Increased Endophily by the Malaria Vector Anopheles arabiensis in Southern Zambia and Identification of Digested Blood Meals

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Abstract. An increase in Anopheles arabiensis showing endophilic behavior was observed in Macha, Zambia during March 2007. To determine whether this shift in resting behavior was accompanied by a change in feeding preference, an attempt was made to calculate the human blood index. However, only 46.2% of blood meals were successfully identified with existing polymerase chain reaction (PCR) diagnostics. This failure was hypothesized to be caused by the limitations of existing methods that are not capable of identifying host source from anophelines resting for extended time periods. Using an assay we developed that allows for the identification of mammalian host DNA out to 60 hours post-feeding, we were able to successfully determine the host source of 94.3% of recovered blood meals. The data show that, although An. arabiensis in Macha experienced a period of higher endophily, the degree of anthropophily and the sporozoite rate in the population remained comparable to the previous malaria season.

INTRODUCTION

Anopheles arabiensis Patton is a major vector of human malaria. However, unlike other highly efficient vectors of Plasmodium falciparum in sub-Saharan Africa, including An. funestus s.s. Giles and An. gambiae s.s. Giles, An. arabiensis is known to show a high degree of exophagic behavior1–3 and to exhibit a wide range of anthropophily. Human blood indexes (HBIs) of both > 0.85 and < 0.56 have been observed in Kenya,4–6 Ethiopia,7,8 and Nigeria,9,10 and similar studies have shown comparable levels of variation throughout the continent.11–13 Additionally, An. arabiensis is regarded as being more exophilic than its companion vector species. Complete endophily has been observed in Madagascar,14 and exophily as high as 80.7% has been calculated in Tanzania.15 Furthermore, pit shelters in southern Ethiopia captured five times the number of An. arabiensis as were found resting inside sleeping huts,16 suggesting that only a small portion of the population there rests indoors.

The feeding and resting behavior of anophelines has been shown to be malleable. Insecticide-treated bed nets (ITNs) have been reported to shift An. gambiae s.l. biting earlier in the evening and outdoors,17,18 as well as to reduce the numbers of mosquitoes resting inside.17,18 Other stimuli have been shown to promote increased endophily. It has been observed that when outdoor shelters are less available, the indoor HBI of An. arabiensis is lowered as exophilic mosquitoes seek refuge in sleeping houses.5 Likewise, endophily may be driven by climate as observed by an increase in endophilic An. arabiensis during the cool season in northeastern Tanzania.19

During March 2007, unexpectedly high numbers of gravid and half gravid An. arabiensis were captured resting indoors by routine pyrethrum spray catch (PSC) in Macha, Zambia. Because an increase in endophily might potentiate increased vector/human contact, we attempted to look for changes in the HBI of the population. However, nearly one half of the blood-fed mosquitoes collected had blood meals that were unidentifiable using existing polymerase chain reaction (PCR) techniques. Diagnostics designed to identify mammalian blood meals from mosquitoes have only been shown to be sensitive up to 24–30 hours after feeding.20–23 Beyond this time point, digestion of serum components prevents the identification of blood meals by immunologic techniques, and as cellular DNA becomes degraded, it becomes more difficult to amplify larger PCR products. Consequently, to recover data on feeding preferences from our collection, we developed a unique PCR primer set and restriction digest assay that was used to identify host source based on a small fragment of mammalian mitochondrial cytochrome b DNA recovered from An. gambiae s.l. blood meals out to 60 hours after feeding. The assay also proved useful for identifying the smaller blood meals of An. longipalpis and Culicoides spp., two hematophagous arthropods often collected alongside An. arabiensis in Macha. Here we present the development of this small blood meal assay as a means of understanding our observations on the ecology and feeding behavior of An. arabiensis during a period of increased endophily in Macha.

MATERIALS AND METHODS

Mosquito collection and handling. Field specimens of An. arabiensis Patton were collected by PSC24 in Macha, Zambia (16.39292 S, 26.79061 E) during March 2006 and 2007. Collections were performed in village-areas within 10 km of the Johns Hopkins Malaria Research Institute in an area of the Southern Province described previously.13 Each house was sampled once per month. Approximately 90 sleeping houses were sampled during 2006 and 180 during 2007. An. longipalpis Theobald were captured in the same region by PSC and Centers for Disease Control (CDC) light traps between April and June 2007. Mosquitoes were identified by morphology and were individually placed in tubes containing silica gel desiccant (Fisher Scientific, Fair Lawn, NJ) and cotton for stable storage at room temperature until processing 2–4 months later. It has been shown previously that mosquitoes stored in this manner for up to 7 months have DNA viable for diagnostic purposes.20 Colony mosquitoes used in the time course analysis were killed by freezing at designated times after feeding and were kept frozen until processed together once all time points were taken. Heads/thoraces were separated from abdomens for all mosquitoes. Only DNA isolated
from engorged abdomens was used for blood meal analysis and diagnostic assay development. Unfed An. arabiensis were included as negative controls for potential nonspecific amplification.

**DNA isolation and PCR.** Before homogenization, dried specimens were rehydrated at room temperature in 20 μL of double-distilled water for 10 minutes. No rehydration step was needed for freshly killed colony specimens. DNA was extracted from mosquito heads/thoraces and abdomens by a modified salt procedure as described previously. DNA pellets were resuspended in 50 μL of double-distilled water. Extraction quality was assessed by PCR using arthropod-specific primers to amplify a portion of the mitochondrial NADPH dehydrogenase subunit 4 (ND4) gene, and all blood meals were first attempted to be discerned using the Kent and Norris multiplexed diagnostic, followed by the Kent avian primers. The identity of all specimens morphologically identified as An. gambiae s.l. was confirmed by PCR on the head and thorax extractions. Additionally, head/thorax DNA was screened for Plasmodium falciparum by nested PCR. Whole blood was extracted with a DNeasy Blood and Tissue Kit (Qiagen Sciences, Germantown, MD) according to the manufacturer’s instructions and eluted in 100–200 μL of double-distilled water.

**Culicoides processing.** Blood-fed Culicoides spp. were captured in January 2008 while testing BG-Sentinel traps in Macha, Zambia. DNA was extracted from whole specimens with the same modified salt procedure as above.

**Blood sample sources and time course analysis.** Whole blood samples were used to optimize the restriction digest assay. Whole blood was acquired from Pel-Freez Biologicals (Rogers, AR) and Pocono Rabbit Farm and Laboratory (Canadensis, PA). Cow (Bos taurus), pig (Sus scrofa), and goat (Capra hircus) blood was preserved with one part sodium citrate to nine parts blood. Dog (Canis familiaris) and human (Homo sapiens) blood contained K$_3$ EDTA anticoagulant.

Mosquitoes used in the time course analysis were generated at the Johns Hopkins Bloomberg School of Public Health (Baltimore, MD). For the time course, colonized An. gambiae s.s. were fed on a human blood meal until fully engorged and held for various lengths of time while they digested the blood meals. Three to five mosquitoes at 12, 24, 48, 60, 66, and 72 hours after feeding were frozen at −20°C until processed.

**Small product amplification.** From a multiple alignment of cytochrome b sequences, a novel mammalian forward primer (UNIFORA: 5'TCCAAACACCA[G/A][A/C]AGCAT-AATATT-3') was designed to amplify a small 98-bp product when paired with a previously designed “universal reverse primer” (UNREV1025). Sequences were acquired from GenBank: human (H. sapiens AY495285), cow (B. taurus AB090987), dog (C. familiaris NC_002008), goat (C. hircus AB110597), pig (S. scrofa AC273513). Primer3 software was used to check for melting temperature (Tm) compatibility. The PCR was optimized to amplify templates from each potential vertebrate host. An initial denaturation of 5 minutes at 95°C was followed by 40 cycles at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The final extension was carried out at 72°C for 7 minutes. Each 25-μL reaction contained 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 0.01% gelatin, 0.1 mmol/L of each dNTP, 2 units Taq polymerase, and 50 pmol of UNREV1025 and UNIFORA. For positive control reactions using whole blood DNA extracts, 15 ng of template was more than sufficient for successful amplification. DNA amplifications were subjected to electrophoresis and visualized on ethidium bromide–stained 3% agarose gels. GeneRuler 100-bp DNA mass marker (Fermentas, Glen Burnie, MD) was included on all gels.

**Restriction digests.** Restriction enzymes (New England BioLabs, Ipswich, MA) were chosen that would specifically digest the small blood meal product based on the same multiple alignment of cytochrome b sequences used in primer design (Table 1). Although enzymatic digestions are specific for host source, the resulting small fragments are difficult to differentiate on agarose. Therefore, the host source was determined by separate restriction enzyme digests on 1 µL of UniA PCR product, in a stepwise fashion starting with what was expected to be the most common host (human) and working to the least common host (pig) based on prior studies. For Fnu4HI and BanI, each 25-µL reaction contained 2.5 μL of 10× NEBuffer 4 and 1 unit of enzyme. MspI digests included NEBuffer 2 and 2 units of enzyme. NsiI reactions contained NEBuffer 3 and 1 unit of enzyme, whereas SpeI digests were incubated with NEBuffer 2, 1 unit of enzyme, and bovine serum albumin (BSA). All digestions were carried out at 37°C overnight for 16 hours.

**Exophily and HBI calculations.** The HBI was determined for indoor-resting An. arabiensis and was based on all blood meals successfully identified by the Kent and Norris PCR and our small fragment approach. Mixed blood meals were treated as two separate blood meals. Degree of exophily (DE) was calculated as reported previously, DE = 1 – (1/F:HGG), where F is the number of fed mosquitoes and HGG is the sum of the gravid and half gravid mosquitoes collected by PSC.

**RESULTS**

During March 2007, 289 An. arabiensis were collected by PSC in Macha, Zambia, including 160 blood-fed mosquitoes and 90 gravid or half gravid specimens. PSCs from March 2006 collected 98 mosquitoes, including 93 blood-fed and 3 gravid or half gravid An. arabiensis. Comparing the 2 years, the number of mosquitoes resting indoors increased by 28%. The degree of exophily in the population as calculated by Ameneshewa and Service decreased from 96.77% in 2006 to 43.75% in 2007. Of the blooded specimens collected in 2007, the host source of 76 blood meals was determined using the Kent and Norris multiplexed PCR diagnostic, leaving 84 samples unidentified. Between the two rainy seasons under consideration, there was an increase of 35% in the number of indistinguishable host sources by PCR. It was hypothesized that with the observed increase in endophily, the number of blood meals taken at a time point > 30 hours after collection
would have likewise increased, necessitating the design of a novel diagnostic.

We developed a mammalian primer set that produced an expected 98-bp amplicon from the cytochrome b gene of a select group of hosts on which *An. arabiensis* in the region commonly feed. This small product was PCR amplified from field-collected mosquitoes that were engorged on human, cow, dog, goat, or pig blood. No product was obtained from unfed mosquitoes or from mosquitoes that had fed on an avian host (data not shown). A time course analysis was performed to determine how long after imbibing human blood the small amplicon could be detected. Although the human product from the Kent and Norris multiplexed PCR could only be visualized in mosquito abdomens frozen at 12 and 24 hours after feeding, the 98-bp fragment was detectable up to 60 hours after feeding (Figure 1). Moreover, using whole blood controls, we designed a novel restriction enzyme assay to differentially digest the small amplicons based on host source (Figure 2).

DNA from abdomen extractions of those mosquitoes collected in 2007, for which blood meal identification was not previously possible, was used as template for the small product mammalian PCR. Seventy-five (89%) of the blood-fed mosquitoes that failed to be identified with the multiplexed assay produced an amplicon with our mammalian primer set to produce a small cytochrome b fragment. Because humans are the most common blood source from indoor-resting mosquitoes in Macha, the fragments were first incubated with *Fnu*4HI. From these digestions, the host blood from 61 specimens was identified as human. DNA from the remaining 14 samples was digested with *Ban*II, because cattle are the next most common host in the region. Five mosquitoes were determined to have ingested cow blood. Subsequently, the small products from the remaining mosquitoes were subjected to digestion with *Msp*I, *Nsi*I, and, *Spe*I. Of those, two blood meals were recognized as dog, whereas the digest pattern of seven samples could not be discerned.

Sequencing of the small cytochrome b amplicons from these seven specimens showed that six blood meals were from human hosts, with the final sample having been obtained from cattle. On inspection of the sequences, the restriction site for *Fnu*4HI was determined to be intact for each of the human samples. Presumably, there was too little PCR product generated to definitively visualize the 39- and 59-bp restriction fragments by agarose gel electrophoresis. For the last blood meal that was taken from cattle, there was a point mutation in the *Ban*II restriction site. Instead of a consensus guanine, the nucleotide at position 52 was identified as adenine. However, even with these limitations, the restriction digest assay was able to identify 90.7% of the small products generated with our mammalian primer set, and with the ability to sequence the remaining few samples, the host sources of 94.3% of all blood meals taken during March 2007 were successfully identified. Additionally, the utility of our novel blood meal diagnostic was shown on field-collected *An. longipalpis* and *Culicoides* spp. On samples of these species where the Kent and Norris multiplexed PCR was used unsuccessfully, the new small fragment approach was able to identify all blood meals as cattle.

When all of the blood feeding data were complied for the period of increased endophily observed during March 2007, the HBI was calculated to be 0.872. This value is similar to the previously reported HBI of 0.923 for the Macha region. There was no statistically significant difference between the proportion of mosquitoes that fed on humans (*P* = 0.06). Last, the sporozoite rate calculated in the *An. arabiensis*
population for March 2007 was 0.028, similar to a rate of 0.031 observed during March of 2006 ($P = 0.88$).

**DISCUSSION**

During March 2007, we observed a period of increased endophily among *An. arabiensis* in Macha, Zambia. Although an increase in endophilic behavior has the potential to raise rates of vector/human contact, it also offers the potential for greater effectiveness of indoor vector control measures, such as the use of insecticide-treated bed nets and indoor residual spraying. Increased endophily in Macha resulted in an increase in the proportion of gravid and half gravid mosquitoes collected by PSC, but the cause of this shift in behavior is unclear. There were no extreme weather events noted during the month. However, it is possible that a few days of colder or wetter than normal weather might have conspired to keep endophagic mosquitoes indoors. It does not seem as if larger numbers of exophagic mosquitoes were sheltering inside because no statistically significant change in HBI was observed.

To effectively determine the HBI, it was necessary to devise a new blood meal diagnostic because more than one half of the blood meals sampled were unidentifiable using existing techniques. The length of time after feeding that a blood meal is detectable is related to the length of a mosquito’s gonotrophic cycle. Murkabana and others examined the effect of digestion on the ability to effectively profile human blood from *An. gambiae s.s.* The group determined that amplification success decreased steadily and significantly in mosquitoes held for > 8 hours after feeding, falling to < 50% from 15 hours after ingestion. Cytochrome b PCR-based assays, designed to amplify avian DNA products between 200 and 400 bp, have differentiated blood meals from *Culex pipiens* and *Cx. tarsalis* out 3–7 days after feeding, but a time course analysis of the Kent and Norris meals from products between 200 and 400 bp, have differentiated blood specimens held for at least 15 hours after ingestion. The greater sensitivity afforded by the small fragment diagnostic is particularly suited to such samples. The small blood meal assay also proved successful on field-caught *An. longipalpis*, which predominately feed on cattle. In situations where humans are not the primary host, the order in which the restriction digests are performed can be shuffled so that the most likely hosts are tested first. Although the order of the digests has the potential to restrict the detection of multiple blood meals, the frequency of multiple blood meals for *An. arabiensis* in the Macha region is very low, between 2% and 3%. Based on the number of samples analyzed with the small blood meal assay, it would be expected that only one or two specimens would contain blood from two different hosts. The few mosquito blood meals that remained unidentified were tested with avian-specific primers, but no chicken or guinea fowl hosts were detected. Those blood meals were either obtained from a host not compatible with existing assays or host DNA was too degraded even for our new detection method.

Based on a combination of assays for the best efficiency, the host sources of 94.3% of all blood-fed mosquitoes caught by PSC during March 2007 were identified. Additionally, based on comparisons between the HBI and sporozoite rate calculated in this study and those determined from previous collections in the Macha region, it does not seem that there was an increase in vector/human contact despite increased endophily by *An. arabiensis*. The shift toward endophilic behavior in the population remains an intriguing observation that, in conjunction with local weather data, will need to be monitored further.

Received May 9, 2008. Accepted for publication September 5, 2008.

Acknowledgments: The authors thank Shadrack Habbianti and Harry Hamapumbu for time and effort spent coordinating field team operations in Zambia. We also thank Haggard Mushutula, Winna Muchimba, Fines Mwaanga, Malony Mulota, Fidelis Chanda, Guide Hansumo, and Cliff Singanga for collecting mosquitoes and Musapa Mulenga for managing our collections in Macha.

Financial support: This research was supported in part by funding to DEN from the Johns Hopkins Malaria Research Institute and a Johns Hopkins Bloomberg School of Public Health Sommer Scholarship award to CMF.

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