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Molecular Xenomonitoring for assessing malaria parasite prevalence as a proxy to human cases and vector blood-feeding patterns in South Ethiopia

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Research Article

Keywords: Plasmodium infections, Blood meal sources, southern Ethiopia

Posted Date: May 29th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9415206/v1

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Additional Declarations: No competing interests reported.

Abstract

Introduction: Identifying mosquito blood-meal sources and elucidating parasite transmission dynamics are essential for designing effective malaria control strategies. This study aimed to characterize the blood-meal origins of fed mosquitoes and to determine the prevalence of *Plasmodium* parasites in both the blood meals and salivary glands of mosquitoes collected from five malaria-endemic villages across three zones in southern Ethiopia.

Methods: *Anopheles* mosquitoes were collected monthly using indoor and outdoor CDC light traps, and pyrethrum spray catches (PSCs) from 10 randomly selected households per method in five villages across three zones. Samples were collected at each site six times between April 2022 and August 2023, at three months intervals. *Plasmodium*parasites and origins of the blood meals in the abdomens of fed *Anopheles* mosquitoes were determined using the nested polymerase chain reaction (PCR) technique. *Anopheles* mosquito heads and thoraxes were tested for *P. falciparum*, *P. vivax*-210, and *P. vivax*-247 circumsporozoite proteins (CSPs) using the Enzyme-Linked Immunosorbent Assay (ELISA).

Results: A total of 1,593 female *Anopheles* mosquitoes were collected, with *Anopheles gambiae s.l*comprising 98%. PCR screening of 510 blood-fed mosquito abdomens detected *Plasmodium* parasites in 18.1% (92/510) of the samples. *P. falciparum* was the most prevalent species, identified in 9.4% (48/510) of fed mosquitoes' abdomens, followed by *P. vivax* in 4.2% (21/510). Mixed infections involving both species were detected in 4.5% (23/510) of the samples. ELISA-based circumsporozoite protein detection identified CSPs in 0.25% (4/1,593) for *P. falciparum* and 0.44% (7/1,593) for *P. vivax*-210, with no mixed infections detected. Among the 510 blood-fed specimens, 84% (n = 427) had fed on one or multiple vertebrate hosts, including humans and domestic animals. Humans were the predominant blood source (64%; n = 326), followed by goats (55.3%; n = 282) and cows (12%; n = 60). Single-host meals accounted for 21% (human; n = 109), 7.5% (goat; n =38), and 5% (cow; n =25), with some mosquitoes feeding on three or four hosts. Human blood indices were 65% for indoor CDC light traps, 62% for pyrethrum spray catches, and 52% for outdoor CDC light traps.

Conclusions: These findings show that vectors frequently interact with malaria-infected hosts, leaving susceptible humans at continued risk of infectious bites. Effective control requires interventions that target both indoor and outdoor-feeding mosquitoes to minimize parasite transmission.

Background

Ethiopia is home for both *Plasmodium falciparum* and *Plasmodium vivax* malaria, with about 68% of the total population at risk of infection [1]. Significant progress was made in reducing the malaria burden between 2000 and 2016 [2]. However, the decline in malaria incidence has slowed since 2017 [3], and since 2023, it has surged at an alarming rate, placing Ethiopia among the countries with the highest rise in malaria cases [4]. Additionally, more areas were classified as endemic in 2022 than in 2017 [5].

Over 47 species of *Anopheles* mosquitoes have been recorded across the country so far [6, 7]; however, *Anopheles arabiensis* (*An. arabiensis*) is widely regarded as the principal malaria vector, accounting for the majority of malaria transmission [8, 9]. While *An. pharoensis* is the confirmed secondary vector, and *An. coustani* is an emerging vector showing susceptibility to *Plasmodium* infection and expanding its role in malaria transmission [10, 11]. The invasive Asian malaria vector, *An. stephensi*, has recently been documented from different parts of the country [12–14] and is reported to be susceptible to local *Plasmodium* infection [15].

In Ethiopia, major malaria control measures rely heavily on insecticide-based indoor interventions such as insecticidal-treated nets (ITNs) and indoor residual spraying (IRS), along with accessible point-of-care diagnosis and prompt treatment [1]. The IRS and LLINs remain the frontline public health interventions against infectious exposure responsible for the major reduction in malaria burden [16]. These intervention tools have been proven to be most effective against indoor biting and resting malaria vectors [17, 18]. However, the emergence and rapid spread of insecticide resistance and behavioural adaptations among vectors may reduce mosquito susceptibility, thereby reducing their public health impact [17, 19] and affecting the effectiveness of IRS and ITNs [3].

Residual malaria transmission persists in regions with extensive coverage of IRS and ITN coverage, underscoring the influence of vector behaviours and the need for supplementary vector control strategies to achieve transmission interruption [20]. *Anopheles arabiensis* exhibits diverse feeding and resting behaviours; it may bite people in the early evening before they go to bed [21–24]. This behavioural plasticity makes the species less susceptible to conventional control methods.

Achieving effective malaria control requires ongoing surveillance of the feeding behaviour of local malaria mosquitoes within a specific geographic area, which is vital for evaluating intervention effectiveness and developing tailored vector control strategies [25]. In addition, the resurgence of malaria cases across the country underscores the need for advanced surveillance tools to facilitate early detection and monitoring of malaria parasites in human populations [5].

Malaria surveillance has traditionally relied on symptomatic individuals seeking care at health facilities and, in some cases, on active screening of human populations for malaria parasites [26]. These data are essential for planning and implementing interventions to prevent further transmission. Molecular xenomonitoring offers an alternative approach by utilizing vectors to monitor and survey human malaria parasites [27]. Polymerase chain reaction (PCR) methods using mosquito abdomens can detect parasite DNA from recent blood meals and parasite

developmental stages in the mosquito gut. This approach likely indicates malaria infection in human hosts rather than identifying infectious mosquitoes. It is a noninvasive method to identify malaria parasites and serves as a proxy for human infection in malaria surveillance [28, 29]. This approach can also assess how effectively current malaria interventions prevent mosquito bites and parasite transmission. This study aimed to assess the prevalence of *Plasmodium* DNA in the abdomens of blood-fed *Anopheles* mosquitoes and to identify their blood meal sources in malaria-endemic villages across three zones of southern Ethiopia.

Materials and methods

Study area description

This study was conducted in selected districts in three zones of the South Ethiopia regional state: South Omo, Gamo and Wolaita (**Fig. 1**).

Salamago Woreda (equivalent to district), located at 5°32'-6°46' N and 35°81'-36°45' E, lies within the altitudinal range of 500 and 2500 meters above sea level (masl). The area has arid-to-sub-humid climatic conditions, with annual temperatures ranging from 20°C to 41°C and an average annual rainfall of approximately 977 mm.

Dasenech district spans between 4°45' and 4°99' N and 35°81' and 36°41' E, with an altitudinal range of 272 to 655 masl. The climate is predominantly arid, with annual average rainfall of 400-600 mm and annual mean temperature of 28°C to 42°C.

The Damot Weyde district is located approximately at 6°43’–7°33’ N and 37°28’–37°59’ E. The area features an altitude range of 1400 to 1750 masl. The average monthly temperature ranges from 17°C to 24°C and the average annual rainfall is about 904 mm.

Kindo Koysha district is located between 6°48’ and 7°04’ N and 37°24’ and 37°38’ E. Elevations range from 687 to 2164 masl. The area has a semi-arid climate, with average maximum and minimum temperatures of 30.7°C and 18.2°C, respectively, and annual rainfall of 400-800 mm.

Arba Minch is a rapidly growing town in southern Ethiopia, situated in the Rift Valley between 5°30' and 6°50' N and 37°32' and 37°36' E. The town lies at an altitude of 1200 to 1300 masl and experiences a hot, humid climate, with an average annual rainfall of 889.7 mm and a mean temperature of 23.9°C.

Plasmodium falciparum is most prevalent, and its transmission peaks mainly following the rainy seasons [29]. *Anopheles arabiensis* is the primary malaria vector in the region [9,30,31]. *Anopheles stephensi* is reported from Arba Minch [13], and its role in malaria transmission remains unknown. LLINs and IRS are the main interventions for controlling malaria vectors in the region [1].

Study design

A longitudinal entomological survey was conducted at approximately three-month intervals from April 2022 to August 2023. The selection of districts, villages, and households was conducted using a multistage sampling design. Briefly, from the twelve zones in the Southern regional state, three Zones were selected via purposive sampling: Wolaita, Gamo, and South Omo. From the three selected zones, two malarious districts per zone were purposively selected based on the zonal malaria reports, except for the Gamo zone, which included only one district. Based on malaria prevalence, one village (the lowest administrative unit in Ethiopia) was purposively selected from each district; five villages were included in the study.

Adult *Anopheles* mosquitoes were collected following the procedures described elsewhere[14]. Potential mosquito breeding habitats were identified before household selection, and a kilometre-by-kilometre grid was established around breeding sites. Four households were purposefully selected within each quadrant, and four additional households were randomly chosen in each direction, approximately 100 meters apart. Twenty households per village were included. If a selected household head was unavailable or declined, a nearby volunteer household was chosen as a replacement.

Mosquito sampling methods

Adult female *Anopheles* mosquitoes were collected using CDC light traps (Model 512; John W. Hock Company, Gainesville, Florida, USA) and pyrethrum spray catches (PSCs). Of the 20 households in each village, 10 were assigned to CDC light traps and 10 to PSCs. In the quadrant near breeding sites, two of four households used CDC light traps and two used PSCs. In each village, eight of sixteen households outside the quadrant were randomly assigned to either CDC light traps or PSCs.

The CDC light traps were installed indoors and outdoors in each household at intervals of 2 to 3 months. The indoor CDC light traps were hung approximately 1.5 m above the ground at the foot end of the a person (s) sleeping under the existing bed nets [32]. Outdoor CDC light traps were positioned approximately 5 m from the indoor collection house, at a height of 1.5 m above the ground. The traps were set up and run from 6:30 pm to 6:00 am. The collection team retrieved collection bags between 7:00 am and 8:00 am.

We collected indoor-resting *Anopheles* mosquitoes between 6:00 am and 9:00 am using PSCs, following the World Health Organization [33] standard protocol. The same households were used for mosquito collections throughout the study period.

Mosquito processing

All *Anopheles* mosquitoes collected with CDC light traps and PSCs were morphologically identified using a key [6]. Female *Anopheles* mosquitoes were sorted and placed in individually labelled Eppendorf tubes containing silica gel desiccant. Samples were transported to the Arba Minch University Advanced Medical Entomology and Vector Control Laboratory for storage at -20°C, pending analysis for blood meal source identification, circumsporozoite protein assays, and detection of malaria parasites in the abdomens of fed mosquitoes.

Determination of blood meal origins

Blood meal identification was performed on all fed female *Anopheles* mosquitoes using a multiplex PCR assay targeting host mitochondrial cytochrome b DNA gene, as described by Kent and Norris [34]. Genomic DNA was extracted from each abdomen using the CTAB protocol [35]. PCR products were visualized on a 2% agarose gel (Benchmark Scientific, USA), and blood meal sources were identified by the specific band sizes produced by each primer.

Plasmodium DNA detection in the fed mosquito's abdomen

Nested PCR targeting the 18S rRNA gene was used to detect *P. falciparum* and *P. vivax* in fresh-fed and half-gravid *Anopheles* mosquitoes following the protocol described by Snounou and Singh [36]. In the first step, *Plasmodium*-specific primers were used to detect the parasite. In the second step, species-specific primers identified *P. falciparum* and P. vivax. PCR amplification was conducted using a Biometra thermocycler (the Netherlands). Positive DNA samples of *P. falciparum* and *P. vivax* were included as positive controls in each PCR run, and PCR-grade water served as the negative control.

Plasmodium sporozoites detection

All female Anopheles mosquitoes were individually tested for Plasmodium falciparum, P. vivax-210, and P. vivax-247 circumsporozoite proteins (CSPs) using a sandwich ELISA [37].

Data analysis

All data were entered and cleaned in Microsoft Excel 2010 and then exported to R (version 3.51) for analysis (R Core Team, 2024). The human blood index (HBI) was calculated as the proportion of *Anopheles* mosquitoes that fed on humans among the total number tested for blood meal origin [38]. The blood meal indices of other vertebrate hosts were also determined in a similar way. Mixed-blood meals were included in the calculation of blood meal indices [39].

The sporozoite rate (SR) was estimated as the fraction of *Anopheles* species detected positive for *P. falciparum and P. vivax* CSPs over the total samples tested [40]. The annual entomological inoculation rate (EIR) for *Anopheles* mosquitoes was calculated from CDC light traps using the formula 1.605 × (number of CSPs-positive ELISA results from CDC light traps/number of mosquitoes tested) × (number of mosquitoes collected from CDC light traps/number of trap nights) × 365 days [32].

Results

Anopheles mosquito species composition

A total of 1,593 female *Anopheles* mosquitoes were collected during the study period: 789 (49.5%) from Belle, 590 (37%) from Billate, 151 (9.5%) from Hana, 45 (2.8%) from Arba Minch town, and 18 (1.1%) from Omorate (Table 1).

Anopheles gambiae s.l. was the predominant species across all study areas, accounting for 98.2% (n = 1,565). The remaining 1.8% comprised *An. pretoriensis*, *An. pharoensis*, and *An. parensis*. *An. gambiae* s.l. was found at all sites. *Anopheles pharoensis* was identified at Billate and Hana, while *An. parensis* and *An. pretoriensis* were collected at Arba Minch town and Belle, respectively (Table 1).

Of the 1,419 *Anopheles* mosquitoes collected using CDC light traps, 1,210 (85.3%) were captured indoors (Table 1). Only 174 (10.9%) mosquitoes were sampled using the PSC method. Overall, 92.8% (375 of 404) of fresh-fed mosquitoes were collected indoors.

Table 1. Species composition of *Anopheles* mosquitoes by collection methods across the five study sites in South Ethiopia.

Sites	Species	CDC light trap						PSC			Total (%)
		Indoor			Outdoor						
		UF	FF	HG	UF	FF	HG	UF	FF	HG	
Belle	*An. gambiae s.l.*	431	150	30	54	13	0	34	43	12	767 (97.2)
	An. pretoriensis	5	0	0	16	1	0	0	0	0	22 (2.8)
Billate	*An. gambiae s.l.*	300	117	31	71	8	4	22	30	5	588 (99.7)
	An. pharoensis	2	0	0	0	0	0	0	0	0	2 (0.3)
Arba Minch	*An. gambiae s.l.*	25	3	2	11	3	0	0	0	0	44 (97.8)
	An. parensis	0	0	0	1	0	0	0	0	0	1 (2.2)
Omorate	*An. gambiae s.l.*	3	0	0	0	0	3	9	1	2	18 (100)
Hana	*An. gambiae s.l.*	72	23	13	16	4	4	9	7	0	148 (98)
	An. pharoensis	2	1	0	0	0	0	0	0	0	3 (2)
Total		840	294	76	169	29	11	74	81	19	1593

Keys: CDC: Centers for Disease Control; PSC: Pyrethrum spray catches; UF: unfed; FF: Fresh-fed; HG: Half-gravid.

Detection of *Plasmodium* DNA in fed *Anopheles* mosquitoes

Our nested PCR analysis revealed that 18% (92/510) of the fed *An. gambiae* s.l. had *Plasmodium* parasite DNA in their abdomens. Of these, *P. falciparum* was detected in 9.4% (48/510), *P. vivax* in 4.1% (21/510), and mixed infections in 4.5% (23/510). The overall prevalence of *Plasmodium* infection varied across study sites, ranging from 0% in Arba Minch and Omorate to 20.5% in Belle (Table 3).

Table 2 PCR-based *Plasmodium* infection rates in the abdomen of blood-fed *Anopheles* mosquitoes collected from five malarious sites in South Ethiopia.

Sites	Number tested		Number of positive mosquitoes (%)						Overall prevalence, n (%)
	Indoor	Outdoor	Indoor			Outdoor			
			Pf	Pv	Pf + Pv	Pf	Pv	Pf + Pv	
Belle	235	14	19	13	15	1	1	2	51 (20.5)
Billate	183	12	25	7	6	0	0	0	38 (19.5)
Arba Minch	5	3	0	0	0	0	0	0	0 (0)
Omorate	3	3	0	0	0	0	0	0	0 (0)
Hana	44	8	3	0	0	0	0	0	3 (5.8)
Total	470	40	47 (10)	20 (4.3)	21 (4.5)	1 (2.5)	1 (2.5)	2 (5)	92 (18)

Key: PF = *Plasmodium falciparum*, Pv= *Plasmodium vivax*

Regarding the collection method, 20.3% (75/370) of *Plasmodium*-positive *An. gambiae* s.l. collected by indoor CDC light traps, 10% (4/40) by outdoor CDC light traps and 13% (13/100) from PSC, but there was no statistically significant difference (Fig. 2).

Circumsporozoite protein and entomological inoculation rates

Of 1,593 *Anopheles* mosquitoes screened for *Plasmodium* circumsporozoite proteins (CSPs) by ELISA, 0.69% (11 specimens), all *An. gambiae* s.l., tested positive. Four were positive for *P. falciparum* and seven for *P. vivax*-210.

CSP-positive *An. gambiae* s.l. were collected exclusively using CDC light traps. In Belle, 0.88% (6/678) of mosquitoes tested positive for *Plasmodium* CSPs, with three (0.44%) identified as *P. falciparum* and three (0.44%) as *P. vivax*. In Billate, 0.56% (3/531) of CSP-positive *An. gambiae* s.l. were found, including one (0.19%) *P. falciparum* and 2 (0.38%) *P. vivax*. In Hana, the CSP rate for *An. gambiae* s.l. was 1.52% (2/132), both identified as *P. vivax*. No *Anopheles* mosquitoes from Arba Minch town or Omorate tested positive for CSPs, and no mixed CSP infections were detected in all sites.

The daily EIR for *An. gambiae* s.l. was 0.096 infectious bites per person per night in Belle, 0.048 in Billate, and 0.032 in Hana. The annual EIR varied from 0.00 in Omorate and Arba Minch town to 35.04 in Belle (Table 3).

Table 3: Overall circumsporozoite proteins and entomological inoculation rates of *Anopheles gambiae* s.l. collected by CDC light traps in South Ethiopia.

Site	Number tested	CSPs infection rate n (%)	Daily EIR	Annual EIR
Belle	678	6 (0.88)	0.096	35.04
Billate	531	3 (0.56)	0.048	17.52
Arba Minch	44	0 (0.00)	0.00	0.00
Omorate	18	0 (0.00)	0.00	0.00
Hana	132	2 (1.52)	0.032	11.68
Overall	1391	11 (0.79)	0.035	12.78

Key: CSP =Circumsporozoite proteins, EIR = Entomological inoculation rate

Table 4 presents the indoor and outdoor EIR of malaria vectors at the study sites. The number of EIR varied between sites and between indoor and outdoor locations. The overall yearly EIR of *An. gambiae* s.l was higher indoors (11.67 infectious bites/person/year) than outdoors (2.61infectious bites/person/year). Belle had more infectious bites than the other sites, with 36.5 infectious bites/person/year indoors and 7.3 infectious bites/person/year outdoors.

Table 4. The circumsporozoite protein infection and entomological inoculation rates of *Anopheles gambiae s.l* collected indoors and outdoors across study sites in South Ethiopia

Site	Collection location	Mosquitoes tested (n)	Sporozoite positive	Sporozoite rate, %	No catches	Daily EIR	EIR annual
Belle	In	611	5	0.82	50	0.1	36.5
	Out	67	1	1.19	50	0.02	7.3
Billate	In	448	3	0.67	50	0.06	21.9
	Out	83	0	0	50	0	0
Arba Minch	In	30	0	0	55	0	0
	Out	14	0	0	55	0	0
Omorate	In	3	0	0	60	0	0
	Out	3	0	0	60	0	0
Hana	In	108	1	0.9	65	0.0154	5.621
	Out	24	1	4.17	65	0.0154	5.621
Total	In	1200	9	0.75	280	0.032	11.73
	Out	191	2	1.05	280	0.0072	2.61
	Overall	1391	11	0.79	560	0.0196	7.16

Blood meal origins of *Anopheles* mosquitoes

Figure 3 presents the overall blood meal indices of *Anopheles* mosquitoes in the study area. Fed (n = 404) and half-gravid (n = 106) *Anopheles* mosquitoes were tested for blood meal sources using multiplex PCR. Blood meal analysis showed that 83.7% (n = 427) had fed on one or more vertebrate hosts, including humans, goats, cows, sheep, dogs, pigs, and horses. The remaining mosquitoes could not be identified. Among those identified, 34.3% (n = 175) fed on a single host, while 49.4% (n = 252) fed on multiple hosts. Humans, goats, and cows were the most common sources, accounting for 63.9%, 55.3%, and 11.8% of blood meals, respectively. A smaller proportion of mosquitoes had blood from three or four different vertebrates in their abdomens.

Anopheles gambiae s. l. infected with *P. falciparum* (93.8%, 45/48), *P. vivax* (81%, 17/21), or mixed infections (100%, 23/23) parasites DNA fed on human blood, either exclusively or multiple vertebrate hosts. In addition, 9.5% (2/21) of *P. vivax*-infected mosquitoes tested positive for goat and cow blood. Blood meal origins of three *P. falciparum* and two *P. vivax*-infected mosquitoes were not identified.

Table 2 presents the blood meal indices of *Anopheles* mosquitoes across the five study sites. Most of the *Anopheles* mosquitoes from indoor (65.4%) and outdoor (52.5%) CDC light traps had blood meals from humans, followed by goats. The majority (73%) of indoor resting *Anopheles* mosquitoes had human blood, followed by goat blood.

Table 5. Blood meal indices of *Anopheles* mosquitoes across the five malarious sites in South Ethiopia

Sites	Species	CDC light trap								PSC			
		Indoor				Outdoor							
		N	HBI	GBI	BBI	N	HBI	GBI	BBI	N	HBI	GBI	BBI
Belle	*An. gambiae s.l.*	180	59.4	72.2	12.8	13	46.2	69.2	0	55	54.5	74.5	20
	An. pretoriensis	0	0	0	0	1	0	100	0	0	0	0	0
Billate	*An. gambiae s.l.*	148	69.6	36.5	10.8	12	58.3	33.3	16.7	35	74.3	48.6	17.1
Arba Minch	*An. gambiae s.l.*	5	100	60	0	3	100	100	0	0	0	0	0
Omorate	*An. gambiae s.l.*	0	0	0	0	3	33.3	66.7	33.3	3	0	33.3	0
Hana	*An. gambiae s.l.*	36	72.2	27.8	0	8	50	12.5	12.5	7	100	71.4	0
	An. pharoensis	1	100	100	0	0	0	0	0	0	0	0	0
Overall		370	65.4	53.5	10.5	40	52.5	50	10	100	73	32	17

HBI: Human blood meal index; GBI: Goat blood meal index; BBI: Bovine blood meal index

Discussion

A substantial proportion of Anopheles mosquito abdomens contained *Plasmodium* DNA, indicating frequent contact with infected humans and confirming ongoing malaria transmission at the study sites. Blood meal analysis further revealed that these vectors feed on a wide range of hosts, particularly in humans and goats. This finding underscores the potential utility of xenomonitoring in assessing malaria transmission dynamics.

Of the engorged *An. gambiae* s.l mosquitoes collected from five malaria-endemic sites and analyzed by nested PCR, 18% tested positive for *P. falciparum*, *P. vivax,* or mixed parasite DNA. Previous studies in Ethiopia reported parasite prevalence rates ranging from 2.2% to 4.6% [24,41]. Parasite DNA detected in fed mosquitoes may originate from the most recent human blood meal or from a previous feeding on an infected person, as parasite DNA remains detectable throughout several mosquito developmental stages. The parasite develops to the oocyst stage in the vector's abdomen [42].

The high prevalence of *Plasmodium* DNA in the abdomens of fed *Anopheles* mosquitoes indicates substantial human exposure to mosquito bites and ongoing parasite transmission. When using this measure as a proxy for human malaria prevalence, two main factors must be considered. First, a single human can infect multiple mosquitoes, potentially leading to an overestimation of malaria prevalence in humans. Second, a mosquito may infect several individuals if its feeding is interrupted or if parasites increase its likelihood of biting multiple hosts simultaneously or over time, potentially leading to underestimation. Repeated blood feeding on different individuals also increases the number of sporozoites injected, maintains infectivity, and enhances parasite transmission [43]. Despite these limitations, parasite prevalence in the abdomens of fed mosquitoes can be used as a proxy for human prevalence [28]. The ease of collecting *Anopheles* mosquitoes and detecting malaria parasites in their abdomens provides a practical, non-invasive method for monitoring active parasite circulation in human populations. Using this approach as an early warning and surveillance tool can support timely, evidence-based decisions and the implementation of targeted malaria control interventions.

Although most *Plasmodium*-positive samples contained human blood, some mosquitoes had blood meals from non-human hosts and from unidentified sources. This indicates that the parasite's DNA may persist in the mosquito's midgut, likely originating from the oocyst stage, which takes a longer time to mature than other stages in the parasite's life cycle within its vector [42,43]. *Plasmodium falciparum* and *P. vivax* mixed parasite infections were identified in some fresh-fed mosquitoes. These mosquitoes might have fed on a human host with a mixed infection or on multiple individuals carrying different parasite species in the same feeding cycle or at different times [44,45], given that both *P. falciparum* and *P. vivax* are endemic in the study region. Unfortunately, none of the mosquitoes tested for CSP showed mixed infections of both parasites in the salivary glands. Although a few experimental studies report efficient transmission of *P. falciparum* and *P. vivax* to mosquitoes [46], there is little or no field evidence [45]. Future research should examine why parasites found in mosquito abdomens do not develop into infectious stages in the mosquito's salivary glands.

Anopheles gambiae s.l. feeds on a diverse range of blood meal sources, including humans, goats, cows, sheep, dogs, pigs, and horses. Such diverse blood-feeding behaviour of *Anopheles* mosquitoes has been documented in the region [23,24]. Humans are the most common blood meal source of *Anopheles* mosquitoes in the area, followed by goats and cows. This elevated human exposure likely increases the risk of human contact with

infectious mosquitoes, thereby potentially intensifying malaria transmission. A significant proportion of meals from multiple mixed-blood sources suggests interrupted feeding, likely due to defensive host behaviour or parasite-driven vector manipulation. Mixed blood meals in *Anopheles* mosquitoes have already been documented in the country [24,47]. In contrast, other studies found that *An. arabiensis* primarily feeds on bovine blood, indicating zoophagy [22,48,49], as determined by ELISA. Additionally, opportunistic feeding behaviour has been reported in other research [21,50]. Detecting malaria parasite DNA in the abdomens of fed *Anopheles* confirms active transmission and frequent human-vector contact. Additional human data further validates these results.

Conclusions and recommendations

A significant proportion of freshly fed *Anopheles* mosquitoes carried *Plasmodium* DNA in their abdomens, confirming ongoing transmission. The elevated rate of infectious contact between humans and vectors is notable, with humans and goats identified as the primary blood meal sources. It is recommended to enhance access to infection prevention measures and to strengthen community behavioural change communications to promote the use of prevention tools.

Abbreviations

BBI: Bovine blood index

CDC: Center for disease control and prevention

CSP: Circumsporozoite protein

DNA: Deoxyribonucleic acid

EIR: Entomological inoculation rate

ELISA: Enzyme-linked immunosorbent assay

GBI: Goat blood meal index

HBI: Human blood index

IRS: Indoor residual spraying

ITNs: Insecticide-treated nets

PCR: Polymerase chain reaction

PSC: Pyrethrum spray catch

WHO: World Health Organization

Declarations

Acknowledgements

We thank the household heads for permitting mosquito collection. We also appreciate the zonal and district health departments for their cooperation and assistance in community communication.

Authors contributions

T.A., Y.A.E., E.G. and F.M.: Conceptualization. U.K., Y.T. and F.M.: Data curation and formal analysis. S.C., B.L., E.G. and F.M.: Funding acquisition. U.K., T.A. and A.K.: investigation. N.E.: Laboratory analysis. T.A., Y.A.E., E.G. and F.M.: Methodology. B.L., E.G. and F.M.: Supervision. U.K. and FM.: Writing draft manuscript. U.K., T.A., N.E., A.K., N.N., Y.T., Y.A.E., SC., B.L., E.G. and F.M.: Writing, reviewing and approving final draft. All authors have reviewed and approved the final version for submission.

Funding

The Norwegian Programme for Capacity Development in Higher Education and Research for Development (QZA-21/0162) funded the laboratory analysis. The Armauer Hansen Research Institute (AHRI) through funding obtained from government supported fieldwork.

Availability of data and materials

Most data are included in the manuscript. The data file is also provided as a supplementary file.

Declarations

Ethical approval and consent to participate

Ethical approval for the study was obtained from the AHRI/ALERT ethics committee (PO/16/21) and the National Research Ethics Review Committee (NRERC) of the Federal Ministry of Education of Ethiopia (Reference: 7/1-229/ m259/35). The objectives of the study were clearly communicated to the heads of the households at each study site prior to recruitment. Verbal consent was obtained as authorization to conduct PSCs or CDC light-trap mosquito collections in their compounds.

Consent for publication

Each author contributed to the preparation of the manuscript and approved the final version for submission.

Competing interests

The authors declare no competing interests

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Figures

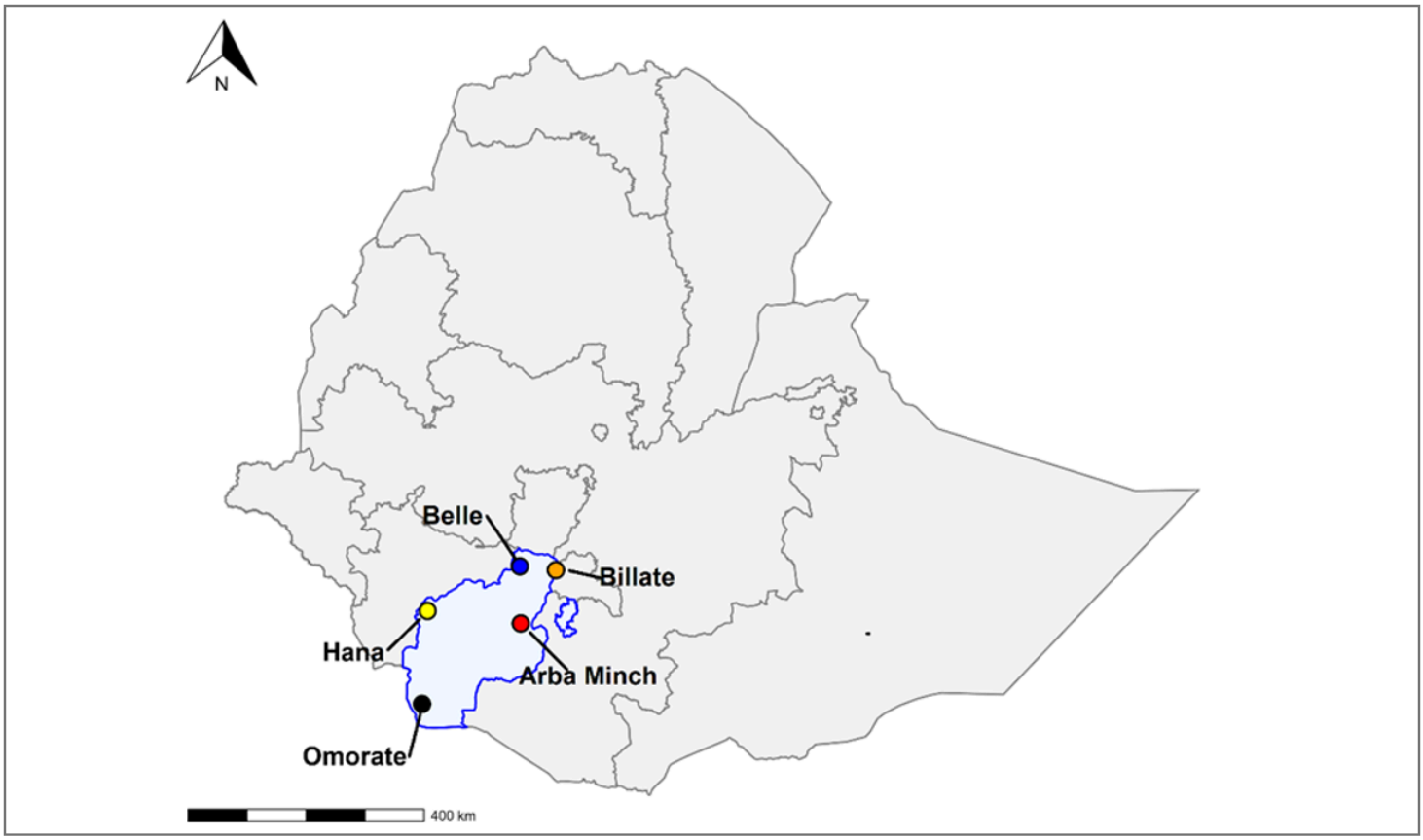

Figure 1

Map of the study sites in South Ethiopia

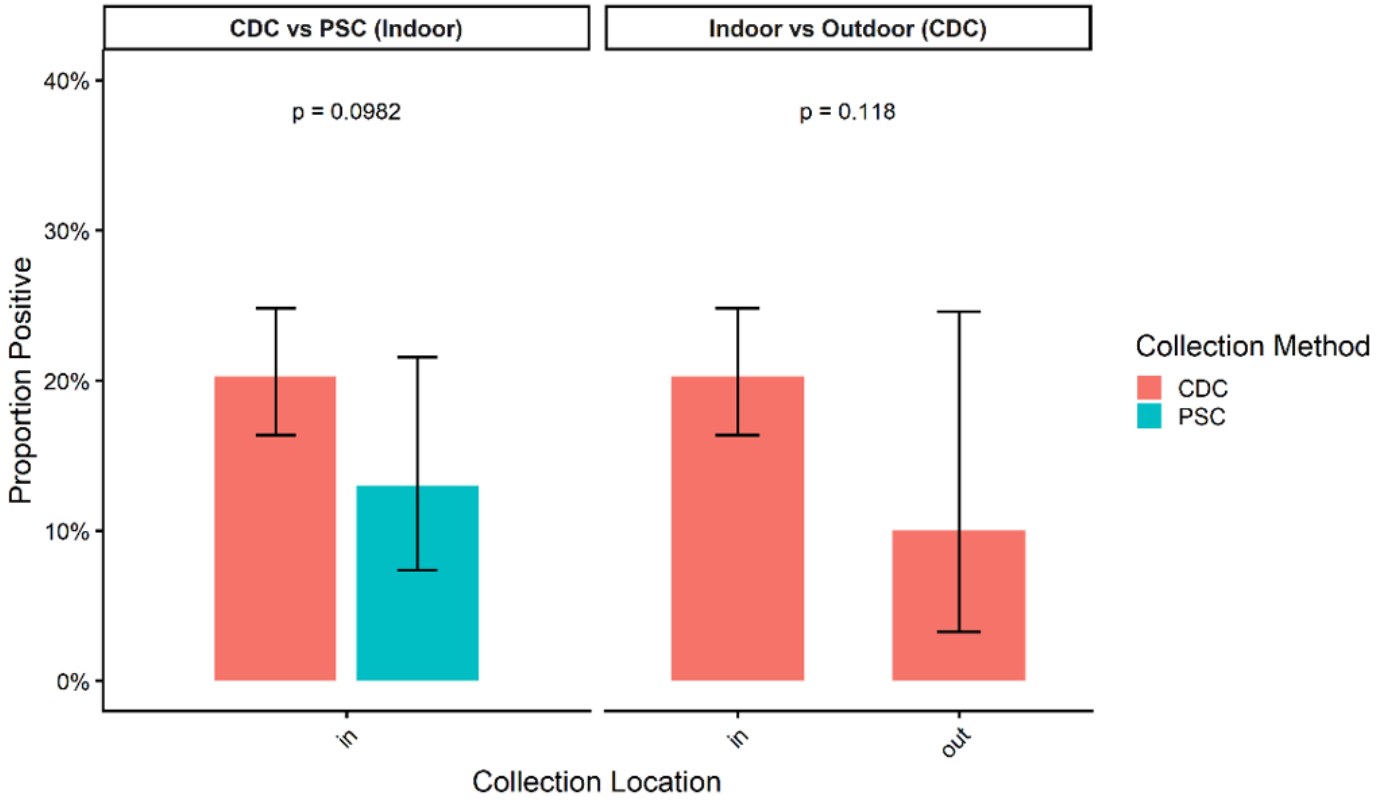

Figure 2

Proportion of blood-fed *Anopheles gambiae* s.l. mosquitoes testing positive for *Plasmodium*parasite DNA by the collection method in malaria-endemic sites of South Ethiopia.

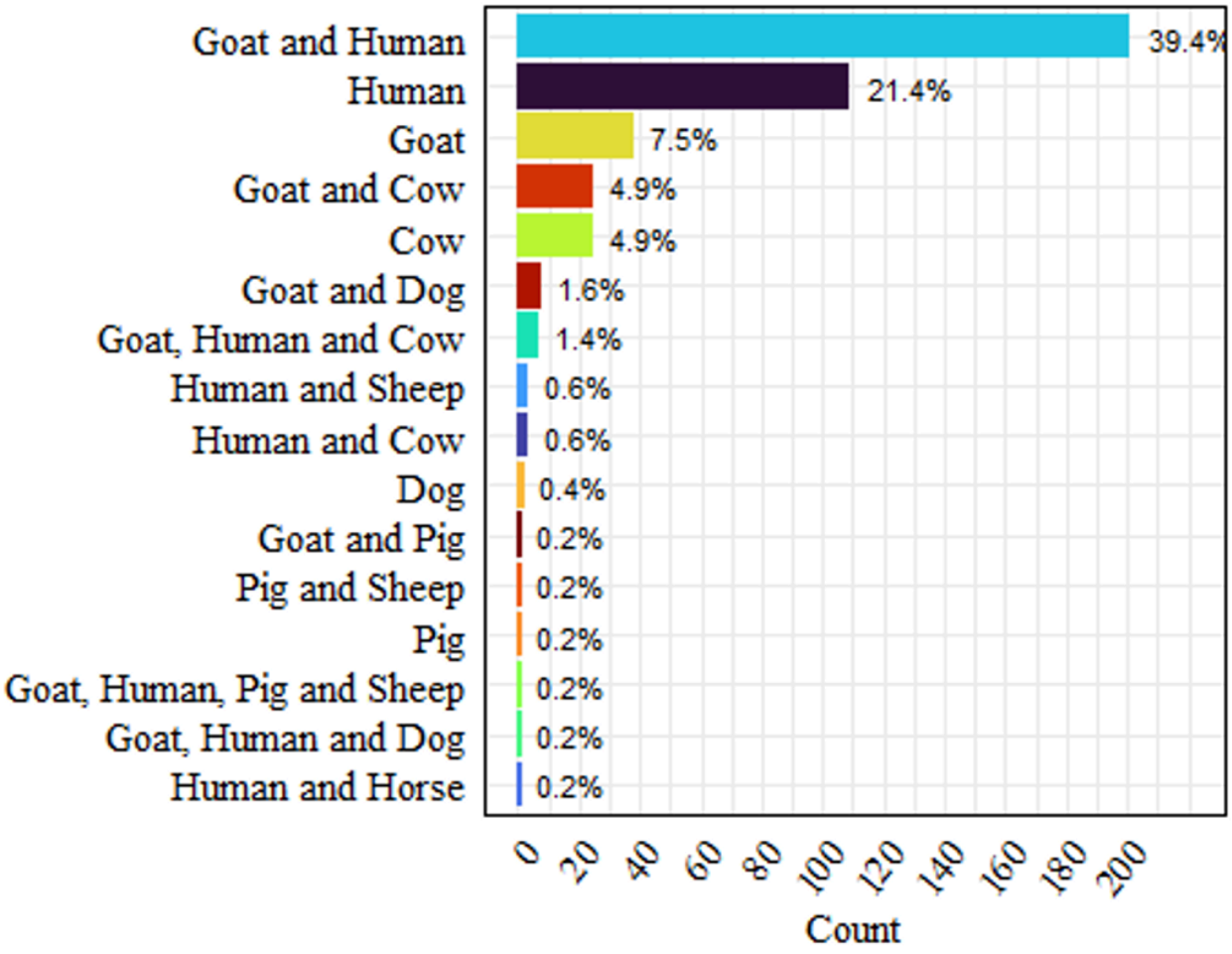

Figure 3

The number and percentage of blood meal sources of *Anopheles* mosquitoes collected across study sites in South Ethiopia

Supplementary Files

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- Supplementaryfiledata.xlsx