections.

The results reported here provide several insights regarding the use of the tool for quantifying and assessing the impact of these questions for the epidemiology of vector-borne infections. First, the finding that intrinsic host factors may account for a major proportion of the observed variability in biting by mosquitoes in our study population is of special relevance to attempts for understanding and modeling the impact of differential host selection by vectors on the population dynamics of infection. Specifically, the present result indicates that in small communities (such as the present study community, which comprised a few families living on neighboring streets), the effects of variable biting of hosts by vectors may be adequately addressed by simply specifying and incorporating age- and sex-dependent biting in models of infection. Whether such intrinsic host factors may play a similarly large role in the observed variation in host biting rates in larger villages or towns—where large spatial effects in mosquito biting could be expected to play a bigger role in host biting heterogeneity—remains to be seen, but it is clear that the developed PCR tool will provide an important means to aid the successful investigation of this topic.

The results show that Culex quinquefasciatus biting and resting behavior may also contribute to the household variation in bites by this mosquito vector in urban endemic areas. The analysis presented here shows that such variability may derive in part from the propensities of a significant proportion of mosquitoes caught resting within households to have either taken bloodmeals elsewhere or to have fed on several people within households. Although the study was not designed to examine the causes of these findings, it is pertinent to note that such vector biting and resting behaviors are related to host densities, vector densities, or both at the household level. Such density-dependent vector behavioral patterns may play important roles in the transmission dynamics of vector-borne diseases. Increased multiple feeding by mosquito vectors on different hosts at higher human densities could clearly increase the transmission rate of vector-borne infections within endemic communities. It could also increase the rate at which humans are infected with multiple parasite clones or clusters, which patently will have profound implications for the rate of parasitie outcrossing and hence for the likely development and spread of drug resistance.

By contrast, the propensity of a portion of house-resting mosquitoes to have taken bloodmeals elsewhere means that there is unlikely to be a simple correlation between levels of infection among house-resting mosquito populations and host infection levels within the same household. This implies that caution must be exercised when house-resting catches are used to measure the actual exposure or biting rates of people and could clearly help explain the discrepancies that arise when inoculation rates of malaria in people, for example, are estimated entomologically (via assessment of infection levels in house-resting mosquitoes) and parasitologically.

These findings and conclusions imply that if the transmission dynamics and control of mosquito-borne infections are to be better understood and quantified, more thorough studies of mosquito biting and resting biology at both the individual and household levels will be required. The present results suggest that the exquisite sensitivity and high-throughput capability of the newly developed PCR-based technique for DNA fingerprinting of mosquito bloodmeals may provide a valuable molecular epidemiological tool in aiding the successful design and execution of such studies.
References


