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A Novel Assay for Simultaneous Assessment of Mammalian Host Blood, Mosquito Species, and *Plasmodium* spp. in the Medically Important *Anopheles* Mosquitoes of Madagascar

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Abstract. *Anopheles* mosquitoes vary in habitat preference, feeding pattern, and susceptibility to various measures of vector control. Consequently, it is important that we identify reservoirs of disease, identify vectors, and characterize feeding patterns to effectively implement targeted control measures. Using 467 anopheline mosquito abdomen squashes captured in Madagascar, we designed a novel ligase detection reaction and fluorescent microsphere assay, dubbed Bloodmeal Detection Assay for Regional Transmission (BLOODART), to query the bloodmeal content, identify five *Anopheles* mosquito species, and detect *Plasmodium* infection. Validation of mammalian bloodspots was achieved by preparation and analysis of known hosts (singular and mixed), sensitivity to degradation and storage method were assessed through mosquito feeding experiments, and quantification was explored by altering ratios of two mammal hosts. BLOODART identifications were validated by comparison with mosquito samples identified by sequenced portions of the internal transcribed spacer 2. BLOODART identification of control mammal bloodspots was 100% concordant for singular and mixed mammalian blood. BLOODART was able to detect hosts up to 42 hours after digestion when mosquito samples were stored in ethanol. A mammalian host was identified in every field-collected, blood-fed female *Anopheles* mosquito by BLOODART. The predominant mosquito host was cow (n = 451), followed by pig (n = 26) and human (n = 25). Mixed species bloodmeals were commonly observed (n = 33). A BLOODART molecular identification was successful for 318/467 mosquitoes, with an overall concordance of 60% with all field-captured, morphologically identified *Anopheles* specimens. BLOODART enables characterization of large samples and simultaneous pathogen detection to monitor and incriminate disease vectors in Madagascar.

INTRODUCTION

The World Health Organization (WHO) Global Technical Strategy for Malaria 2016–2030 (GTS) sets key benchmarks in the fight against malaria, seeking a reduction in global malaria mortality and incidence by 90% compared with that of 2015.[1] Furthermore, this plan seeks to eliminate malaria in at least 35 countries and prevent re-establishment of transmission in malaria-free countries. To succeed, the WHO has built a strategic framework supported by three pillars, the first of which seeks to ensure universal access to malaria prevention, diagnosis, and treatment. A fundamental facet of this pillar is effective vector control and surveillance. Malaria is transmitted by the *Anopheles* mosquito. Female *Anopheles* mosquitoes require vertebrate blood[2] to complete their gonotrophic cycle. The acquisition of this bloodmeal allows the mosquito to serve as the bridge for transmission of the *Plasmodium* species that cause malaria.[3] Species within *Anopheles* differ in their habitat preferences,[4,5] feeding patterns,[4,6–9] susceptibility to insecticides,[10–12] and response to non-insecticidal measures of control.[13–16] Consequently, it is important that we identify reservoirs of disease, properly identify vectors, and characterize their feeding patterns to effectively implement targeted control measures.

With collection techniques such as the barrier screen[17] capturing upward of several hundred blood-fed *Anopheles* mosquitoes per sampling night, we need a monitoring strategy that is both financially and temporally feasible. This strategy should also align with WHO GTS priorities of assessing the presence of vector species, abundance and seasonality, time and place of biting, vector behavior, insecticide susceptibility status, and underlying resistance mechanisms.[1] Therefore, the primary objective of this study was to address several of these priorities by developing an efficient, sensitive protocol for *Anopheles* identification, querying of hosts in the bloodmeal, and detection of *Plasmodium* parasites likely to be used in malaria-endemic regions. To do so, there are several hurdles we must address.

Morphological mosquito identification is notoriously difficult, and taxonomic keys are sporadic in their availability and degree of comprehensiveness. For example, the key in use for mosquito identification in much of Madagascar does not include sufficient illustrations to effectively identify mosquitoes.[18] The more comprehensive works by Gillies and De Meillon[19] and Gillies and Coetzee[20] include detailed illustrations, but the size of the geographic region covered can lead to a cumbersome number of characters to determine. Consequently, much of the identification is achieved with unpublished localized keys and personal communication with experienced culicid taxonomists. Furthermore, proper vouchering and archival specimen storage is often lacking in non-taxonomic fields, a serious detriment to reproducibility and reanalysis in light of new data.[21] Increasingly, molecular diagnostic strategies are being used for mosquito identification and epidemiological studies. Examples of these assays include several multiplex assays for the identification of medically important *Anopheles*, such as cocktail polymerase chain reactions (PCRs) with distinguishable band size differences (typically internal transcribed spacer 2 [ITS2] focused),[22–24] real-time PCR probes,[25–27] and ligase detection reaction–fluorescent microsphere assays (LDR-FMAs) (in Papua New Guinea[28,29]). For bloodmeal assessment, techniques include multiplexes with band size differentiation, "barcode" marker sequencing (e.g., cytochrome oxidase I [COI] and cytochrome B),

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group-specific PCR primers, heteroduplex analysis, restriction fragment length polymorphism, real-time PCR, reverse line blot hybridization, and DNA profiling.[30] More recently, next-generation sequencing was used to detect mosquito hosts.[6] These methods vary in their ease of use and sensitivity, and none are equipped to concurrently identify mosquito species and detect *Plasmodium* parasites.

Here, we provide a post-PCR mosquito bloodmeal assay that simultaneously queries the *Anopheles* mosquito and a broad range of hosts in the bloodmeal. This novel assay can be used in conjunction with a previously developed assay to detect malaria-causing *Plasmodium* parasites.[31] This approach demonstrates the ability to evaluate hundreds of samples efficiently, and is compatible with technology available in many molecular diagnostic laboratories of malaria-endemic countries. The Bloodmeal Detection Assay for Regional Transmission (BLOODART) uses LDR-FMA technology. This technique uses species-specific and fluorescently labeled conserved sequence probes that ligate to a PCR-generated target amplicon. These species-specific probes contain unique tags that correspond to anti-tag labeled microspheres that emit unique fluorescent signals. These signals are detected using a Bio-Plex array reader. Probe sets for multiple amplicon targets can be combined in a reaction, allowing for simultaneous assessment of multiple genetic targets in a single sample.

To demonstrate the efficacy of BLOODART, we performed controlled laboratory experiments, followed by an assessment of a field-collected sample. Absence of cross-reactivity was demonstrated through artificial mixtures of blood from known mammalian hosts. Sensitivity to DNA degradation, as well as determination of the most effective storage method for BLOODART analysis, was assessed using laboratory-reared *Aedes aegypti* fed on known mammalian hosts. We assessed the ability of BLOODART to detect low volumes of host DNA by diluting whole blood in phosphate buffered saline (PBS) and lowering the number of PCR cycles. To determine whether BLOODART has the ability to quantify the proportion of blood consumed from recently fed-upon hosts, we mixed human and cow blood in various proportions before analysis.

METHODS

DNA extraction. DNA was derived from several sources: bloodspots (mammalian blood controls and mosquito abdomen squashes) or abdomens pulled from whole mosquitoes in RNAlater® (Thermo Fisher Scientific, Waltham, MA) or ethanol. Individual squashes were hole-punched (three circles, 3 mm in diameter), and these materials were used for genomic DNA extraction using a QIAMP 96 DNA blood kit (QIAGEN, Venlo, The Netherlands; following the supplementary protocol for dried blood spots with 12-hour incubation in ATL buffer). Abdomens stored in RNAlater or ethanol were carefully removed using forceps, and the same bloodspot protocol was used for DNA extraction.

PCR amplification. Mosquito DNA amplification was achieved using primers[28] anchored in conserved regions of the nuclear 5.8S and 28S rDNA, which flank the highly variable ITS2 region.[32,33] Internal transcribed spacer 2 is routinely used to distinguish closely related species and cryptic complexes in medically important mosquito genera, including *Aedes*,[34] *Anopheles*,[22,35,36] and *Culex*.[37] A subset of bands from the field-collected mosquitoes was chosen for sequencing based on ITS2 amplicon length (on an agarose gel) to establish a benchmark for anopheline diversity in our sample (*Anopheles arabiensis* [*n* = 1, GenBank MK129246]; *Anopheles coustani* [*n* = 1, MK129245]; *Anopheles funestus* [*n* = 2, MK129243]; *Anopheles maculipalpis* [*n* = 2, MK129244]; *Anopheles rufipes* [*n* = 2, MK129242]; and *Anopheles* sp. *unk.* [*n* = 2, MH560267]). These ITS2 sequences were used to perform Basic Local Alignment Search Tool (BLAST) searches to assess within-species diversity (*An. arabiensis*, among 100 sequences identified [e.g., Nigeria, Kenya, and Tanzania] 100% were concordant with the BLOODART probe; *An. coustani*, among three sequences identified [e.g., Kenya and Zambia] 100% were concordant with the BLOODART probe; *An. funestus*, among 62 sequences identified [e.g., Cameroon, Kenya, Zambia, and South Africa] 100% were concordant with the BLOODART probe; *An. maculipalpis*, among three sequences identified [e.g., Kenya and Zambia] 100% were concordant with the BLOODART probe; and *An. rufipes*, among five sequences identified [e.g., Kenya, Zambia, and Madagascar] 100% were concordant with the BLOODART probe). The sequences generated from ITS2 amplicons were BLAST-searched to determine the species. As a secondary species marker, we amplified COI using primers previously optimized for mosquito COI amplification[38] (Supplemental Table 1). As this product does not differ significantly in size, COI was sequenced for representative species identified by ITS2 to aid in species delimitation. Mammalian DNA was amplified using mammal 16S rDNA primers previously demonstrated to amplify the vast majority of mammals present in the National Center for Biotechnology Information (NCBI) database.[6] *Plasmodium* DNA was amplified using primers for the small subunit ribosomal RNA (SSU rRNA) gene[39] (Supplemental Table 1).

Bloodmeal Detection Assay for Regional Transmission. We designed a post-PCR LDR-FMA based on GenBank-mined and sequenced portions of field-collected mosquito ITS2 and Genbank-mined mammal 16S sequences (Supplemental Table 2). Hosts were chosen based on the fauna described by National Malaria Control Program (NMCP) technicians who participated in the collection. This was combined with a previous assay for the detection of *Plasmodium* species.[31] These probes were designed to simultaneously decipher the mosquito species, mammalian host(s), and *Plasmodium* infection from an individual abdomen squash. The ITS2 (mosquito), 16S (mammal), and SSU rDNA (*Plasmodium*) PCR products (PCRs were performed separately) were added to the LDR, wherein species-specific upstream classification probes, with anti-tag complementary sequences corresponding to Luminex microsphere sets, ligate to downstream 3′biotinylated reporter probes. The LDR and detection[31] followed previously established methods using a Bioplex array reader (Bio-Rad Laboratories, Hercules, CA). Each run included controls for four *Plasmodium* species: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*. A detailed protocol for performing the BLOODART analysis is provided in the Supplemental Material.

Validation of BLOODART probes. Using whole blood collected from six mammalian host species (Lampire Biological Laboratories, Pipersville, PA), we verified the specificity of the assay. A small amount of whole blood (~3 uL) in ethylenediaminetetraacetic acid (EDTA) was pipetted onto filter

paper. This was either from a single mammalian host or from a premixed sample of equal proportions from two to three mammalian hosts (n = 24; Supplemental Table 3). The resolving power of BLOODART was assessed by diluting whole blood in PBS (150, 125, 100, 10, and 2 µL of blood) for a standard Qiagen blood extraction (total 200 uL), and PCR amplification was set at 15, 20, and 25 cycles (n = 18; Supplemental Table 4). To determine the effect of variable blood ratios in the bloodspot, we prepared various proportions of mixed human and cow blood to form bloodspots. The proportions were as follows: 1:1, 4:3, 2:1, 4:1, 6:1, and 1:0 for a series with cow blood held at a constant volume, and also with human blood held at a constant volume (n = 12; Supplemental Table 5). Mosquito species probes were validated by performing the BLOODART assay on mosquito samples identified by sequencing of the ITS2 locus.

Mosquito rearing, storage, and preservation. Mosquitoes used for preliminary testing of mammalian probes were raised from desiccated *Ae. aegypti* eggs in an insectarium in the Department of Microbiology and Molecular Genetics, Michigan State University. The strain was received from the Florida Medical Entomology Laboratory and originates from White City, FL.

We tested the maintenance of host DNA over a period of digestion in *Ae. aegypti* bloodmeals. Mosquitoes were assessed using BLOODART, providing insight into the assay's sensitivity to degraded DNA and optimal storage methods for future surveys. Adult female mosquitoes were allowed to feed on human blood for 30 minutes, with various individuals starting and finishing their feeding during that time (n = 35). Any individuals that were not visibly engorged were removed via aspiration. Mosquitoes were sampled at 18, 24, and 42 hours, with 3–5 mosquitoes collected at each timepoint per storage method. Individuals were knocked down by freezing at –20°C for 10 minutes, then stored in 95% ethanol (EtOH), RNAlater, or squashed on Whatman 3 MM CHR filter paper (Sigma-Aldrich, St. Louis, MO; product typically used for gas chromatography). For the EtOH and RNAlater, the mosquito was submerged in a 2-mL screw cap tube, and a small piece of cotton was placed on top to prevent the mosquito from contacting air. The abdomen squash was performed by removing the abdomen from the mosquito with a fine pair of forceps and gently pressing it with a plastic pestle onto the filter paper, taking care to preserve the entire abdomen and the full bloodmeal. The tubes were subsequently stored at –20°C, and the filter paper was stored at room temperature until DNA extraction 5 days later. As a control, we created two bloodspots on Whatman filter paper following a finger prick (n = 2), designating the collection time as 0 hours. All samples were extracted as described in the DNA extraction section.

Field-collected mosquitoes. Wild-caught mosquitoes were collected by Madagascar NMCP entomologists and technicians in March of 2017 in the villages of Amparihy (lat. 19°23.112′S, long. 046°08.186′E; 763 m above sea level) and Ambolodina (lat. 19°22.460′S, long. 046°07.459′E; 808 m above sea level), located in the fokontany of Kambatsoa (Commune Maroharona, Tsiroanomandidy Health District). Entomological and parasitological surveys are routinely performed by the Madagascar NMCP and are consistent with protocols approved by the Madagascar Ministry of Health (No. 099-MSANP/CE). In addition, community and household approvals were obtained following fokontany-based meetings before initiating all study activities.

Collection methods included the barrier screen[17] (n = 453 mosquitoes), with one round of pyrethroid spray catch (n = 14 mosquitoes). These sites exhibit a seasonal transmission trend (beginning in November–December and peaking in April–May) and are largely characterized by submicroscopic infection.[40] The barrier screen technique was used near Malagasy Zebu (*Bos taurus indicus*) corrals nestled within the villages. Pyrethroid spray catch was conducted by clearing a human dwelling, laying down a white sheet, and applying pyrethroid insecticides to the walls and ceiling. Blood-fed mosquitoes knocked down by the insecticide were aspirated from the sheet. Blood-fed mosquitoes collected by both approaches were morphologically identified,[18–20] squashed onto Whatman 3 MM CHR filter paper, and allowed to dry. These bloodspots were subjected to BLOODART analysis.

Data analysis. Data analyses, histograms, and scatterplots were generated in R[41] using the compilation package "Tidyverse."[42]

RESULTS

Validation of BLOODART probes. In each case, BLOODART correctly identified the host(s) present in the bloodspots prepared from known mammalian hosts (Supplemental Table 3). Diluting a sample in PBS did not alter the BLOODART signal at either 25 or 20 cycles of PCR. However, a measurable drop in signal occurred for the more diluted hosts at 15 cycles but not below the limit of detection of < 500 mean fluorescent intensity (MFI) (Supplemental Table 4). Mixing various proportions of human and cow blood did not significantly alter the BLOODART signal (Supplemental Table 5).

The sensitivity and specificity of mosquito species probes were assessed by performing the BLOODART assay on mosquito samples identified by sequencing of the ITS2 locus. Each sample was correctly identified by BLOODART, and background signal on these samples was sufficiently low (< 500 MFI) that no false positives were scored (Supplemental Figure 1).

Mosquito storage and preservation. The mosquito was buoyant in the RNAlater, and the addition of cotton caused the mosquito to become entangled and difficult to manipulate without loss of morphological integrity. Individual mosquitoes stored in 2-mL tubes filled completely with ethanol minimized damage. We revealed a precipitous drop in BLOODART MFI as bloodmeal digestion time increased (Supplemental Figure 2). All three storage methods: 95% EtOH, RNAlater, and Whatman filter paper, allowed for successful detection of human DNA for most of the samples at 18 (n = 11/12) and 24 (n = 7/9) hours. At 42 hours, however, only samples stored in 95% EtOH had detectable human DNA in the bloodmeal (n = 3/14). A logistic regression, using human BLOODART signal as the outcome and storage method as the predictor, revealed that storage in ethanol led to a significantly higher BLOODART signal ($P < 0.01$; Supplemental Figures 3 and 4).

Field-collected mosquitoes. The barrier screen procured 824 mosquitoes (*Aedes* [n = 5], *Anopheles* [n = 600], *Coquillettidia* [n = 3], *Culex* [n = 160], and *Mansonia* [n = 56]), 467 of which were blood-fed females subjected to BLOODART analysis. A BLOODART mosquito identification was successful for 318/467 samples (Figure 1), with an overall concordance of

60% (192/318) between BLOODART and morphologically identified specimens, excluding the negative BLOODART identifications (Table 1). Of the total mosquitoes, 149 were unable to be identified by BLOODART. Of the mosquitoes with a negative species identity via BLOODART, 95% were morphologically identified as *Anopheles squamosus*. Subsequent studies will include a probe for this species. Concordance between morphology and ITS2 molecular diagnosis among individual species (excluding the negative *An. squamosus*) was 97% for *An. coustani*, 85% for *An. rufipes*, 83% for *An. maculipalpis*, 59% for *An. gambiae* s.l., and 0% for *An. funestus*.

The bulk of the sample was identified by BLOODART as *An. rufipes* (n = 123), *An. coustani* (n = 93), and *An. maculipalpis* (n = 51). Two specimens sequenced at the ITS2 locus revealed a novel sequence (< 90% similarity to existing sequences). A probe was designed to detect this ITS2 signature based on sequenced field-specimen data alone, and henceforth specimens identified with this BLOODART probe will be referred to as *An.* sp. *unk* (n = 10). These specimens were not consistently assigned to any morphological species (Table 1). Across all of our sequenced mosquito samples, the marker COI (91–99% similarity to any existing sequence on NCBI) was less precise than ITS2 (99–100% similarity), despite using primers that capture the standard region used by the Barcoding of Life Database.[38]

Mammalian DNA was amplified in all samples. The primary bloodmeal constituent was bovine (n = 451), with some pig (n = 26), human (n = 25), and mixtures of the three (n = 33) (Figure 2). Host preference was cow for most species, differing only for *An. funestus*, *An. gambiae* s.l., and *An.* sp. *unk*. *Anopheles funestus* and *An. gambiae* s.l. were skewed toward human and mixed human–cow bloodmeals. *Anopheles* sp. *unk.* exhibited a higher proportion of pig and human–cow bloodmeals than other anopheline species sampled. Most of the *An. funestus* were captured via pyrethroid spray catch (13/15), and this method also captured the highest proportion of human blood-fed mosquitoes in this dataset. We did not find any pattern relating the position of the mosquito on either side of the net to species captured, level of engorgement, or host. Of the five genera recorded, *Anopheles* was the most likely to be engorged (Figure 3). No mosquitoes were found to be *Plasmodium* positive in this collection.

DISCUSSION

Our results demonstrate the ability of BLOODART to efficiently identify mosquito species, the capacity to uncover the contents of their bloodmeals, and test for the presence of *Plasmodium* parasites. We show a high degree of zoophilic feeding behavior across our sample, consistent with our earlier study that evaluated bloodmeals by an Illumina sequencing strategy.[6] We also demonstrate a disproportionate representation of the known malaria vectors indoors, and an absence of *Plasmodium* parasites in this collection. Furthermore, we demonstrate the utility of the barrier screen in the highland fringe of Madagascar.

Validation of BLOODART probes. BLOODART successfully identified single and mixed species bloodspots. The assay is semiquantitative,[43] and our laboratory-based studies suggest that further assessments of quantification are difficult to determine (Supplemental Tables 4 and 5, Supplemental Figure 1). By 20 cycles of PCR, the BLOODART signal was essentially saturated across samples ranging from 200 µL of extracted blood to just 2 µL diluted in 198 µL of PBS. This suggests that our standard protocol of 30 cycles will likely produce a similar signal for each host species, regardless of the amount of starting blood material. Furthermore, our bloodspots containing mixed proportions of human and cow blood did not alter the signal from either host. These experiments suggest that we cannot provide an estimation of quantity of blood in the mosquito abdomen or an estimation of proportion of each host in the original bloodmeal.

Mosquito storage methods. In-laboratory testing of mosquito storage methods suggests that placing specimens immediately into 95% EtOH is the most effective strategy for preserving mammalian DNA in the mosquito bloodmeal,

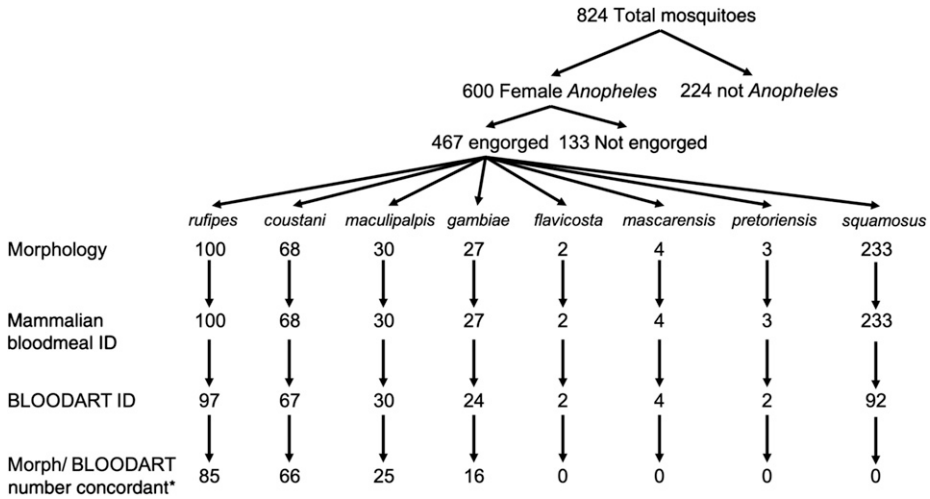

FIGURE 1. Schematic showing the distribution of the 824 mosquitoes captured on the barrier screens. Six hundred of the total catch were female *Anopheles* mosquitoes, 467 of which were blood-fed females squashed onto Whatman filter paper. The top row indicates the morphological identification of the specimen. The second row indicates the number of specimens with the morphological identification in the first row that had a host assigned by BLOODART analysis (100%). Row 3 displays the number of specimens with the morphological identification in row 1 that were assigned a molecular species ID (68%) via BLOODART (although not necessarily the same species displayed in row 1). Row 4 shows the number of specimens that were concordant for morphology and BLOODART identifications. *For full concordance information, refer to Table 1.

TABLE 1
Concordance between morphological identification and BLOODART species determination of Malagasy *Anopheles* mosquitoes

Morphology	BLOODART						
	Anopheles rufipes (*n* = 123)	*Anopheles coustani* (*n* = 93)	*Anopheles maculipalpis* (*n* = 51)	*Anopheles gambiae* (*n* = 26)	*Anopheles funestus* (*n* = 15)	*Anopheles* sp. *unk* (*n* = 10)	Neg (*n* = 149)
An. rufipes (*n* = 100)	**85**	2	2	1	7	0	3
An. coustani (*n* = 68)	1	**66**	0	0	0	0	1
An. maculipalpis (*n* = 30)	3	2	**25**	0	0	0	0
An. gambiae (*n* = 27)	0	0	0	**16**	5	3	3
An. funestus (*n* = 0)	0	0	0	0	**0**	0	0
Anopheles flavicosta (*n* = 2)	0	0	0	0	1	1	0
Anopheles mascarensis (*n* = 4)	1	0	0	0	1	2	0
Anopheles pretoriensis (*n* = 3)	0	0	2	0	0	0	1
Anopheles squamosus (*n* = 233)	33	23	22	9	1	4	**141**

BLOODART = Bloodmeal Detection Assay for Regional Transmission. Bold numbers indicate where morphology and BLOODART identifications were concordant. Cell in the last row/column is highlighted to indicate that the majority of morphologically identified An. *squamosus* were negative by BLOODART.

with human DNA detected in lab-fed mosquitoes up to 42 hours after ingestion. The caveat being that this has the potential to damage certain morphological characters. Observation of mosquito specimens stored in such a manner for several months, however, revealed that minute characters (such as fine wing scales) were wholly intact. However, dismemberment of some or all of the legs was common, some of which can be attributed to the specimen becoming entangled in the cotton. High-resolution photography of specimens before abdomen excision and extraction may constitute a viable means of downstream morphological investigation. It should also be noted that Whatman 3 MM CHR filter paper for gas chromatography, rather than Whatman Flinders Technology Associates (FTA) Blood Cards (Sigma-Aldrich), was used in this comparison. The latter would likely perform better, as they are designed specifically for preservation of nucleic acids.

Mosquito collection recommendation. The following workflow optimizes the utility of the sample for further epidemiological and taxonomic analyses. Collected mosquitoes were killed with ethyl acetate, chloroform, or by freezing, and keyed to species. Rather than squashing the mosquito sample, the specimen was stored directly into 95% ethanol. Although this is not ideal for the preservation of taxonomic characters, it maximizes the utility of the sample for future DNA extraction[44] and retains many important aspects of insect morphology for future systematic analysis or potential description of new diversity. Extraction by removal of the entire abdomen with fine-tipped forceps ensured that the maximum content of the bloodmeal was captured, and further guarantees

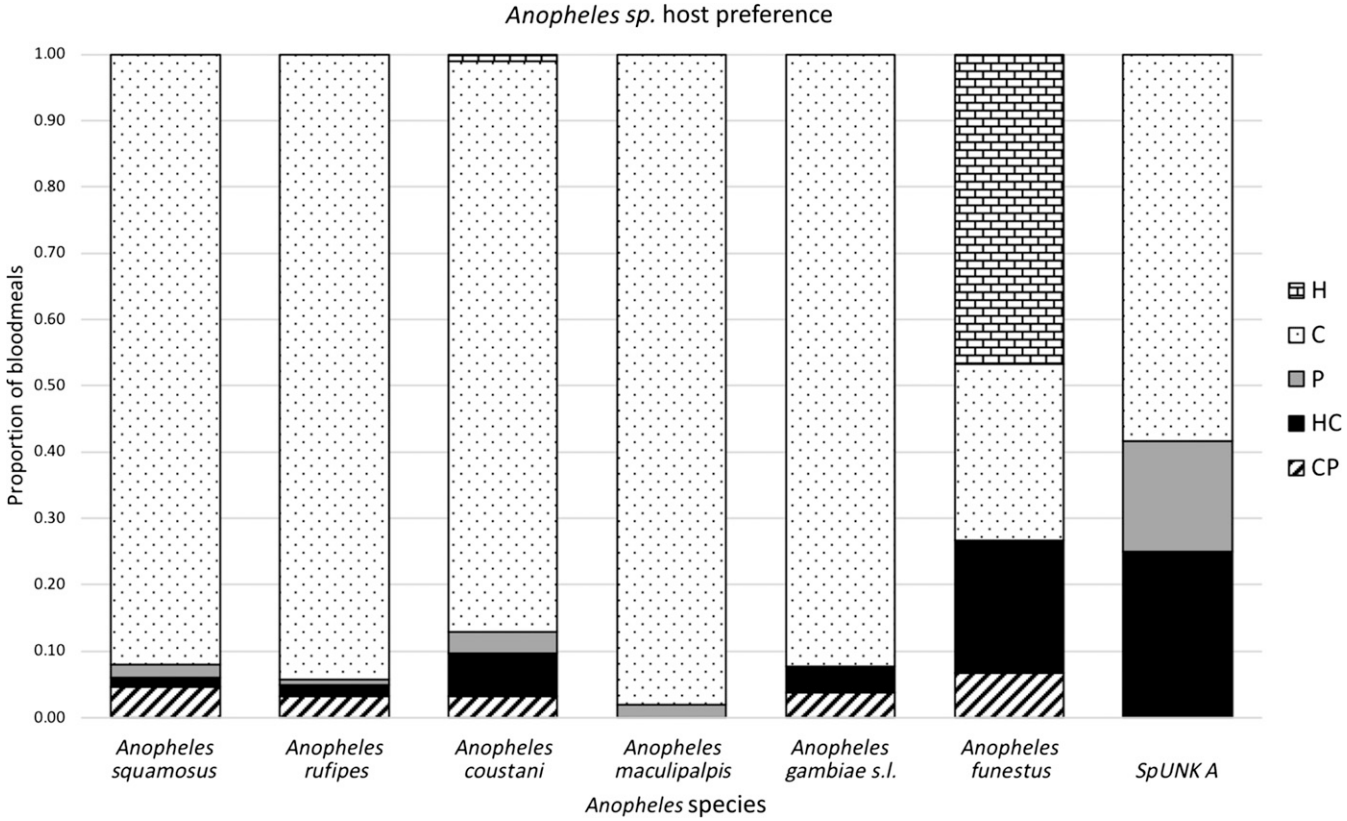

FIGURE 2. Bar graph displaying the bloodmeal content frequency for *Anopheles* mosquitoes collected in Kambatsoa. C = cow; H = human; P = pig.

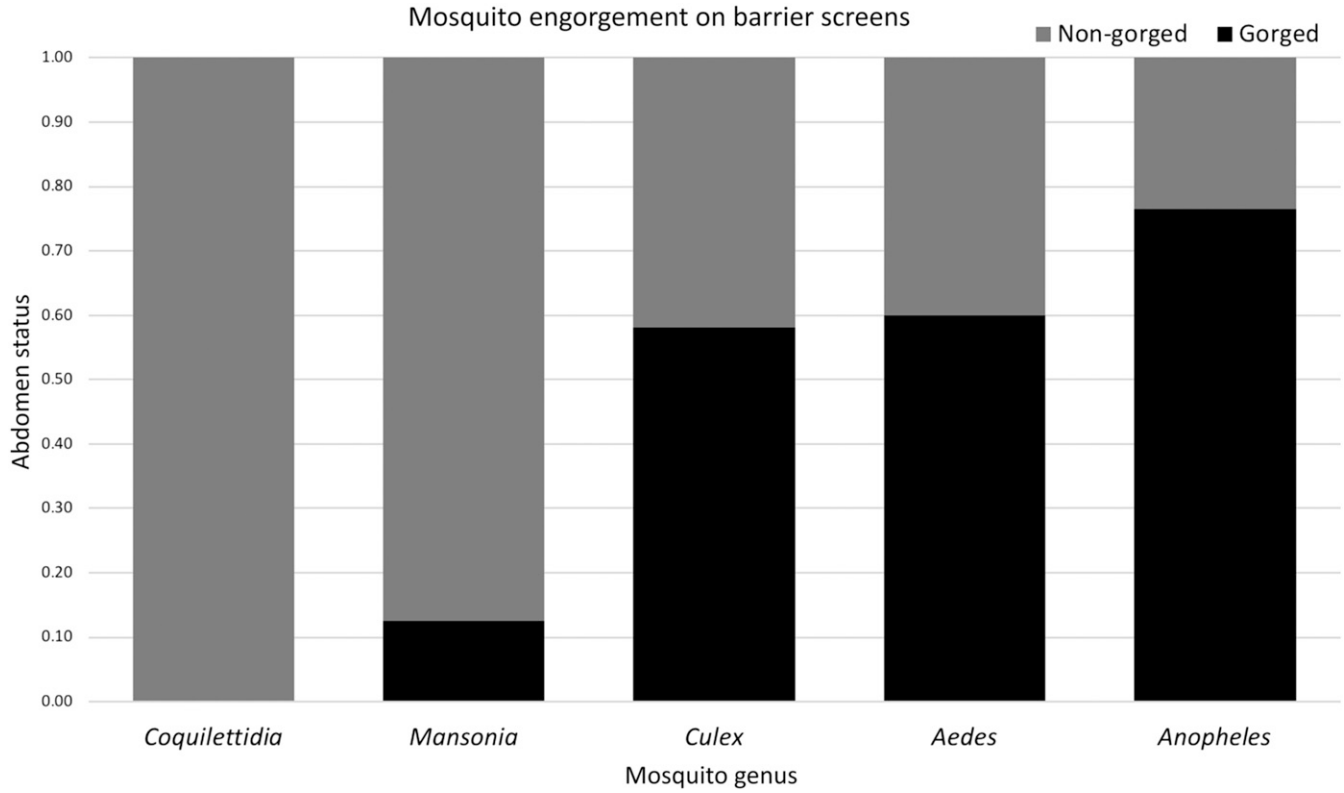

FIGURE 3. Abdomen engorgement of Kambatsoa mosquitoes. These data primarily represent capture by barrier screen. *Anopheles* mosquitoes represented most of the sample and were also the most likely to be engorged.

that sufficient mosquito tissue is present for PCR amplification. Using a unique voucher for each specimen, the remaining mosquito carcass can be linked to the bloodmeal. Samples prepared in this manner are observed to remain stable for at least 2 months[45]; for longer term storage, samples are stored frozen at –20 to –80°C.

Bloodmeal Detection Assay for Regional Transmission field-collected mosquitoes. The BLOODART signal distinguished the species for which probes were designed. As most of the BLOODART-negative specimens were morphologically identified as *An. squamosus*, it is likely that these specimens were not identified by BLOODART simply because a probe for this species was not included in this study. Subsequent analyses will include a probe for *An. squamosus* and other *Anopheles* species known to exist in Madagascar. Low concordance between morphological and BLOODART identifications for several species (particularly *An. gambiae* s.l. and *An. funestus*) can likely be attributed to the insufficient dichotomous keys and the difficulty associated with identifying mosquitoes in the field. By sequencing ITS2 for species confirmation, we demonstrated the accuracy of BLOODART over morphology in this setting. Lack of similarity for COI data can primarily be attributed to a lack of concurrent coverage and secondarily to potential novel polymorphism in Malagasy *Anopheles*.

This study also revealed a novel ITS2 sequence that could not be linked to a known species. It is not known whether this mosquito is a competent vector. Given the number of human positive bloodmeals for these specimens, it is important to screen for this species in future epidemiological studies. Because of the current limitations of the abdomen squash technique, we do not have the ability to morphologically describe these specimens, preventing their association with an existing species or description as a new one. Using the suggested workflow described earlier, we plan to more effectively characterize novel mosquitoes discovered during ongoing epidemiological surveys in Madagascar. The probes for *An. gambiae* and *An. funestus* should theoretically only work on *Anopheles funestus* sensu stricto and *Anopheles gambiae* sensu stricto, respectively; but a control experiment using known members of the cryptic species complexes composing these two vector species was beyond the scope of this study. Future work will seek a probe design with the capacity to differentiate the various members of these cryptic complexes.

Every mosquito sample was successfully assigned a mammalian host. Considering the small size of the mammalian PCR product (~100 bp) relative to the size of the ITS2 product (400–800 bp), it was expected that mammalian DNA amplification would be more successful. Furthermore, the diversity of potential mammalian hosts is less than the number of potential *Anopheles* species, and a robust search of species queried in silico for these mammalian primers suggested that these primers should amplify more than 95% of mammalian 16S RNA genes present in the NCBI database.[6]

No *Plasmodium*-positive mosquitoes were recorded in this sample. However, BLOODART analysis of mosquitoes collected in other parts of Madagascar revealed *Plasmodium* positivity rates ranging as high as 6–10% (R. E. Tedrow, unpublished data).

The BLOODART technique used in this study successfully and efficiently analyzed a large sample of mosquito

bloodmeals, as probes can easily be added or subtracted to optimize multiplex capacity. The BLOODART output is numerical, allowing for rapid analysis of every sample from raw data, or instantaneous empirical analysis of large datasets using simple computational programs or spreadsheet equations. There are some limitations to the assay. As shown by the negative BLOODART result for morphologically identified *An. squamosus*, absence of a probe means no detection. However, sequencing the amplicon for a specimen with a negative or inconclusive BLOODART result will provide insight into potential new mosquito/host species, for which new probes can be added to the assay. Although we performed cross-reactivity analyses for numerous potential hosts, we cannot eliminate the possibility that unaccounted-for hosts in a wild setting would cross-react with our probes. The performance of this assay will be optimal in areas with well-characterized mosquito, host, and pathogen diversity.

Comparison to existing detection methods. Currently, there are several technologies with the capacity to identify mosquitoes, hosts, and *Plasmodium* parasites efficiently. Next-generation sequencing provides a wealth of information, providing insight into the DNA present without the limit of specific probes, and with the ability to parse out multiple targets much more effectively than Sanger sequencing.[6] However, next-generation sequencing may not be as efficient as our BLOODART strategy. Another promising technology is matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF), which has the capacity to detect both mammalian DNA in the bloodmeal and mosquito species using extracted peptides to identify both microorganisms and multicellular organisms. The device can independently detect mosquito species[46,47] and mammalian host,[48] or simultaneously detect mosquito species and *Plasmodium* infection status.[49] However, MALDI-TOF may not be appropriate for simultaneous detection of mosquito species and mammalian host, as insect abdomens tend to elicit different spectra within species because of variable gut contents.[46] The extraction process for MALDI-TOF does not yield a sample with future utility comparable to standard genomic DNA, and the high acquisition price of the platform is not feasible for many resource-challenged countries in which these analyses would be most important.

This vector-focused BLOODART contributes a powerful epidemiological tool for assessing the feeding dynamics and diversity of medically important Malagasy *Anopheles* mosquitoes. There is extensive operational utility for this technology in local public health facilities in Madagascar. The Madagascar NMCP currently possesses the platform and could feasibly incorporate this assay into their vector monitoring program. Assessments of local mosquito populations that lead to vector implication can quickly and efficiently inform control strategies. Inclusion of future mosquito collections will facilitate expansion of both the mosquito and host probes, providing a more comprehensive picture of vector diversity and host choice. With an acquisition price of ~$20,000, and a training time of approximately 1 week (for those already proficient in PCR), the approach used here can be applied to novel locations at risk of mosquito-borne diseases.

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