Received: 11 December 2023 | Accepted: 1 May 2024

DOI: 10.1111/mve.12725

ORIGINAL ARTICLE

Medical and Veterinary Entomology

Next generation sequencing improves the resolution of detecting mixed host blood meal sources in field collected arboviral mosquito vectors

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Funding information
Norwegian Agency for Development Cooperation, Grant/Award Number: RAF-3058 KEN-18/0005; Swedish International Development Cooperation Agency (Sida); Swiss Agency for Development and Cooperation (SDC); Australian Centre for International Agricultural Research (ACIAR); Norwegian Agency for Development Cooperation (Norad); German Federal Ministry for Economic Cooperation and Development (BMZ); Government of the Republic of Kenya

Associate Editor: Marco Pombi

Abstract

Accurate knowledge of blood meal hosts of different mosquito species is critical for identifying potential vectors and establishing the risk of pathogen transmission. We compared the performance of Miseq next generation sequencing approach relative to conventional Sanger sequencing approach in identification of mosquito blood meals using genetic markers targeting the 12S rRNA and cytochrome oxidase I (COI) genes. We analysed the blood meals of three mosquito vector species (*Aedes aegypti*, *Aedes simpsoni* s.l. and *Culex pipiens* s.l.) collected outdoors, and compared the frequency of single- versus multiple-blood feeding. Single host blood meals were mostly recovered for Sanger-based sequencing of the mitochondrial 12S rRNA gene, whereas Miseq sequencing employing this marker and the COI marker detected both single and multiple blood meal hosts in individual mosquitoes. Multiple blood meals (two or more hosts) which mostly included humans were detected in 19%–22.7% of *Ae. aegypti* samples. Most single host blood meals for this mosquito species were from humans (47.7%–57.1%) and dogs (9.1%–19.0%), with livestock, reptile and rodent hosts collectively accounting for 4.7%–28.9% of single host blood meals. The frequency of two or more host blood meals in *Ae. simpsoni* s.l. was 26.3%–45.5% mostly including humans, while single host blood meals were predominantly from humans (31.8%–47.4%) with representation of rodent, reptile and livestock blood meals (18.2%–68.2%). Single host blood meals from *Cx. pipiens* s.l. were mostly from humans (27.0%–39.4%) and cows (11.5%–27.36%). Multiple blood meal hosts that mostly included humans occurred in 21.2%–24.4% of *Cx. pipiens* s.l. samples. Estimated human blood indices ranged from 53%–76% for *Ae. aegypti*, 32%–82% for *Ae. simpsoni* s.l. and 26%–61% for *Cx. pipiens* s.l. and were consistently lower for Sanger-based sequencing approach compared to Miseq-based sequencing approach. These findings demonstrate that Miseq sequencing approach is superior to Sanger sequencing approach as it can reliably identify mixed host blood meals in a single mosquito, improving our ability to understand the transmission dynamics of mosquito-borne pathogens.

KEYWORDS
Aedes aegypti, *Aedes bromeliae*, arbovirus vectors, blood feeding patterns, *Culex pipiens* s.l., next generation sequencing

INTRODUCTION

Blood feeding by mosquitoes is an essential biological process that fulfils the reproductive need of adult females. However, this process is also associated with transmission of several viral, protozoan and bacterial pathogens that cause diseases that afflict mankind. Examples include malaria, lymphatic filariasis, yellow fever, dengue, chikungunya, Rift Valley fever vectored by mosquito species in the genera *Anopheles*, *Aedes* and *Culex*. Blood feeding is one of the sensitive parameters of vectorial capacity, a metric that estimates pathogen transmission potential of a vector (Tchouassi et al., 2021; West et al., 2020). The centrality of blood feeding in pathogen transmission highlights the goal of vector control interventions to interrupt the cycle of human-vector contact. Investigations on mosquito blood feeding can provide critical insights into the epidemiology and dynamics of vector-borne diseases such as identifying potential reservoirs. Furthermore, the mechanisms that underlie interactions between mosquitoes and suitable hosts can be exploited in the development of vector monitoring or control tools including attractants or repellents (Torto & Tchouassi, 2024).

Several techniques are available for identification of blood meal hosts of mosquitoes and other hematophagous arthropods. Serological techniques targeting species-specific proteins are among the oldest commonly utilized methods (Beier et al., 1988; Kent, 2009), although availability of antibodies/antigens for certain hosts especially wild species can be challenging. Conventional DNA-based approaches have improved the sensitivity and specificity of blood meal sources using a priori species-specific primers to identify putative hosts (Kent, 2009) or sequencing of PCR amplicons followed by comparisons of blood meal DNA sequences in reference databases (Kamau et al., 2023; Reeves et al., 2018; Tchouassi et al., 2021). A major limitation of the latter method is the poor ability to resolve multiple host-feeding in individual blood meals (Kent, 2009; Tchouassi et al., 2021). These limitations can be overcome through cloning and sequencing of PCR products, but this method can be expensive, time-consuming and biased towards newly ingested blood meals as older blood meals may be digested by the vector.

More modern methods employ next generation sequencing (NGS) as a cost-effective approach to identify host blood meals of field collected mosquitoes (Estrada-Franco et al., 2020; Logue et al., 2016; Muturi et al., 2021). We recently described an NGS method for identifying blood meals including multiple hosts feeding from *Aedes aegypti* artificially fed on mixed blood from different hosts (Muturi et al., 2021). Here, we extend this approach to compare with Sanger sequencing to analyse blood meals from wild mosquito species: *Ae. aegypti*, *Aedes simpsoni* s.l. and *Culex pipiens* s.l. The mosquito species are the primary vectors of dengue virus, yellow fever virus and lymphatic filariasis, respectively (Torto & Tchouassi, 2021). Specifically, we compared the frequency of single versus multiple feeds in these field collected mosquitoes, analysed on both sequencing platforms.

MATERIALS AND METHODS

Mosquito samples

Engorged adult mosquitoes that were trapped during surveys as part of arbovirus surveillance activities were analysed. The surveys were carried out in two contrasting areas of Kenya: Kerio Valley (with history of yellow fever outbreak) in the Rift Valley; and Rabai in coastal Kenya (endemic for dengue). Blood-fed *Ae. aegypti* and *Ae. simpsoni* s.l. were collected between 6:30 AM and 6:00 PM using CO_2-baited BG Sentinel traps, while blood-fed *Cx. pipiens* s.l. were collected using CO_2-baited CDC light traps between 6:00 PM and 6:30 AM. The traps were set at different time points between August 2019 and February 2020. A detailed description of the study sites, trapping design and mosquito processing is reported elsewhere (Kamau et al., 2022, 2023).

DNA extraction and Sanger sequencing for host blood meals

Genomic DNA was extracted from the abdomen of individually engorged *Cx. pipiens* s.l. using the ISOLATE II Genomic DNA Kit (Bioline, Meridian Bioscience, Germany) following the manufacturer's protocol. A 500 bp portion of the 12S mitochondrial rRNA gene was amplified by polymerase chain reaction (PCR) in a 10 μL reaction volume as described previously (Kamau et al., 2023; Tchouassi et al., 2021). This comprised 2 μL 2× MyTaq Mix (Bioline, Germany), 10 μM of each primer, 0.2 U of Mytaq DNA polymerase and 1 μL of the template DNA (~20 ng). The cycling conditions included an initial denaturation at 95°C for 3 min followed by 40 cycles at 95°C for 20 s, 59°C for 30 s and 72°C for 30 s and a final extension at 72°C for 7 min. Amplicons were separated on 1.2% agarose gel electrophoresis against a 100 bp DNA HyperLadder (Bioline, Meridian Bioscience, Tennessee, USA). Purified PCR products (using SureClean Plus kit, Bioline, Meridian Bioscience) were Sanger sequenced at Microgen (Netherlands). Cleaned DNA sequences were queried using BLASTn tool against the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and taxonomic identity to species level assigned when sequence similarity was ≥98% (Kamau et al., 2023; Tchouassi et al., 2021).

Next generation sequencing of blood meals

For additional blood meal analysis DNA samples from *Cx. pipiens* s.l, *Ae. simpsoni* s.l. and *Ae. aegypti* were sequenced on an Illumina Miseq platform at the United States Department of Agriculture, Peoria, IL. The samples were PCR amplified using two sets of primers targeting the mitochondrial 12S rRNA and cytochrome oxidase 1 genes (Table 1). The 25 μL PCR reaction volume consisted of 2.5 μL genomic DNA, 5 μL each of forward and reverse primers (1 μM) and 12.5 μL of 2× KAPA HiFi HotStart ready mix. Thermocyling conditions for the

TABLE 1 Summary of primers used in this study.

Target region	Primer name	Primer sequence (5′–3′)	Reference
12S	12S3F	GGGATTAGATACCCCACTATGC	Roca et al. (2004)
	12S5R	TGCTTACCATGTTACGACTT	
CO1	Mam-F	TGAYATGAAAAAYCATCGTTG	Kim et al. (2009)
	Mam-R	TGTAGTTRTCWGGGTCKCCTA	
Overhang adapters	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	Provided by Illumina
	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	

12S rRNA gene were identical to those reported for the Sanger sequencing above. Thermocycling conditions for amplification of CO1 gene and library preparation and normalization procedures are reported elsewhere (Muturi et al., 2021).

All bioinformatics analysis was performed using CLC genomics workbench v21.01 (Qiagen inc). The demultiplexed reads were quality trimmed to a base calling confidence of 95%. The trimmed reads were de novo assembled into contigs with the following parameters, 50% read length and 95% nucleotide identity. The trimmed reads were mapped back to the contigs to normalize coverage between individual libraries. Only contigs with at least 1% of the reads of an individual library were processed for identification. The consensus sequences were extracted from these reads and BLASTed against the NCBI nucleotide database. Sequences with a BLAST bit score <250 were discarded. Sequences were manually examined and those not belonging to the Animal kingdom were discarded.

Ethical approval

The study was approved by the Scientific Ethics Review Unit (SERU) of the Kenya Medical Research Institute (Protocol NO. SSC 2787). Additionally, consent was sought from household heads to set up traps around their homesteads.

Statistical analyses

Human blood index (HBI) was calculated as the proportion of mosquitoes fed on humans (both single and mixed blood meals) divided by the number of mosquitoes analysed. Statistical differences in estimated HBI for the marker types were compared using Chi-square (χ^2). Analyses were performed in R v. 4.1.2 at the significant level of $p \leq 0.05$.

RESULTS

Blood meal source identification

A total of 129 specimens were analysed (*Ae. aegypti* = 52, *Ae. simpsoni* s.l. = 28; *Cx. pipiens* s.l. = 49). Not all blood meal samples were successfully amplified/identified across the markers used (Figure 1). This resulted in disparity in sample size for the specimens analysed for each species. The Sanger method employing 12S gene marker (12S_Sanger) mainly detected single host blood meal sources in individual mosquitoes tested. This was consistent across the three mosquito species examined. Mixed host blood meals were observed for all the species only when analysed by NGS targeting 12S rRNA (12S_Miseq) or *COI* (COI_Miseq) (Tables 2–4).

Ae. aegypti host feeding profile

Blood meal source was determined for 42–45 *Ae. aegypti* samples (out of 52 analysed) based on marker type (Figure 1; Table 2). The highest number of single host feeding was on human and the proportion ranged from 47.7% (21/44) to 57.1% (24/42) based on analytical method (Table 2). The second most common host detected in single blood meals was dog ranging from 9.1% to 19.0%. Single blood meals from livestock, reptile and rodent hosts varied from 4.7% to 28.9% based on the marker (Table 2). Sanger-based analysis exclusively recovered single host blood while NGS-based marker analysis recovered both single and mixed blood meals that included a human plus one or two other hosts (Table 2). The common mixed blood meals included humans plus either lizard, cow, domestic dog, African giant pouched rat with one detection of a blood meal sample consisting of three hosts including human, lizard and cow (Table 1). Patent multiple meals (double and triple) in *Ae. Aegypti*, which mostly included humans, were detected in 19.0% (8/42)–22.7% (10/44) of the specimens.

Ae. simpsoni s.l. feeding pattern

Typing of host blood meals was achieved in 28 *Ae. simpsoni* samples of which 19–22 were successful based on marker type (Table 2). Blood meals from single species varied by methods with mixed blood meals only evident for the NGS-based analysis. The highest single feed was on humans and the proportion ranged from 7/22 (Sanger_COI) to 9/19 (12S_Miseq) (Table 3). Other hosts detected in single blood meals included domestic cat, dog, grass rat, squirrel and African giant pouched rat. All multiple meals included a human plus one or two other hosts (Table 3).

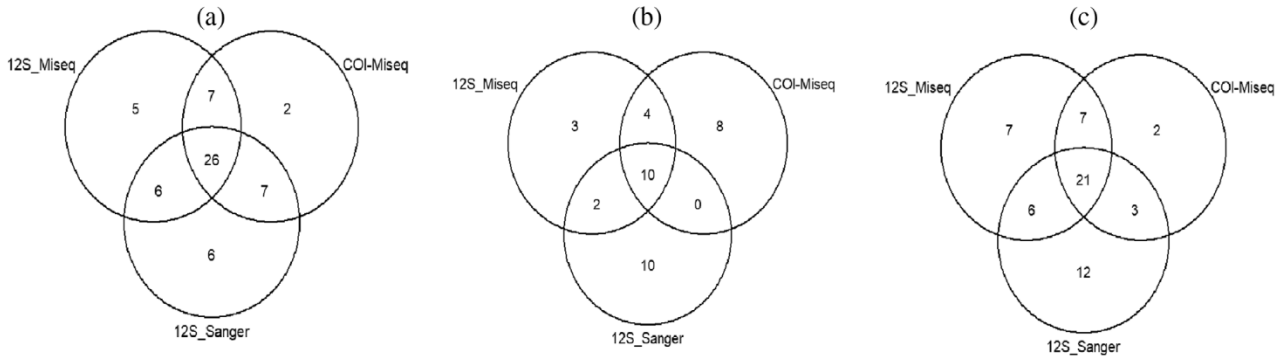

FIGURE 1 Disparity in successful host meal profiled using three marker types among three mosquito vectors. (a) *Aedes aegypti*, (b) *Aedes simpsoni* s.l., (c) *Culex pipiens* s.l. COI, cytochrome oxidase I.

TABLE 2 Summary of *Aedes aegypti* feeding patterns computed using the three marker types.

Host meal sources	12S_Miseq	COI_Miseq	12S_Sanger
African giant pouched rat + human	2	3	0
Cow + lizard + human	1	0	0
Lizard + human	1	0	0
Cow + human	1	1	0
Domestic cat + human	1	1	0
Domestic dog + human	4	3	0
Domestic dog	4	8	8
African giant pouched rat	2	1	4
Goat	4	0	2
Human	21	24	24
Domestic cat	0	0	2
Lizard	3	0	3
Mongoose	0	0	1
Bat	0	0	1
Cow	0	1	0
Total	44	42	45

Abbreviation: COI, cytochrome oxidase I.

Cx. pipiens s.l. trophic pattern

Of the 33–42 *Cx. pipiens* successfully processed (out of 49) for blood meal host identification (Table 4), most had single host feeds on human (except cow for Sanger sequencing) and the rate differed by methods (14/41, 13/33 and 7/26, for 12S_Miseq, COI_Miseq and 12S_Sanger, respectively) (Table 4). The next most common hosts detected in single blood meals were cows (10/41; 9/33; 3/26; 12S_Miseq, COI_Miseq and 12S_Sanger, respectively) followed by low frequencies of domestic cat, domestic dog, grass rat, passerine birds, chicken, lizard and African giant pouched rat. Similarly, most multiple blood meals included human and one or more other hosts, which was observed in 7/33–9/41 of the specimens (Table 4). All mixed blood meals included two hosts and were exclusively recovered via NGS analysis (Table 4). The proportion of feeds with mixed blood meals ranged from 9/41 (12S_Miseq) to 7/33 (COI_Miseq). Based on presence of humans in each blood meal (single and multiple), the estimated HBI ranged from 0.26 to 0.61 (Figure 2) and differed by marker type and analysis method.

HBI comparisons by analytical method

Estimated HBI based on frequency of single or mixed host feeding that included humans was lowest for each species where samples were analysed by Sanger sequencing (12S_Sanger) compared to the NGS methods. HBI ranged 0.53 (12S_Sanger), 0.70 (12S_Miseq) to 0.76 (COI_Miseq) for *Ae. aegypti*. Estimates for *Ae. simpsoni* s.l. were 0.32 (12S_Sanger), 0.74 (12S_Miseq) and 0.82 (COI_Miseq). For *Cx. pipiens* s.l., HBI estimate was the highest for COI_Miseq (0.61), followed by 12S_Miseq (0.59) and then 12S_Sanger (0.26). Thus, estimated HBI was influenced by the method of analysis and marker type.

TABLE 3 *Aedes simpsoni* s.l. feeding patterns computed by three marker types.

Host meal sources	12S_Miseq	COI_Miseq	12S_Sanger
Squirrel + Maxomys mice + human	0	1	0
Cow + human	1	0	0
African giant pouched rat + human	2	3	0
Cow + human	1	0	0
Human + squirrel	1	2	0
Domestic cat + human	0	1	0
Goat + human	0	1	0
Human + mongoose	0	2	0
Human	9	8	7
Domestic cat	2	1	2
Grass rat	1	1	1
Squirrel	1	1	3
African giant pouched rat	1	0	2
Cow	0	0	1
Goat	0	0	2
Mongoose	0	0	2
Mastomys mouse	0	0	1
Lizard	0	0	1
Domestic dog	0	1	0
Total	19	22	22

Abbreviation: COI, cytochrome oxidase I.

TABLE 4 *Culex pipiens* feeding patterns computed by three marker types.

Cx. pipiens host meals	12S_Miseq	COI_Miseq	12S_Sanger
Chicken + human	1	0	0
Cow + human	2	4	0
Domestic dog + human	1	0	0
Gecko + human	1	1	0
Human + domestic cat	3	2	0
Passerine bird + human	1	0	0
Mus mouse + passerine bird	1	0	0
Human	14	13	7
African giant pouched rat	1	1	1
Chicken	2	0	3
Cow	10	9	3
Gecko	1	0	4
Lizard	3	0	2
Domestic dog	0	2	2
Domestic cat	0	0	3
Sheep	0	1	0
Passerine bird	0	0	0
Bird (bush warbler)	0	0	1
Total	41	33	26

Abbreviation: COI, cytochrome oxidase I.

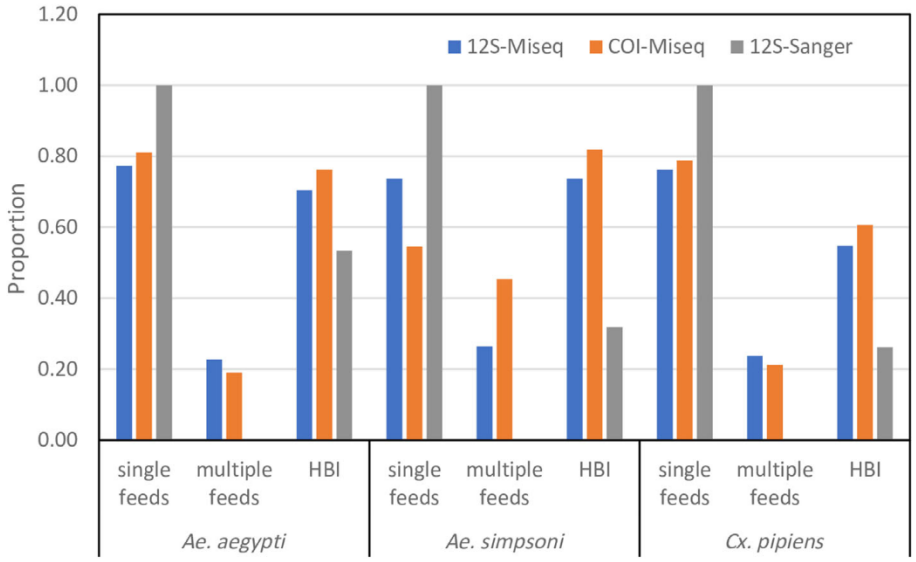

FIGURE 2 Frequency of single and mixed vertebrate hosts in single mosquito blood meal and estimated human blood indices for Sanger versus next generation sequencing analysis. COI, cytochrome oxidase I; HBI, human blood index.

The probability of feeding on human host either as single or in patent mixed meal ranged from 53% to 76% (Figure 2) in *Ae. aegypti*. The HBI for this species differed significantly between the COI_Miseq and 12S_Sanger analysis only (32/42 vs. 24/45; $\chi^2 = 4.00$, df = 1, $p = 0.045$). Estimated HBI differed significantly between the three protocols for *Ae. simpsoni* s.l. (14/19 vs. 18/22 vs. 7/22; $\chi^2 = 13.262$, df = 2, $p = 0.001$) and *Cx. pipiens* s.l. (23/41 vs. 20/33 vs. 7/26; $\chi^2 = 7.63$, df = 2, $p = 0.02$).

DISCUSSION

Here, we describe a novel high-throughput sequencing method that enables analysis of 129 mosquitoes simultaneously and provides a comprehensive and quantitative perspective on the composition of each blood meal. Although *Ae. aegypti* principally from coastal Rabai fed on a variety of hosts, almost all engorged mosquitoes contained human blood. Even when multiple meals were taken, they always included at least one feeding on a human. Detection of human blood meals was affected by the method utilized.

We compared the frequency of single versus multiple host feeds in engorged field collected mosquitoes individually analysed using different marker-sequencing platform combination. Consistently, targeting 12S or COI, we detected both single and mixed host blood meals using NGS marker analysis. On the other hand, Sanger-based method (12S_Sanger) exclusively recorded single host blood meal sources in individual mosquitoes. Poor resolution of mixed blood meals by PCR and direct amplicon sequencing compared to MiSeq methods is well-documented (Estrada-Franco et al., 2020; Logue et al., 2016; Muturi et al., 2021). Improved resolution of NGS methods has been ascribed to deriving each sequence read from a single source DNA molecule (Metzker, 2010). Our findings indicate that the sequencing method and type of marker can affect the resolution or detection of hosts implicated in the feeding habits of disease vectors. Therefore, previous studies likely underestimated the frequency of *Ae. aegypti* feeding on multiple vertebrate species (Kamau et al., 2022, 2023).

PCR and direct sequencing of the amplicon (Sanger sequencing)

High anthropophagy has a direct relationship with vectorial capacity which measures the risk of human pathogen exposure (Kamgang et al., 2012; Tchouassi et al., 2022). For the three vector species, estimated HBI was consistently lower for PCR-Sanger sequencing than recorded for the NGS methods. Effectiveness of *Ae. aegypti* or other species to human amplified arboviral pathogens (e.g., dengue, chikungunya and Zika viruses) is linked to its high human feeding habits (Kamgang et al., 2012; Tchouassi et al., 2022). In this study, HBI up to 76% was estimated for *Ae. aegypti* consistent with its high human feeding rates (Ponlawat & Harrington, 2005; Sene et al., 2022). The rate far exceeds previous levels for this species for populations in different ecological areas of Africa (Agha et al., 2019; Chepkorir et al., 2018). The difference may be due to the high-resolution analytical method employed in this study. *Ae. aegypti* is known to selectively feed on humans, and even in cases where multiple blood-feeding occurred, at least one human host was included (Harrington et al., 2001). Noteworthy, the feeding habits of this species indicated that dog was the second most utilized host among non-human hosts. The domestic dog represents a primary alternate host for this species (Agha et al., 2019; Estrada-Franco et al., 2020; Olson et al., 2020). While previous blood meal analysis showed that *Ae. aegypti* predominantly fed on human, feeding on non-human hosts calls into question

the relative importance of host availability versus innate preference in driving host feeding patterns (Tchouassi et al., 2022).

Ae. simpsoni s.l. accounted for HBI of up to 82%. This value supports the highly anthropophagic behaviour of *Aedes bromeliae*, a sibling species of *An. simpsoni* s.l. that is dominant in the study areas (Kamau et al., 2022) and is a primary vector of yellow fever virus in East Africa (Ellis et al., 2012; Mukwaya, 1974). Previous HBI value of 33% may have been an underestimation that was limited by the low resolution of Sanger sequencing (Kamau et al., 2022). *Culex pipiens* s.l. had maximal estimated HBI value of 61%, which is in agreement with frequent human feeding by indoor populations of this species in parts of Kenya (>0.80) (Beier et al., 1988) compared to outdoor populations (Muturi et al., 2008). Members of the *Cx. pipiens* complex are important vectors of arboviral pathogens (e.g., West Nile virus) and parasitic nematodes that cause lymphatic filariasis (e.g., *Wucherera bancrofti*) (Torto & Tchouassi, 2021). The feeding pattern including diverse hosts suggest opportunistic feeding by both mosquito vectors, and the underlying basis should be investigated further.

An important knowledge gap is the consequence of multiple host blood feeding on the risk of pathogen transmission. Mixed blood feeding could modulate outcome of vector competence to viruses through effect of successive meals (Armstrong et al., 2020) or changes in midgut microbiota composition (Muturi et al., 2019). A viral infection acquired in the initial blood meal may enhance or inhibit the ability of a second virus acquired via a subsequent blood meal. The latter is termed superinfection interference and is commonly reported among closely related viruses (Muturi et al., 2017; Muturi & Bara, 2015).

There were instances of discordance where data for a given host presence in a blood meal was not uniformly detected across the three methods investigated. This accounted for the disparity in sample size for the specimens analysed for each species. Blood meal data was first processed by PCR-Sanger sequencing before shipment for NGS analysis 3 months later. This time lapse may have affected the quality of DNA and hence success of subsequent NGS runs on same samples. Data obtained for samples that failed via PCR-Sanger likely indicate the high sensitivity of NGS protocols over the latter method in detecting and resolving host feeding profiles. Also, evident were instances of discordance in host blood meal identification even between the marker types for Miseq sequencing. This finding underscores the need for employing a combination of markers for better appraisal of host-feeding patterns. Using the present NGS-based method, other parameters such as vector species identity, pathogen infection, microbiome could be integrated to facilitate simultaneous detection and so gain better insights into epidemiology of vector-borne diseases. Adapting this approach using the portable MinION device as a cost-effective and point-in-need mobile sequencing platform could benefit research especially in disease endemic areas.

In summary, we found better ability of Miseq over Sanger sequencing in resolving mixed host blood meals in field collected mosquito vectors namely *Ae. aegypti*, *Ae. simpsoni* s.l. and *Cx. pipiens* s.l. The analysis also translated to higher estimates of human blood feeding rates for each of these species indicating that studies that employ single markers through Sanger sequencing to determine blood meal patterns may underestimate the HBI. Finally, instances of discordance in host meal identification between the marker types for Miseq sequencing underscores the need for employing a combination of markers for better appraisal of host-feeding patterns.

AUTHOR CONTRIBUTIONS

David P. Tchouassi: Conceptualization; investigation; methodology; validation; funding acquisition; visualization; writing – review and editing; writing – original draft; formal analysis; project administration; resources. **Robinson O. Kisero:** Investigation; writing – review and editing; methodology. **Gilbert Rotich:** Investigation; methodology; writing – review and editing. **Christopher Dunlap:** Investigation; methodology; validation; writing – review and editing; formal analysis. **Baldwyn Torto:** Investigation; methodology; writing – review and editing; resources; funding acquisition; project administration. **Ephantus J. Muturi:** Investigation; resources; writing – original draft; writing – review and editing; methodology; validation; formal analysis.

ACKNOWLEDGEMENTS

We acknowledge the local administration at the counties of Kilifi and Baringo, the local levels and the communities for support and cooperation during implementation of field activities.

FUNDING INFORMATION

This study was conducted under the project Combatting Arthropod Pests for better Health, Food and Climate Resilience (CAP-Africa) funded by the Norwegian Agency for Development Cooperation (Norad) (Grant number: RAF-3058 KEN-18/0005). The authors gratefully acknowledge the financial support for this research by the following organizations and agencies: Swedish International Development Cooperation Agency (Sida), Swiss Agency for Development and Cooperation (SDC), Australian Centre for International Agricultural Research (ACIAR), the Norwegian Agency for Development Cooperation (Norad), the German Federal Ministry for Economic Cooperation and Development (BMZ) and the Government of the Republic of Kenya. The views expressed herein do not necessarily reflect the official opinion of the donors. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study was approved by the Scientific Ethics Review Unit (SERU) of the Kenya Medical Research Institute (Protocol NO. SSC 2787). Further consent was sought verbally from the local administration and household heads to set up traps around their homesteads.

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How to cite this article: Tchouassi, D.P., Kisero, R.O., Rotich, G., Dunlap, C., Torto, B. & Muturi, E.J. (2024) Next generation sequencing improves the resolution of detecting mixed host blood meal sources in field collected arboviral mosquito vectors. *Medical and Veterinary Entomology*, 1–9. Available from: https://doi.org/10.1111/mve.12725