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Application of a reverse dot blot, DNA-DNA hybridization method to quantify host-feeding tendencies of two sibling species in the *Anopheles gambiae* complex

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Abstract

A DNA-DNA hybridization method, reverse dot blot analysis (RDBA), was used for identification of *Anopheles gambiae* s.s. and *An. arabiensis* hosts. Of 299 blood fed and half gravid *An. gambiae* s.l. collected from Kisian, Kenya, 244 individuals were identifiable to species; 69.5% were *An. arabiensis*, and 29.5% were *An. gambiae* s.s. Host identifications with RDBA were comparable to conventional PCR followed by direct sequencing of amplicons of the vertebrate mitochondrial cytochrome B gene. Of the 174 amplicon-producing samples used for comparison of these two methods, 147 were identifiable by direct sequencing, and 139 of these same by RDBA. *An. arabiensis* blood meals were mostly (>90%) bovine in origin, whereas *An. gambiae* s.s. fed upon humans > 90% of the time. RDBA detected that 2 of 112 *An. arabiensis* had blood from more than one host species, whereas PCR and direct sequencing did not. Recent insecticide-treated bednet (ITN) use in Kisian has likely caused the shift in the dominant vector species from *An. gambiae* s.s. to *An. arabiensis*. RDBA provides an opportunity to study changes in host-feeding by members of the *An. gambiae* complex as a response to the broadening distribution of vector control measures targeting host-selection behaviors.

Keywords

Anopheles gambiae; blood meal; host selection; DNA-DNA hybridization; reverse dot blot

Introduction

Host-selection patterns influence transmission in vector-borne disease systems, and increase efficiency of pathogen transmission among host populations when vectors exhibit high host

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fidelity (McCall and Kelly 2002). Analyzing vector populations for patterns of host-selection achieves several objectives: 1) identifies hosts important to pathogen amplification (Molaei *et al.* 2006a, Hamer *et al.* 2009), 2) determines the relative contribution of vector species to disease transmission (Apperson *et al.* 2004, Molaei *et al.* 2006b), and 3) elucidates seasonal host-use shifts (Edman and Taylor 1968, Kilpatrick *et al.* 2006). Enzyme-linked immunosorbent assays (ELISA) replaced the earlier precipitin and related immunological methods to assess host-selection patterns (Beier *et al.* 1988, Scott *et al.* 1993, Rubio-Palis *et al.* 1994, Bøgh *et al.* 1998, Diatta *et al.* 2001, Mwangangi *et al.* 2003, Apperson *et al.* 2004, Lefèvre *et al.* 2009). More recently, DNA-based methods have been successfully used for vector blood meal analysis in order to increase specificity of host identification and sensitivity for more digested blood meals (Lee *et al.* 2002, Cupp *et al.* 2003, Kilpatrick *et al.* 2006, Molaei *et al.* 2006a, Molaei *et al.* 2006b, Hamer *et al.* 2008, Hamer *et al.* 2009, Kent 2009). While such methods are efficient and the results more reliable than those of ELISA, the equipment used for direct sequencing is not widely available in most laboratories and sequencing is expensive. Methods that obviate the need for sequencing but retain the sensitivity and specificity would circumvent this issue.

The mosquitoes *Anopheles gambiae sensu stricto* and *An. arabiensis* Patton are highly important to the perpetuation of the human malaria transmission cycle in sub-Saharan Africa. While host selection by *An. gambiae s.s.* (hereafter *An. gambiae*) tends toward anthropophily (White and Rosen 1973, White 1974, Coluzzi *et al.* 1979), and *An. arabiensis* tends toward zoophily (Highton *et al.* 1979, Duchemin *et al.* 2001), regional differences in host selection by *An. gambiae* (Diatta *et al.* 1998, Bøgh *et al.* 2001, Sousa *et al.* 2001) and *An. arabiensis* (Tirados *et al.* 2006), as well as phenotypic plasticity (Lefèvre *et al.* 2009) have recently been described. Vector control measures executed within human dwellings, such as insecticide-treated bed nets (ITNs) preclude human blood meal acquisition (Molineaux and Gramiccia 1980, Gimnig *et al.* 2003, Killeen and Smith 2007), thereby causing *An. gambiae* populations (White 1974, Bayoh *et al.* 2010), and malaria transmission (Lindblade *et al.* 2004) to decline. Some studies have detected shifts in the ratio of *An. gambiae* and *An. arabiensis* abundance after the distribution of ITNs into communities (Lindblade *et al.* 2006, Bayoh *et al.* 2010), but the influence of ITNs on host selection by these vectors remains understudied.

In the present study, we adapted a novel blood meal analysis tool, reverse dot blot analysis (RDBA), previously used in Phlebotomine sand flies (Abbasi *et al.* 2009) to the *An. gambiae s.l.* system, and examined host selection in an area of high ITN use. We postulated that the acquisition of human blood meals by *An. gambiae* and *An. arabiensis* following a mass distribution of ITNs in Kisian in 2006 would decline, and the proportion of blood meals taken from non-human hosts would increase. Moreover, we postulated that the diversity of hosts utilized by *An. gambiae* and *An. arabiensis* would increase as human hosts become unavailable.

Methods

Study site

Sampling was conducted in Western Kenya, in the village of Kisian (Nyanza Province), during June - July, 2008. Mutuku *et al.* (2006) previously described the demography, physiography, and geography of this locale. ITNs were owned by *ca.* 68% of households within the sampling area (Hightower *et al.* 2010).

Mosquito collection and species identification

Blood fed mosquitoes, ranging from freshly-fed to sub-gravid (Sella stages 2-6) were collected indoors from the walls of human dwellings, and outdoors from clay pots (Odiere *et al.* 2007) via mouth aspirator. The clay pots had been placed by local residents to collect roof water and were typically situated near the houses. Mosquitoes were collected on 16 dates from 57 housing compounds. After collection, mosquitoes were frozen, and then morphologically identified as *An. gambiae s.l.* Abdomens were separated from the thorax with a scalpel, then placed individually in vials and dried in a desiccator containing Drierite. DNA was extracted from the mosquito abdomen (DNeasy Tissue Kits; Qiagen, Valencia, CA) using sterile technique, for a final elution volume of 100 µL. Quantitative PCR was used for species identification according to Walker *et al.* (2007).

Experiment 1: Initial blood meal identification via PCR and direct sequencing

Host DNA was initially identified using an established protocol for blood meal identification following Hamer *et al.* (2009). Extracted DNA used in mosquito identification was amplified via PCR with primers directed against the mitochondrial cytochrome B gene. Initially, the primer pair “mammal A” (5’-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3’ and 5’-TGT AGT TRT CWG GGT CHC CTA-3’; hereafter referred to as Mam A) was used to amplify host DNA. Any samples that failed to produce amplicons using Mam A primers were subjected to a second round of PCR using “avian A” (5’-GAC TGT GAC AAA ATC CCN TTC CA-3’ and 5’-GGT CTT CAT CTY HGG YTT ACA AGA C-3’; hereafter referred to as Av A). When a PCR produced amplicons, host DNA was purified and then directly sequenced (ABI Prism 3700 DNA Analyzer; Applied Biosystems, Foster City, CA). For host identification, sequences were confirmed for adequate quality and queried in GenBank (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) with a nucleotide BLAST search. Host identity was accepted for a sample when the following criteria were met: 1) the sample produced an amplicon after PCR, and 2) the sample sequence was matched with >95% similarity to a single vertebrate according to BLAST results.

Experiment 2: Comparison of RDBA with PCR and direct sequencing

After identification of host DNA according to Hamer *et al.* (2009), these same DNA samples were used to examine the adaptability of Reverse Dot Blot Analysis (Abbasi *et al.* 2009) to the *An. gambiae s.l.* system. Host DNA was amplified using a single Cyto primer pair (Abbasi *et al.* 2009), which produces a shorter amplicon from the same region of the mitochondrial cytochrome b gene as does the Mam A primer pair. Any amplicons produced were 1) purified and directly sequenced (ABI Prism 3700 DNA Analyzer; Applied Biosystems, Foster City, CA) as above, as well as 2) hybridized to known membrane-bound probes in a Reverse Dot Blot Analysis (RDBA). In cases where PCR with Cyto primers failed to produce an amplicon, host DNA was not used for comparison of these two methods. Host identification, and success rates of RDBA and direct sequencing following DNA amplification with Cyto primers was compared.

Host DNA amplification with Cyto primer pair

The forward and reverse primers from Abbasi *et al.* (2009) (hereafter, “Cyto” primers) are universal primers that amplify a 344 bp segment in a conserved region of the mitochondrial cytochrome b gene. Cyto primer 1 (5’ –CCA TCA AAC ATC TCA GCA TGA TGA AA-3’) and Cyto primer 2 (5’ –CCC CTC ATA ATG ATA TTT GTC TCT-3’), forward and reverse primers respectively, were modified by the addition of a biotin group at the 5’ end, and examined for *An. gambiae* complex host identification suitability. Extracted host DNA was amplified in a 100 µl reaction (4 µl DNA extract with a final primer concentration of 300nM) using the Failsafe PCR system (Epicenter Biotechnologies, Madison, WI), under

the following reaction conditions: an initial denaturation step of 5 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and an elongation step at 72°C for 1 min. Electrophoresis (E-gel system; Invitrogen, Carlsbad, CA) revealed amplicons that would be submitted for identification via direct sequencing and RDBA. Amplicons were visualized and scored based upon band intensity (0 = no product and 4 = bold product). An aliquot (45 µL) of samples yielding a visible amplicon (*ie.* a score of 1-4) was purified (QIAquick PCR Purification Kit; Qiagen), submitted for direct sequencing, and identified as above. The remaining 45 µL of amplified and unpurified host DNA was directly subjected to RDBA.

Reverse dot blot analysis – membrane preparation

Vertebrate host-specific oligonucleotide probes (Table 1) were diluted with Tris-EDTA buffer to a concentration of 5 pmol/µL, and imprinted onto nylon membranes in a row, as follows. Probes were spotted onto Pall Biotodyne B nylon membranes (Nagle Nunc International, Rochester, NY) using a 96 pin high-density replicating tool on a Biomek 2000 robot (Beckman Coulter, Inc.). Probes were fixed to the printed membranes for 30 minutes at 80°C.

RDBA - DNA hybridization and detection

Membranes were cut into strips containing each probe (Fig. 1), and strips were placed individually into a 15 mL conical tube. A 2xSSC [0.15 M NaCl, 0.015 M sodium citrate, 0.1% sodium dodecyl sulfate (SDS)] solution was added to the tube to prepare the membrane strips for hybridization with DNA samples. Tubes were held at 45°C for 30 min, and gently rocked in a controlled temperature, incubator-shaker (Boekel Incubator Shaker II, Model 136400, Boekel Scientific, Feasterville, Pennsylvania, USA) to allow the prehybridization solution to wash over the membranes. Amplicons from blood meal host DNA were suspended in a total volume of 45 µL, denatured at 94°C for 5 min, and then added to the conical tubes for hybridization at 43°C for 1 hr in the incubator-shaker. Following hybridization, the membrane was washed with a 0.7xSSC solution for 20 min at room temperature. Hybridization of the biotinylated amplicons was then visualized using a Biotin Chromogenic Detection Kit (Fermentas International Inc., Burlington, Ontario, Canada).

RDBA – Probe accuracy and specificity

Prior to testing field-collected samples, membrane-bound probes were tested for accuracy and cross-reactivity using DNA samples extracted from 5 µL blood samples of known origin. DNA was extracted from the blood (Lampire Biological Laboratories, Pipersville, PA) of mammalian hosts corresponding to probes listed in Table 1. DNA extracted from turkey blood (Lampire Biological Laboratories, Pipersville, PA) was used for testing of avian probes. Extracted DNA was amplified via PCR using Cyto primers, and amplicons were visualized and scored as above. Where amplicons were produced, samples were subjected to RDBA, and strips were examined for hybridization and cross-reactivity. RDBA capacity for mixed blood meal detection was also examined by combining blood from known cattle and goat sources in proportions ranging from 1:7 - 7:1. DNA was extracted from each mixed blood treatment and amplified. Mixed DNA treatments were then subjected to RDBA to determine the sensitivity with which this method detects a mixed blood source.

Thereafter, the remaining 45µl aliquot of amplified host DNA from the initial 100 µl Cyto PCR reaction was identified via RDBA. In 21 of the 299 samples, there was insufficient extracted DNA available for both RDBA and direct sequencing. In these cases, host identity was only determined via direct sequencing following PCR with Mam A/Av A primers, and a comparative blood meal analysis using Cyto primers was not performed. Field-collected

host DNA samples (n = 278) were tested via RDBA in batches of *ca.* 25, were always from the same PCR reaction, and were always run with positive and negative controls. Positive controls were DNA from human or bovine sources that amplified in a Cyto PCR reaction. Negative controls were 1) a combination of PCR mastermix and Cyto primers, purposefully lacking genomic DNA, and 2) always failed to amplify in a PCR reaction. For RDBA reactions where the positive control failed to hybridize, all samples were subjected to another round of RDBA. While we intended to rerun samples from RDBA reactions where hybridization was visualized for negative controls, this never occurred. No samples were subjected to RDBA or direct sequencing if negative controls run in the same PCR 96-well plate produced amplicons.

Statistical Analyses

Association between *An. gambiae s.l.* species distribution and resting site was examined using a Yate's corrected Pearson's χ^2 test with 1 degree of freedom. To examine host choice as it varied by vector species and resting site, host identity was specified as binomially distributed, and modeled using logistic regression as follows::

$$H_1: y = \beta_0 + \beta_1 (\text{spp}) + \beta_2 (\text{rs}) + \beta_3 (\text{spp} * \text{rs}) + \varepsilon_i$$

where y = host, spp = species, and rs = resting site.

The contributions of species and resting site to the success of host DNA identification for RDBA were also assessed as above, with the following exception: y = a binomial response, where 0 = failure and 1 = amplicon or host identity produced. In order for host DNA to be identified by RDBA, host DNA must 1) amplify in a PCR reaction, and 2) bind to a probe in a hybridization reaction. Therefore, analysis of RDBA success was broken down into two different models. The contributions of species and resting site to 1) the success of amplicon production by Cyto primers were examined first, followed by 2) the success of amplicon hybridization to the membrane strips in RDBA. For all logistic regression models, parameter significance was always determined by model reduction with likelihood ratio tests using a chi-squared distribution, with an *a priori* $\alpha = 0.05$. A Yate's corrected Pearson's χ^2 test (1 df) was used to compare host identification success rates of RDBA and direct sequencing.

Results

An. gambiae s.l. species identification

In total, 299 *An. gambiae s.l.* mosquitoes were collected with either fresh blood or a range of Sella stages (from 2 to 6), including subgravid. Of these, 244 (81.6%) successfully reacted in real time PCR to identify sibling species: *An. arabiensis* comprised 69.5% of the mosquitoes collected and identified, while 29.5% were *An. gambiae*. *Culex* spp. were discarded and *An. funestus* was absent. Distribution of *An. gambiae s.l.* species was not independent of resting site ($\chi^2 = 33.9$, df = 1, $p < 0.001$). *An. gambiae* were four times more abundant from indoor resting sites than outdoor resting sites, whereas *An. arabiensis* were twice as abundant from outdoor resting sites (Table 2).

Experiment 1 – *An. gambiae s.l.* blood host identification with Mam A and Av A primers followed by direct sequencing

Of the 299 field-collected *An. gambiae s.l.* in various Sella blood digestion stages, 163 produced amplicons following PCR with Mam A and Av A primers. Direct sequencing identified host DNA in 120 of these samples (Table 3). Of these, 80 were *An. arabiensis*, 15 were *An. gambiae*, and 25 were only morphologically identifiable as *An. gambiae s.l.* Only

3 host animals were found to have been fed upon by *An. gambiae* and *An. arabiensis*: humans, cattle, and birds. Ninety-eight percent of the *An. arabiensis* fed upon cattle, and more than 60% of these individuals were collected from indoor resting sites. One indoor resting *An. arabiensis* fed upon a bird in the genus *Turdus*, and one outdoor resting *An. arabiensis* fed upon a human. Only 2 *An. gambiae* were collected from outdoor resting sites, and both fed upon bovine hosts. Of the 13 *An. gambiae* collected from indoor resting sites, 6 contained bovine blood meals and 7 contained human blood meals.

Experiment 2 – Equiprobable host identification by direct sequencing and RDBA

In initial tests of probe accuracy and cross-reactivity, DNA extracted from blood of known animal origin always hybridized to its corresponding probe. No cross-reactivity was observed for the 10 probes selected in the present study. However, visibility of DNA hybridization to Bov 1 (Table 1), which is specific to Zebu cattle (*Bos indicus*) was weak in initial tests using blood from *Bos taurus* donor cattle (Lampire Biological Corp., Pipersville, PA). For this reason, a second probe, Bov 2 (Table 1), was designed for the detection of European cattle breeds (*Bos taurus*). Both probes were imprinted on membrane strips used for the testing of field-collected samples; many African cattle are *Bos taurus-Bos indicus* hybrids (Freeman *et al.* 2006).

Mixed blood sources were identifiable by RDBA. DNA hybridization to its corresponding host probe was visible when 1 blood type represented as little as 1/4th (1.25 μ L) of the total blood source (Figure 2). Visibility of a DNA-bound probe declined as the volume of its corresponding blood source declined relative to the total blood volume, and *vice versa*. DNA hybridization to the probe was not visible for a blood source when it represented 1/7th of the total mixed blood volume.

Of the 278 samples amplified with Cyto primers, 174 produced amplicons (63%). Identification of visible amplicons by direct sequencing and RDBA were equivalent ($df = 1$, $\chi^2 = 0.962$, $p = 0.327$) when Cyto primers were used. Direct sequencing identified the utilized hosts in 147 (84%) of the field collected samples that produced amplicons, whereas RDBA identified 139 (80%) (Table 3). Furthermore, hosts identified in the same samples by both RDBA and direct sequencing with the Cyto primers were completely concordant, with the following exceptions. Three of the field samples analyzed via RDBA revealed mixed blood meals: 2 *An. arabiensis* fed upon both human and bovine hosts, and 1 unidentified *An. gambiae s.l.* individual fed upon human and avian hosts.

In the initial PCR, Cyto amplicon production varied according to species classification ($p = 0.009$), but not by vector resting site ($p = 0.184$), nor the interaction between species and resting site ($p = 0.09$). The observed proportions of samples producing a Cyto amplicon (2.5%, 97.5% non-parametric bootstrapped CI, $N = 5000$) from host DNA were 0.691 (0.617, 0.759) for *An. arabiensis*, 0.611 (0.493, 0.731) for *An. gambiae*, and 0.449 (0.306, 0.592) for individuals only identifiable as *An. gambiae s.l.* Where amplicons were produced by Cyto primers, host DNA identification varied by resting site ($p < 0.001$), but not vector species ($p = 0.403$) nor the interaction of resting site and vector species ($p = 0.372$). In our sample, the observed proportions of blood meals identified by RDBA (2.5% CI, 97.5% bootstrapped CI, $N = 5000$) were 0.914 (0.852, 0.963) for outdoor resting mosquitoes, but only 0.702 (0.606, 0.787) for indoor resting mosquitoes. Percentages of host DNA identified via the two-step RDBA process from each species/resting site combination are reported in Table 4.

Of the ninety field-collected samples that produced amplicons in Mam A/Av A, as well as Cyto PCR reactions, host identifications were concordant for 84 (93%) of these samples. In 6 of these samples, direct sequencing results differed by primer pair. Four indoor-resting and

one outdoor-resting *An. gambiae* were identified as cattle-fed when Mam A/Av A primers were used, but human-fed when Cyto primers were used. One outdoor-resting *An. arabiensis* was identified as human-fed by PCR with Mam A/Av A followed by direct sequencing, yet cattle-fed when Cyto primers were used. In five of these samples (4 for Mam A/Av A and 1 for Cyto) direct sequencing reads were of low quality, with visible mixed peaks. In all of these cases, Cyto primer direct sequencing results were used for statistical tests of host feeding by species and resting site.

When the results of all three methods were pooled, a total of 206 (69%) *An. gambiae s.l.* hosts were identified (Table 5). In total, nearly eighty percent of the total blood meals identified were bovine in origin. *An. arabiensis* blood fed upon cattle 50 times more frequently than upon humans, while most *An. gambiae* fed upon humans (Table 5). After excluding *An. gambiae s.l.* and the “other” category of host, likelihood ratio tests showed a significant effect of mosquito species ($p < 0.001$), but not resting site ($p = 0.374$), nor the interaction of mosquito species and resting site ($p = 0.952$) on host selection.

Discussion

RDBA was as successful at identifying host DNA as was direct nucleotide sequencing when using the mitochondrial cytochrome B gene as the informative gene for the identification system. The two methods consistently returned identical results for our field-collected samples. PCR with Cyto primers produced amplicons for 63% of our samples, which were field-collected in varying stages of blood digestion. The percentage of amplicons identified by RDBA and direct sequencing were 80 and 84% of blood meals, respectively. These findings demonstrate the utility and relative simplicity of this blood meal identification system, in particular, that it retains sensitivity and specificity of a molecular approach, but does not require sequencing. In our hands, the most important developmental aspect was optimization of hybridization conditions. A shaker-incubator is required in field laboratories where this method would most likely be utilized *in lieu* of ELISA, given that membranes with probes would most likely be prepared at a centralized genomics facility but hybridizations would take place in the field lab where test results would be read. The other essential items of equipment are a thermal cycler and an electrophoresis gel rig. Further, DNA extracted from abdomens for blood meal analysis provides template for other molecular assays such as identification of sibling species of mosquitoes and detection of knockdown resistance genes (Mathias *et al.* 2011).

While the rate of hybridization of Cyto amplicons to the membrane-bound probe during RDBA was equivalent to the rate of host identification by direct sequencing, a substantial portion (ca. 36%) of our samples failed to amplify in a PCR reaction with Cyto primers. In the present study, this is likely caused by degradation of host DNA (eg. due to blood meal digestion) rather than an inability to detect unspecified hosts as in ELISA (Service *et al.* 1986). Like ELISA, RDBA is able to detect a specified set of hosts. In the present study, we examined whether potential host species were being overlooked, by comparing host identities produced by sequencing with those of RDBA. Direct sequencing is able to detect any host as long as 1) amplicons are produced by PCR, and 2) host DNA sequences are available in GenBank. In our comparison, no directly sequenced amplicons returned host identities that were undetectable by probes bound to the RDBA membrane. For the *An. gambiae s.l.* sibling species/host relationship in which a relatively few number of potential hosts are involved as was shown here and elsewhere, i.e., humans and bovines (e.g., Beier *et al.* 1988, Githeko *et al.* 1994, Diatta *et al.* 1998, Mwangangi *et al.* 2003, Lefevre *et al.* 2009, Bayoh *et al.* 2010, Fornadel *et al.* 2010), a membrane with a modest number of probes including these two host species provides an adequate platform to implement the method. In other study systems, the number and specificity of the membrane-bound probes can easily

be extended (Abbasi *et al.* 2009). If a larger number of hosts were involved (e.g. Hamer *et al.* 2009), then direct sequencing would be preferable owing to the large number of probes that would otherwise have to be designed into the platform.

Instead, our 63% amplicon production rate highlights general logistical issues for identifying vector host use in the tropics. Amplicon production rates rely heavily upon the availability of host DNA in the mosquito gut, which is typically fully digested within 24-48 h of blood feeding by *An. gambiae* complex mosquitoes in tropical Africa (Service *et al.* 1986). Blood meal sizes, digestion rates, and even nucleation (or lack thereof) of red blood cells in some hosts can contribute to variation in DNA availability, and thereby amplicon production rates across hematophagous insects (Service *et al.* 1986). In one example, mosquito blood meals are 5 times larger than those of Phlebotomine sandflies (Service *et al.* 1986), yet sandflies often digest blood more slowly; fresh blood can still be found in the midgut of sandflies 72 h post ingestion, and blood meal remnants are still present 120 h post ingestion (Service *et al.* 1986, Dillon and Lane 1993). This discrepancy between mosquito and sandfly blood meal digestion rates may, in part, address differences between our host identification rates and those of Abbasi *et al.* (2009). Rapid blood meal digestion by mosquitoes in the tropics presents a challenge for host identification.

In the present study, RDBA host identification was influenced by both vector species designation and resting site. Mosquitoes that were identified as *An. gambiae* and *An. arabiensis* were equally likely to produce an amplicon in a PCR with Cyto primers, yet unclassifiable *An. gambiae* s.l. were less likely to produce an amplicon than *An. arabiensis* in these same PCRs. This association between unidentifiable *An. gambiae* s.l. and reduced amplicon production suggests that overall availability of genomic DNA in these few samples (< 18% of total tested) was insufficient to produce an amplicon in any PCR. Where amplicons were produced, host DNA hybridization to membrane-bound probes during RDBA did not differ by species, but instead mosquito resting site. Amplicons from indoor resting mosquitoes were less likely to hybridize with a probe during RDBA than were amplicons from outdoor resting mosquitoes. We speculate that differences in the timing of host availability, as well as temperatures experienced by indoor vs. outdoor resting mosquitoes might influence blood digestion rates, quantities of fresh blood available in the mosquito gut, and thereby the overall quality of host DNA available for hybridization.

Another advantage of RDBA is that it can detect DNA originating from two or more hosts, as in mixed blood meals, while direct sequencing cannot. In the present study, blood types representing ¼ (1.25 µL fresh blood) of the total blood mixture (5µL) were detectable by RDBA. Nonetheless, the frequency of mixed blood meals detectable by RDBA in field-collected samples was modest compared to Bayoh *et al.* (2010), where *An. gambiae* and *An. arabiensis* were sampled from a community ca. 40 km west of Kisian and mixed human and bovine blood meals were relatively more frequent. In 6 field-collected samples, different hosts were identified via direct sequencing when Mam A/Av A vs. Cyto primers were used. The poor quality of sequencing reads, and presence of mixed peaks in 5 of these cases indicate that these individuals may have taken mixed blood meals, yet poor DNA amplification rendered these mixed feedings below the detectable threshold of RDBA.

Data on the sampling distribution of *An. gambiae* and *An. arabiensis* provided here were consistent with previous studies showing that whereas *An. gambiae* is more frequently collected indoors, *An. arabiensis* was frequently collected both indoors and outdoors (e.g. Githeko *et al.* 1994, Odiere *et al.* 2007). For the latter species, which in our study was much more common than was *An. gambiae*, even indoor collections were dominated by individuals that had fed on cattle blood, thus indoor resting behavior does not necessarily correlate with human host selection.

The proportion of *An. gambiae* relative to *An. arabiensis* collected in June and July of 2008 suggests a shift in the predominant *An. gambiae* s.l. complex vectors present in human dwellings. Prior to 1998, *An. gambiae* comprised 80% of the indoor resting *An. gambiae* s.l. species complex population, and more than 70% of the total *An. gambiae* s.l. population in Kisian village and in nearby villages (Joshi *et al.* 1975, Service *et al.* 1978, Petrarca *et al.* 1991, Githeko *et al.* 1994). In the present study, *An. gambiae* comprised 46% of the indoor resting, and < 30 % of the total population (n = 244) identified to species. This decline confirms observations of a long term trend reported by Bayoh *et al.* (2010) and observed regionally by Mathias *et al.* (2011). Widespread ITN use is likely contributing to this phenomenon, which has been demonstrated in this part of western Kenya (Bayoh *et al.* 2010). Indeed, Hightower *et al.* (2010) reported that homeownership of ITNs approached 70% in the study area. However, increased ITN coverage does not appear to have selected for a shift in host-utilization by *An. gambiae*. Neither *An. gambiae* nor *An. arabiensis* expanded their host range outside of the long term norm, as reviewed above. Over 90% of *An. gambiae* fed upon humans, yet ITNs had been present in nearly 70% of human dwellings since 2006. These results are consistent with those of Fornadel *et al.* (2010), which demonstrated that host selection of an anthropophilic *An. arabiensis* population remained unchanged in the two years following mass ITN distribution. In Burkina Faso, long-term inaccessibility of human hosts (Robert *et al.* 1991) has led to the evolution of phenotypic plasticity, such that nearly 90% of *An. gambiae* feed upon cattle, though preference for human odor-baited traps was maintained in the population (Lefèvre *et al.* 2009). Perhaps the latency between ITN distribution and the present study, household ITN ownership, nightly ITN use, or a combination of these was insufficient to generate selection pressure for phenotypically plastic host-use in the Kisian *An. gambiae* population compared to results presented in Lefèvre *et al.* (2009).

Taken together, the combination of increased frequency of *An. arabiensis* and a high rate of feeding on cattle emphasize the importance of these results to strategies for malaria control. Measures such as treatment of cattle with the systemic insecticides, such as ivermectin (Foley *et al.* 2000, Fritz *et al.* 2009, Fritz *et al.* 2012), or topical insecticides, such as pyrethroids (Hewitt and Rowland 1999) have been demonstrated as an effective way to reduce zoophilic vector survival and longevity. The RDBA method would be useful in determining if the frequency of cattle feeding is high in a given area, thereby signaling that such measures could be appropriately implemented.

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Figure 1.

Schematic of a membrane strip used for reverse dot blot analysis, including placement of the probes. On the right, a membrane strip used in a test reveals that the unknown host was bovine.

Figure 2.

Relative visualization of probes in a mixed blood meal based upon the proportion of DNA from each host. Gel bands demonstrate a roughly equal quantity of DNA per samples, yet relative intensity of the RDBA probe varies based upon the proportion of DNA from each host. In this figure, background brightness for some strips was adjusted to create equivalent background brightness across all strips.

Table 1

Oligonucleotide probes imprinted upon membrane strips for reverse dot blot procedure.

	Species	Oligonucleotide sequence
1	Bovine 1 (<i>Bos indicus</i>)	ATT ATG GGT CTT ACA CTT T
2	Bovine 2 (<i>Bos taurus</i>)	ATT ACG GGT CTT ACA CTT T
3	Brown rat (<i>Rattus rattus</i>)	CAG TCA CCC ACA TCT GC
4	Goat (<i>Capra hircus</i>)	ATA CAT ATC GGA CGA GGT CTA
5	Sheep (<i>Ovis aries</i>)	TCC TAT TTG CGA CAA TAG CTT CCT
6	Domestic mouse (<i>Mus musculus</i>)	TGG AGT ACT TCT ACT GTT CGC AGT
7	Human (<i>Homo sapiens</i>)	ATG CAC TAC TCA CCA GAC GC
8	Domestic dog (<i>Canis domesticus</i>)	CAG ATT CTA ACA GGT TTA
9	General Avian 2	GCC TCA TTC TTC TTC AT
10	General Avian 1	TAC ACA GCA GAC AC

Table 2

Resting sites from which *An.gambiae s.s.* and *An. arabiensis* were collected. Indoor resting sites included the sides and underside of furniture, and the interior walls of houses. Outdoor resting sites were the interior of clay pots used for rain catchment outside of houses. Individuals classified as *An. gambiae s.l.* were morphologically identified as belonging to the complex but could not be identified via qPCR. The distribution of species was associated with resting site ($\chi^2 = 33.9$, $df = 1$, $p < 0.001$)

Species	Collection Site		
	No. Indoor (%)	No. Outdoor (%)	Total (%)
<i>An. gambiae s.s.</i>	58 (19.3)	14 (4.6)	72 (23.9)
<i>An. arabiensis</i>	67 (22.4)	106 (35.4)	173 (57.8)
<i>An. gambiae s.l.</i>	40 (13.3)	15 (5.0)	55 (18.3)
Total (%)	165 (55.0)	134 (45.0)	299

Table 3

Methods used for identification of host blood meals. Samples that produced amplicons after PCR with primers listed below were subjected to either direct sequencing (DS), or Reverse Dot Blot Analysis (RDBA).

Primer pairs	Host Identification Method	n	No. Amplicons Produced by PCR	No. Hosts Identified	Percentage Amplicons Identified	Percentage Sample Identified
Mammal A and Avian A	DS	299	163	120	73.6	40.1
Cyto	DS	278	174	147	84.4	52.8
Cyto	RDBA	278	174	139	79.9	50.0

Table 4

Percentages of a field-collected sample (n = 278) containing host DNA which first amplified in a PCR with Cyto primer pairs, and was then identified in an hybridization reaction via RDBA. Amplicon production with Cyto was influenced by vector species identification ($p = 0.009$), while hybridization of host DNA to the probe was influenced by vector resting site ($p < 0.001$).

Species	Resting Site	No. samples Subjected to PCR with Cyto	No. amplicons produced	Percentage Samples Amplified	No. Hosts Identified	Percentage amplicons identified	Percentage of Samples Identified
<i>An. gambiae</i>	out	9	6	66.7	6	100	66.7
	in	58	35	60.3	25	71.4	43.1
<i>An. arabiensis</i>	out	96	67	69.8	61	91.0	63.5
	in	66	45	68.2	29	64.4	43.9
<i>An. gambiae</i> s.l.	out	10	8	80.0	7	87.5	70.0
	in	39	14	35.9	12	85.7	30.7

Table 5

Number (percent) of *An. gambiae* s.s. and *An. arabiensis* whose hosts were identified by at least 1 method of blood meal analysis. Individuals whose blood meals were mixed or avian were classified as “other”.

Species	Host	Collection Site		Row totals (percent)
		Indoor	Outdoor	
<i>An. gambiae</i>	Bovine	6 (13.0)	2 (4.3)	8 (17.3)
	Human	33 (71.7)	5 (10.9)	38 (91.8)
	Other	0	0	0
Subtotal (percent)		39 (84.7)	7 (15.2)	46
<i>An. arabiensis</i>	Bovine	43 (38.4)	63 (56.2)	106 (94.6)
	Human	1 (0.9)	2 (1.8)	3 (2.7)
	Other	1 (0.9) *	2 (1.8) **	3 (2.7)
Subtotal (percent)		45 (40.2)	67 (59.8)	112
<i>An. gambiae</i> s.l.	Bovine	26 (54.2)	10 (20.8)	36 (75.0)
	Human	10 (20.8)	1 (2.1)	11 (22.9)
	Other	0 (0)	1 (2.1) ***	1 (2.1)
Subtotal (percent)		36 (75.0)	12 (25.0)	48
		120 (58.3)	86 (42.7)	206

* *Turdus* spp.

** Mixed bovine and human blood meals

*** Mixed human and avian blood meal