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Blood meal source of field-caught *Anopheles* vector mosquitoes of malaria from Northern Sri Lanka

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Abstract Host preference in blood-feeding anopheline mosquitoes is crucial in determining malaria vectors. Using a PCR assay that targeted a portion of the Cytochrome b gene of five vertebrates, this study sought to investigate the blood meals of 30 field-caught *Anopheles* mosquitoes from two Northern Districts of Sri Lanka. Study revealed that blood meals of *Anopheles culicifacies* and *Anopheles subpictus* mosquitoes were exclusively from humans (23.3%) and goats (50%), and the remaining 26.6% were mixed blood meals. *An. culicifacies* (0.65) was more anthropophilic than *An. subpictus* (0.31), based on the Human Blood Index. Single-host blood meals were more common in both *An. culicifacies* (70.6%) and *An. subpictus* (76.92%) than multiple-host blood meals. A significant association ($p<0.05$) was observed between the vertebrate source of the blood meal of the mosquito and the frequency of single and multiple-host blood meals taken. While the conventional multiplex PCR assay was more successful in identifying the source of multiple-host blood meals, the nested PCR technique effectively amplified the DNA of single-host blood meals. The mean DNA concentration of mixed-host blood meal extractions amplified by conventional PCR assay (121.8±37.3 ng/µL) was substantially greater ($p<0.05$) than that of the nested PCR (39.5±29.7 ng/µL). This study reveals important findings which are significant for further exploration into the vectorial capacity of *An. culicifacies* and *An. subpictus*, and consequently their transmission potential as malaria vectors in the area. This understanding of the feeding patterns of *Anopheles* vectors will be helpful for long-term planning and development of suitable, situation-specific malaria control measures in a country.

Key words: *Anopheles*, Blood meal, Human Blood Index, Malaria, Mosquitoes

Introduction

Malaria is naturally transmitted by human interaction with *Plasmodium* parasites and *Anopheles* mosquito vectors. Although Sri Lanka was declared malaria-free by the World Health Organization (WHO) in 2016, reports of imported malaria cases persist within the nation because of travel abroad and population migration to and from malaria-endemic regions of the globe (Anti Malaria Campaign [AMC], 2023). *Anopheles culicifacies* is the primary vector of malaria in Sri Lanka (Amerasinghe et al., 1999a). Secondary malaria vectors include *An. subpictus*, *An. annularis*, *An. varuna*, *An. vagus*, *An. tessellatus*, and *An. stephensi*.

An understanding on host preference patterns in blood-feeding anopheline mosquitoes is essential for determining malaria vectors, and several studies from around the world report on blood meal preferences of *Anopheles* mosquitoes (Bashar et al., 2012, Gunathilaka et al., 2016, Adugna et al., 2021, Jeyaprakasam et al., 2022). These studies demonstrate that a wide variety of vertebrates, including humans, wild boars, dogs, monkeys, cattle, cats, pigs, goats, and chickens, serve as the blood meal source of *Anopheles* mosquitoes. Additionally, it has been documented that, during a gonotrophic cycle, *Anopheles* may take a blood meal from a single host or from multiple hosts (Amerasinghe and Amerasinghe, 1999b, Muriu et al., 2008, Jeyaprakasam et al., 2022). Numerous factors influence a mosquito species' potential to transmit human illnesses, such as mosquito population density, the natural incubation period of each pathogen, the probability of daily vector survival, and host-seeking behaviour, (Amerasinghe et al., 1999b).

Although the capacity of *Anopheles* mosquitoes to spread malaria is dependent on their blood-feeding and host-seeking behaviours, only a few

studies report on these aspects with regards to *Anopheles* species in Sri Lanka (Amerasinghe and Amerasinghe, 1999b, Jude et al., 2014, Gunathilaka et al., 2016). Moreover, no recent reports are available to the best of our knowledge. In host preference investigations, precise identification of a vector species' blood meal source can be difficult. Frequently, the source of blood meal of hematophagous mosquitoes has been identified using serological techniques (Amerasinghe and Amerasinghe, 1999b, Zhang et al., 2017, Gonçalves et al., 2023). Some drawbacks of these techniques are their high assay costs, low sensitivity, and requirement for producing high-quality antisera against every species tested. To get around these problems, very sensitive molecular techniques are currently utilised for mosquito blood meal source identification (Borland and Kading, 2021). Therefore, this study used a low-cost PCR assay to determine the blood meal source of *Anopheles* mosquitoes collected from two chosen study locations in the Northern Province of Sri Lanka. It also explored their feeding patterns by investigating whether they tended to take blood meals from one host or several hosts over the gonotrophic cycle.

Materials and Methods

This was a cross-sectional exploratory study. Sentinel sites in the Kilinochchi and Jaffna districts were selected as the study sites (Figure 1). These two districts were selected based on past malaria vulnerability and the presence of *Anopheles* mosquitoes. The study included blood-fed mosquitoes collected by the Anti Malaria Campaign (AMC), Sri Lanka, from 30 houses in each area from August to October 2023.

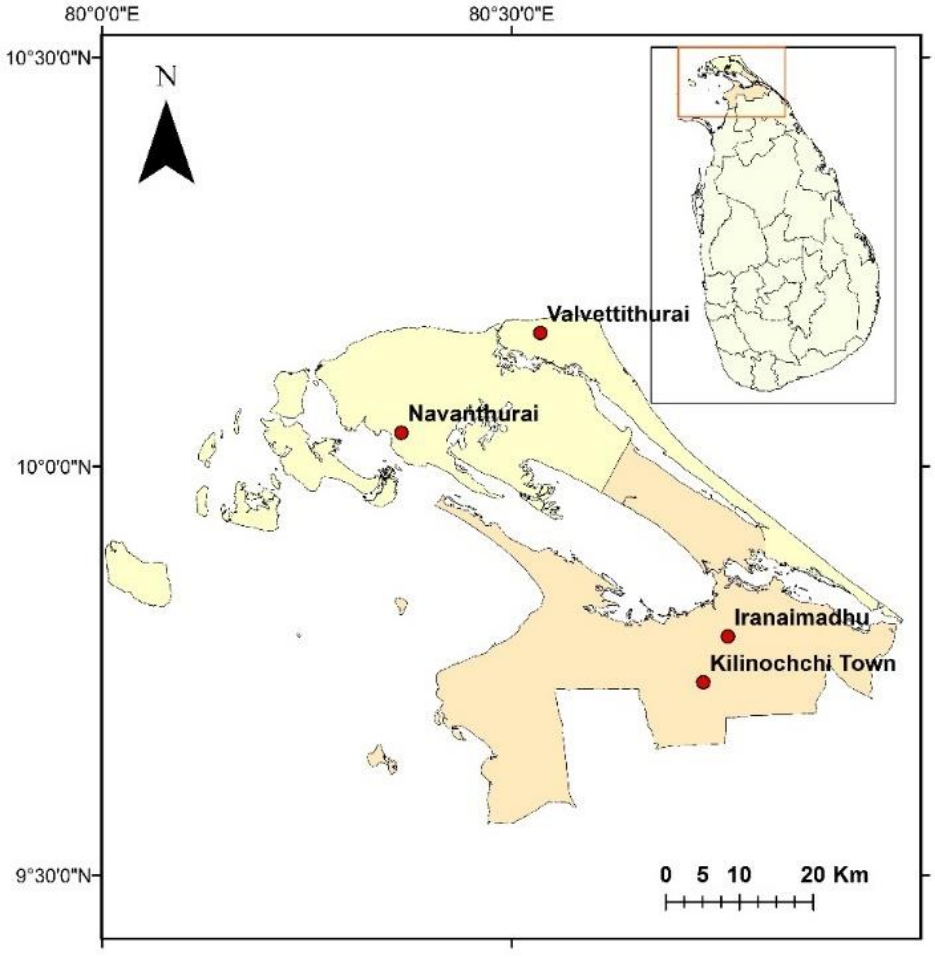

Figure 1: Map showing the study sites in Kilinochchi and Jaffna districts of Sri Lanka.

Mosquito blood meal collection and processing

The entomological assistants of the AMC, Sri Lanka, collected blood-fed female mosquitoes that were resting on different surfaces (cadjan roofs, walls and hanging clothes) inside houses during the morning hours between 6:00 and 8:30 am, using the indoor hand collection (IHC) method as per standard protocols (AMC, 2016). Around five to ten minutes were spent at each house. Female anopheline species were morphologically identified to the species level using taxonomic keys for Sri Lankan anophelines (Gunathilaka, 2017). The blood of each engorged female mosquito was spotted on a Whatman filter paper, carefully separating out the biological material of the mosquito from the blood spot. A reference number, the species, and the date and time of collection were listed on the labels of the filter papers.

DNA extraction and PCR assay

The DNA was extracted using a 5% Chelex 100 solution (Bio-Rad, USA). The concentration of DNA in each extraction was quantified using the Nanodrop spectrophotometer (Nanodrop 2000c, USA). The extracted DNA samples were stored at 4 °C until PCR analysis. The DNA was first processed in a multiplex conventional PCR assay targeting the mitochondrial DNA Cytochrome b gene with vertebrate-specific primers to detect the presence of human, goat, cow, dog, and pig host DNA in the blood meal (Kent and Norris, 2005). The final 25 µL reaction volume included the following: 12.5 µL of 1x GoTaq Green Master Mix (Promega), 2.5 µL of forward and reverse primers (2.5 µM each), 5 µL of nuclease-free water, and 5 µL of DNA (~50 ng). The PCR programme used was: 95 °C for 3 minutes, followed by 35 cycles of 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, with a final extension of 72 °C for 10 minutes. The PCR products were visualised on a 2% agarose gel. One µL of PCR products from the first round were reamplified by a nested PCR assay using the same PCR programme.

Data analysis

The data were analysed using Microsoft Excel and Minitab software. A DNA band of expected size on the agarose gel was taken as positive for the respective vertebrate blood in the mosquito blood meal tested. The percentage of blood meals positive by PCR for each vertebrate host, for each *Anopheles* species, and by study site were calculated. The Human Blood Index (HBI), defined as the proportion of blood meals that mosquito vectors obtained from humans (Garrett-Jones, 1964), was calculated as follows;

$$\text{HBI} = \frac{\text{Number of mosquitoes containing human blood}}{\text{Total number of mosquitoes analysed}}$$

Blood meals of *Anopheles* mosquitoes that were identified to have fed on a single-host or more than one type of host were categorised as single-host and multiple-host blood meals, respectively. A chi-square test was used to examine whether mosquitoes taking a blood meal from a specified vertebrate host tended to take single-host or multiple-host blood meals and whether there was any significant difference (at $p<0.05$) in the number of blood feeds taken between *An. culicifacies* and *An. subpictus* mosquitoes. Student's t-test was used to analyse the success rate of PCR amplifications with respect to mean DNA concentrations in single and multiple-host blood meals to find any variations in relation to conventional and nest PCR assays.

Results

PCR identification of the source of the host blood meal

During the study period, 30 blood meals of *An. culicifacies* (n = 17) and *An. subpictus* (n = 13) female mosquitoes were collected from Kilinochchi and Jaffna. The source of host DNA in the blood meals of all 30 mosquitoes was successfully identified by PCR (Figure 2).

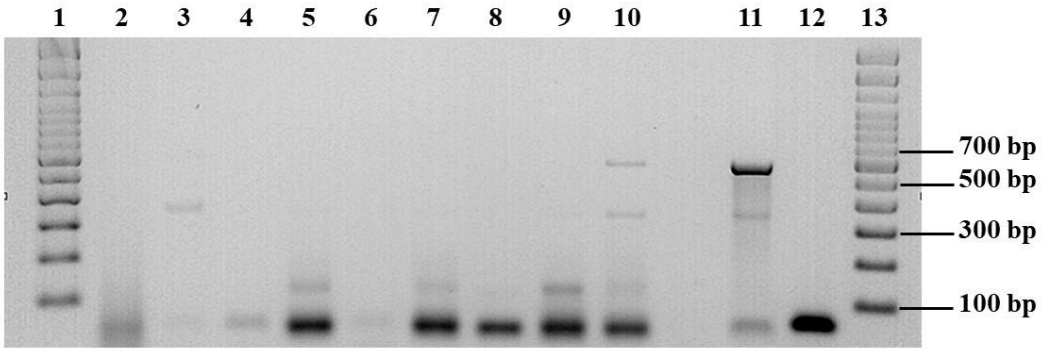

Figure 2: An agarose gel showing host-specific cytochrome b PCR products amplified from some of the *Anopheles* blood meal DNA extractions.

Products amplified from human (334 bp), cow, (561 bp), and goat, (132 bp) are shown. Lanes 1 to 13: Conventional PCR amplification of *An. culicifacies* (2–4) and *An. subpictus* (5-9) blood meal DNA; Nested PCR amplification of *An. subpictus* (10) blood meal DNA; Positive control with known human and cow DNA (11); Negative control (12); 100 bp DNA ladders (1 and 13)

Out of the total blood meals (Table 1), 07 (23.3%) were exclusively human blood meals, 15 (50%) were solely goat blood meals, and 08 (26.6%) were mixed blood meals. None of the mosquito blood meals contained pig or dog DNA. Blood meals of *An. culicifacies* were positive for humans (35.3%), goats (35.3%), and mixed blood meals (29.4%). The majority of blood meals from *An. subpictus* were identified as solely from goats (69.23%), followed by human only blood meals (8%) and mixed blood meal sources (23%).

The HBI of *An. culicifacies* was 0.65, with eleven out of the total 17 blood meals analysed showing human DNA. Comparatively, *An. subpictus* had a lower HBI (0.31), with only four out of the total 13 specimens tested demonstrating the presence of human DNA in their blood meal.

Table 1: Blood meal sources of field-caught *Anopheles* vectors.

Host	**Number of blood meals positive by PCR**						
	An. culicifacies			***An. subpictus***			**Total**
	K (n=17)	**J (n=0)**	**Total (n=17)**	**K (n=4)**	**J (n=9)**	**Total (n=13)**	**(n=30)**
Single-host blood meals							
Human	6	0	6	1	0	1	7
Goat	6	0	6	1	8	9	15
Multiple-host blood meals							
Human + Cow	4	0	4	1	0	1	5
Human + Goat	0	0	0	0	1	1	1
Human + Cow + Goat	1	0	1	1	0	1	2
Total positive blood meals	17	0	17	4	9	13	30
Human Blood Index (HBI)	0.65			0.31			

K: Kilinochchi; J: Jaffna

Blood meal source of Anopheles species according to sampling location

An. subpictus mosquitoes (n = 13) were collected from both Kilinochchi and Jaffna sites, but the majority (n = 9) of them were from the latter location. *An. culicifacies* blood fed mosquitoes (n = 17) were sampled only from Kilinochchi. Both *An. culicifacies* and *An. subpictus* collected from Kilinochchi had consumed blood meals from humans, goats, and cows (Figure 3). At this location, the most frequent blood meal source for *An. culicifacies* (47.83%) and *An. subpictus* (42.86%) species was humans. *An. subpictus* collected from Jaffna showed blood meals from only two hosts; the majority had fed on goats (90%), with a small proportion (10%) showing human blood.

When the host feeding pattern of *An. subpictus* from the two sampling locations was compared, the proportion of *An. subpictus* that fed on goat at Jaffna was higher (90%) compared to the *An. subpictus* mosquitoes at Kilinochchi (28.57%). Whereas the proportion of *An. subpictus* that fed on human hosts at Kilinochchi was higher (42.86%) compared to the *An. subpictus* mosquitoes feeding on humans at Jaffna (10%).

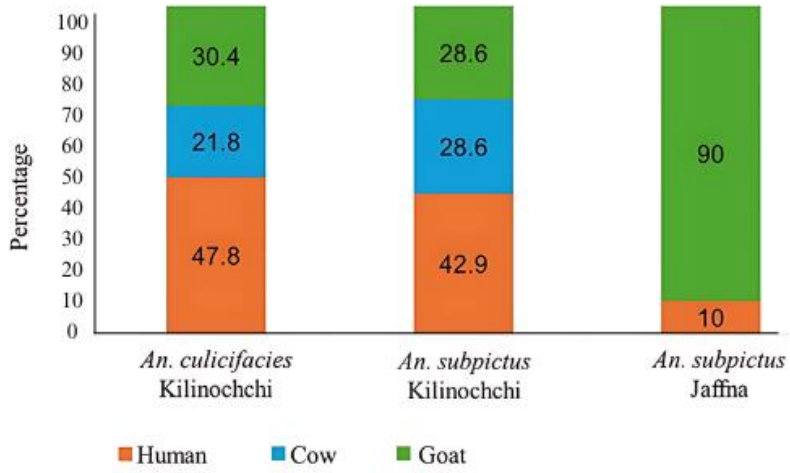

Figure 3: Source of host blood meal of *Anopheles* species according to sampling locations.

Proportion of blood meals derived from vertebrate hosts according to Anopheles species

Among the detected vertebrate sources, humans (47.8%) were the preferred host source over goats (30.4%) or cows (21.8%) for blood meals of *An. culicifacies* (Figure 4). Goats (64.7%) were the most favoured blood meal source of *An. subpictus.*

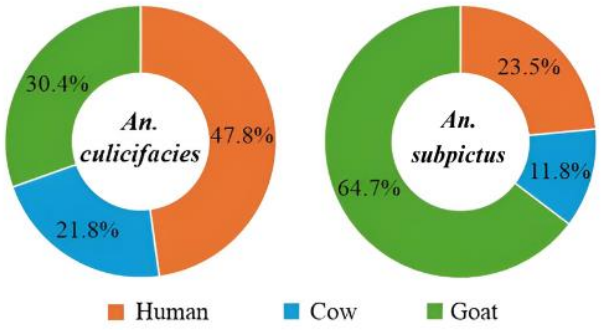

Figure 4: Proportion of human, goat, and cow blood meals of *An. culicifacies* and *An. subpictus*.

Frequency and patterns of single and multiple-host blood meals in Anopheles mosquitoes

An. culicifacies (70.6%) and *An. subpictus* (76.92%) were found to have ingested more single-host blood meals than multiple-host blood meals (Table 2). Out of all the *An. culicifacies* mosquitoes, only 29.4% had fed on two or three hosts. Similarly, a lower percentage (23.1%) of *An. subpictus* mosquitoes were found to have fed on two or three different vertebrates. When considering the blood meals on multiple hosts, both species had fed more often on two hosts than on three. Additionally, the chi-square analysis revealed the number of blood feeds taken by *An. culicifacies* and *An. subpictus* mosquitoes did not differ significantly ($X^2 = 0.151$, $p = 0.697$).

Table 2: Frequency of *Anopheles* mosquitoes with single and multiple-host blood meals.

***Anopheles* species**	**Total number**	**Single host blood meals**	**Multiple host blood meals**		**X^2 statistic**
		n=1 (%)	**n=2 (%)**	**n=3 (%)**	
An. culicifacies	17	12 (70.6%)	4 (23.5%)	1 (5.9%)	$X^2 = 0.151$ df = 1 p = 0.697
An. subpictus	13	10 (76.9%)	2 (15.4%)	1 (7.7%)	

Figure 5 illustrates the frequency of various vertebrates in single-host and multiple-host blood meals of *Anopheles* mosquitoes. For *An. culicifacies*, the single-host blood meals were represented by human (50%) and goat (50%) blood, and for *An. subpictus*, mostly by goat (90%) blood. The frequency of *An. subpictus* mosquitoes taking a blood meal exclusively from human was low (10%). When considering multiple host blood meals, humans (45.5%) and cows (45.5%) were the most common hosts for *An. culicifacies*, followed by goats (9.1%). Humans (42.9%) were the most common host in mixed blood meals of *An. subpictus*, followed by cows (28.6%) and goats (28.6%).

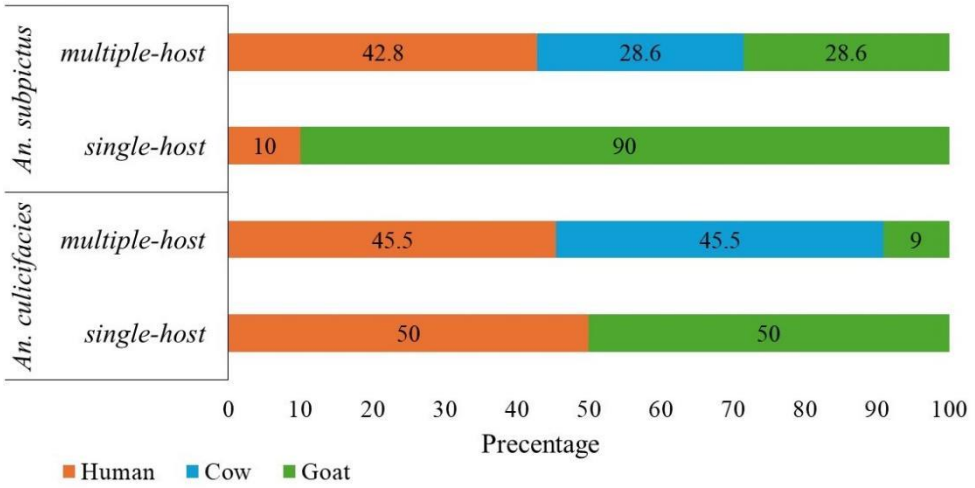

Figure 5: Comparison of different vertebrate host blood meals and frequency of single-host and multiple-host blood meals in *An. culicifacies* and *An. subpictus*.

To find out if mosquitoes feeding on blood from a particular vertebrate host tended to feed from one host or several hosts, a chi-square test was performed (Table 3). The analysis demonstrated a significant association ($X^2 = 14.82$, $p = 0.0006$) between the frequency of single and multiple host blood meals taken by the mosquito and the vertebrate source of the meal. For mosquitoes feeding on a single host, the goats were the preferred source, but for mosquitoes feeding on

many hosts, they were not the preferred host for the blood meal. When taking blood meals from multiple hosts, mosquitoes exhibited a stronger preference for cow hosts.

Table 3: Frequency of vertebrate blood in single-host and multiple-host blood meals of *Anopheles* mosquitoes.

Blood meal source	Single-host blood meals	Multiple-host blood meals	Total	X^2 statistic
Human	7	8	15	$X^2 = 14.82$
Cow	0	7	7	$df = 2$
Goat	15	3	18	$p = 0.0006$*

Performance of conventional and nested PCR assays in detecting host DNA in mosquito blood meals

The performance of PCR in identifying the vertebrate host source in mosquito blood meals is shown in Table 4. The majority of the human (53.3%) and goat (72.2%) blood meals were identified using nested PCR. Most cow blood meals (71.4%) were identified by the conventional PCR assay.

Table 4: Performance of PCR in identifying the vertebrate host source in mosquito blood meals

PCR assay	Number of blood meals (%)		
	Human	Cow	Goat
Conventional PCR	7 (46.7%)	5 (71.4%)	5 (27.8%)
Nested PCR	8 (53.3%)	2 (28.6%)	13 (72.2%)

The nested PCR technique was the primary method (77.3%) identified the sources of the single-host blood meals (Figure 6). Conventional PCR assays accounted for 50% of the identification of the mixed blood meal sources, with the remaining 25% being identified by either nested PCR or by combining the two types of PCR assays.

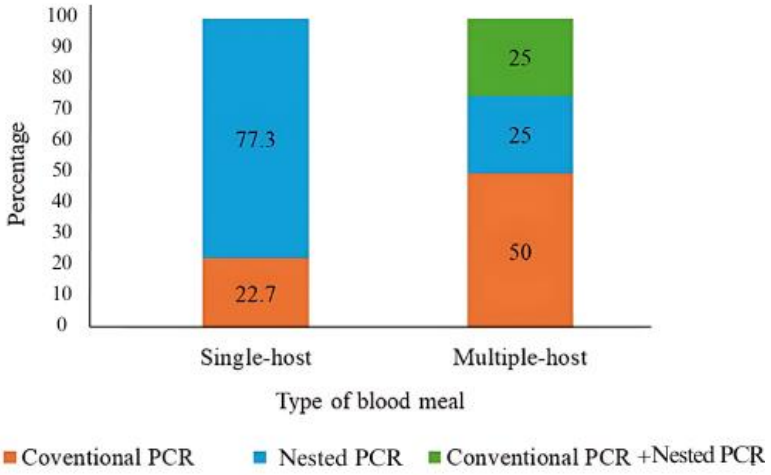

Figure 6: Success rate of conventional and nested PCR assays in detecting vertebrate DNA sources in single and mixed blood meals.

The success rate of PCR amplification with respect to mean DNA concentrations of extracted single and mixed blood meals is shown in Table 5. The mean DNA concentrations of mosquito blood meals tested positive for a single-host source by conventional and nested PCR were 80±26.7 ng/µL

and 79.1±29.8 ng/µL, respectively. The mean DNA concentrations of single blood meals detected by conventional PCR and nested PCR did not differ significantly ($p<0.05$). By contrast, the mean DNA concentration of mixed blood meals identified by the conventional PCR assay (121.8±37.3 ng/µL) was significantly higher ($p<0.05$) than that of the nested PCR (39.5±29.7 ng/µL).

Table 5: Success rate of PCR amplification in single and mixed blood meals.

Blood meal	PCR assay	DNA concentration (ng/µL)		p value
		Mean ± SD	Range	
Single host blood meals	Conventional PCR	80.0 ± 26.7	44.8 – 110.0	p = 0.955
	Nested PCR	79.1 ± 29.8	16.0 – 127.3	
Mixed blood meals	Conventional PCR	121.8 ± 37.3	73.0 - 163.4	p = 0.014*
	Nested PCR	39.5 ± 29.7	3.2 – 64.4	

SD: Standard Deviation; *Significant difference between groups at $p\leq0.05$

Discussion

This study examined the blood-feeding behaviour of field-caught *An. culicifacies* and *An. subpictus* malaria mosquito vectors in Sri Lanka and their potential role in malaria transmission. Because Anopheles mosquitoes' capacity to spread malaria is dependent on their blood-feeding and host-seeking behaviours, determining the source of mosquito blood meals is crucial for epidemiological studies on malaria (Service, 1989). The vertebrate hosts that sustain the survival of the malaria vector population in a given location can be determined with the aid of precise identification of the preferred hosts of malaria vectors. It is also helpful in determining the vectorial capability and the role of each mosquito species in malaria transmission (Garrett-Jone et al., 1980). The transmission dynamics of mosquito-borne conditions corresponding to malaria are largely reliant on the host preference and feeding behaviour of the vector mosquitoes, as well as vector population density and longevity (Garrett-Jones, 1964).

One of the factors that hinders research on blood meal preferences of *Anopheles* mosquitoes is the difficulty in collecting resting mosquitoes. Most blood meal investigations used only visually engorged resting mosquitoes, inadvertently underestimating the proportion of host sources (Escobar et al., 2020). Over the years, techniques for identifying the blood meal source of hematophagous insects have evolved. Laboratories that process large numbers of mosquito specimens for various diagnostic features need to employ and process efficient, cost-effective technologies with a high throughput. In our study, resting *Anopheles* mosquitoes were collected using the indoor hand collection (IHC) method, which has been previously reported as an effective method for blood meal analysis studies (Jeyaprakasam et al., 2022). These blood meals were analysed by a PCR test, which is more sensitive and time-efficient than compared to earlier conventional techniques (Washino and Else, 1972, Boorman et al., 1977, Burkot et al., 1981, Gomes et al., 2001, Kent, 2009).

Even though this study reported 100% identification of the blood meals, other studies (Thiemann et al., 2011, Akirso et al., 2024) report much different levels of host blood meal source identification efficiencies (i.e., 17.5% - 92%). This variation may be due to the differences in the techniques used, species examined, degree of blood meal digestion at the time of analysis, and environmental conditions, etc. As shown in other research (Seyoum et al., 2002, Muriu et al., 2008, Bashar et al., 2012, St. Laurent et al., 2017, Adugna et al., 2021, Akriso et al., 2024), In this study, the *Anopheles* mosquitoes in the northern districts of Sri Lanka were found to feed on a variety of hosts, including humans, cows, and goats. However, none of the mosquitoes were identified to have fed on either dogs or pigs. This can be a result of species-specific host selection. However, it is likely that *An. culicifacies* and *An. subpictus* fed on these hosts because they were readily available in the area. This suggests that the local populations of these mosquito species may be opportunistic feeders (Gunathilaka et al., 2016, Adugna et al., 2021). The availability of hosts in the sites examined may also potentially account for the variations in the host

blood meals of mosquitoes captured from the Kilinochchi and Jaffna research sites. Furthermore, as the PCR primers employed in this study could only identify five possible vertebrate hosts, there might have been additional hosts from which the mosquitoes that were collected had taken blood meals but have not been detected.

Even in areas where the climate is optimal for malaria transmission, the presence of *Anopheles* mosquitoes that do not predominantly feed on humans may prevent malaria from occurring there (Bruce-Chwatt, 1985). Of the two species of *Anopheles* collected, *An. culicifacies* had a higher HBI compared to that of *An. subpictus*. The primary vector of human malaria in Sri Lanka is *An. culicifacies* species E (Surendran et al., 2006), and this species is reported to have the highest anthropophilic index when compared to species A to D (Barik et al., 2009). The HBI of *An. subpictus* was indicative of low anthropophily, like in many other studies (Amerasinghe et al, 1999b, Bashar et al., 2012, Jude et al., 2014). Although the HBI results do not always represent host choice (Boreham and Garrett-Jones, 1973). (Lardeux et al., 2007), the index can be used to predict human biting habits, which is an integral component of vectorial capacity (Lardeux et al., 2007). This index is simple and easy to measure, and it does not require the use of human landing captures to determine the human biting rate of mosquitoes. The human bite rate of mosquitoes can be ascertained by measuring this straightforward and easy-to-use index without the need for human landing captures. Together with other malariometrics like sporozoite and parity indices, the HBI can be periodically estimated to provide valuable insights into the effectiveness of anti-vector interventions.

In Sri Lanka, *An. culicifacies* and *An. subpictus* have been observed to take single and multiple blood meals during a gonotrophic cycle, where multiple-host feeding behaviour is shown mainly in zoophagic and endophilic malaria vectors as a strategy to enhance their human-biting rate and vectorial capacity (Amerasinghe and Amerasinghe, 1999b). According to our research, *An. culicifacies* and *An. subpictus* were more likely to take single-host blood meals than multiple-host blood meals. When each species of mosquito consumed blood from a single host, there were differences in the chosen hosts; humans and goats were the preferred single hosts for *An. culicifacies*, but *An. subpictus* mosquitoes were attracted to goats. *An. subpictus* mosquitoes had fed on cow blood also, as reported by Jude et al. (2014), but only when taking multiple feeds. Further, comparable to earlier research (Amerasinghe and Amerasinghe, 1999b), those feeding on multiple hosts were shown to feed mainly on two different types of hosts. Multiple-host feeding frequency may be influenced by a number of factors, including the nutritional status of the mosquito (Briegel and Horler, 1993) and host defensive behaviour that interrupts feeding (Amerasinghe and Amerasinghe, 1999b). A number of other *Anopheles* species have also been documented to exhibit the multiple-host feeding behaviour (Boreham et al., 1979, Klowden and Briegel, 1994, Wekesa et al., 1995). According to Massebo et al. (2013), when humans and cattle reside close to one another, the likelihood of vectors taking repeated feeds from these hosts increases. Furthermore, the efficacy of *Anopheles* species in inoculating the parasite in many hosts through multiple feeding determines their contribution to the transmission of malaria (Bøgh et al., 2001). This demonstrates the significance of combining mosquito positivity rate assessments with blood meal analysis in malaria epidemiological studies, as these can greatly enhance the comprehension of identifying reservoirs, including zoonotic ones, that may harbour the parasite until the subsequent malaria transmission season. Mosquito species that feed on blood from many hosts over their lifetime may be more likely to spread the malaria parasite to other species and raise the risk that people may contract the disease from animals. Blood meal analysis, therefore, is crucial for identifying changes in feeding behaviour and host selection by vectors. It also emphasises the significance of these factors for assessing malaria control interventions and their incorporation into routine malaria control programmes (Gunathilake et al., 2016).

The multiplex PCR methodology offers a quick, efficient, and economical screening tool to determine host preferences of malaria vector mosquitoes (Osae et al., 2013). Concurrently, the collected DNA can be utilised to verify the species identification of mosquitoes (Ngo and Kramer, 2003). In our research, after utilising a conventional multiplex PCR test, 65.6% of the samples did not yield detectable amplifications after the first round of reaction. However, these samples exhibited discernible DNA bands when 1 µL of the original

PCR results was reamplification utilising a multiplexed nested PCR assay. Our results also show that the nested PCR technique was able to identify many of the sources of blood meals for humans and goats, as well as single hosts. Because the majority of single-host blood meals came from either humans or goats, it is possible that these variations in the amplification success of conventional and nested PCR assays among the various types of vertebrate host blood meal sources. It seems that only the multiplexed nested PCR test has produced successful PCR amplification results from such single-host blood meals. While the multiplexed conventional PCR technique has been successful in identifying the majority of multiple-host blood meals. According to a laboratory-based study done to assess the performance of a PCR assay to identify multiple-host blood meals, the PCR amplified products were less prominent on the agarose gels for the first blood meal compared to the second blood meals (Jeyaprakasam et al., 2022). They concluded that this observation is most likely the result of the mosquito having more time to digest its first blood meal, which results in a lower amount of DNA template being present for effective amplification. It is generally interpreted that several feedings on the same night indicate interrupted feeding as a result of host defensive reactions (Amerasinghe and Amerasinghe, 1999b). Therefore, it is presumed that a mosquito will only feed on its host until it is fully engorged in the absence of host defence, at which point it may decide not to feed on another host during the night. Reasoning to that, more of the single-blood meal is expected to be broken down by the morning resulting in less DNA for PCR amplification. However, when host defence is present, females may try feeding multiple times during the night, which would increase the amount of undigested blood available and, thus, the amount of host DNA that may be amplified.

In line with this explanation, our investigation revealed that the mean DNA quantity of multiple-host blood meals (121.8 ng/µL) available for amplification was higher than that of single-host blood meals (80 ng/µL) among the samples that were successfully amplified by the conventional PCR assay, which requires better quality and quantity of DNA. A nested PCR amplification was only necessary for the multiple-host blood meal samples when the mean DNA concentration was significantly lower (39.5 ng/µL). In contrast, even at significantly greater DNA concentrations (79.1 ng/µL), the single-host blood meals required nesting, most likely because DNA templates in the blood meal were degraded due to digestion. It can be difficult to get fresh samples that are acceptable for PCR since it is not always possible to collect samples in the field and process them right away for PCR analysis, particularly in rural or resource-constrained places. Numerous factors, including digestion of blood or environmental conditions, can cause DNA in mosquito blood meals to deteriorate (Martínez-de la Puente et al., 2013). As the blood is broken down in the mosquito gut, the identification success of its blood meal sources gradually declines (Tuten et al., 2012). According to Mukabana et al. (2002), after 8 hours post-feeding, the probability of producing successful PCR results in *An. gambiae* becomes negatively correlated with the time since blood meal ingestion. After 15 hours post-feeding, the probability of success falls to less than 50%. To get around this problem in our investigation, samples were taken early in the morning, although it was impossible to regulate the interval between the mosquitoes consuming the blood meal and being captured.

This study provides valuable insights into the vectorial potential of *An. culicifacies* and *An. subpictus*, but we are aware that additional research is needed to address some of its shortcomings. Expanding the scope of the sampling, focusing on identifying more vertebrate hosts, and identifying *Anopheles* species that are resting indoors would further strengthen the results. In addition, a comprehensive vertebrate host census at the research sites would allow for the quantification of host preferences through the calculation of forage ratios and host selection indices. Additionally, blood meals could be subjected to DNA profiling to investigate the mosquitoes' propensity to feed on one or more individuals of the same vertebrate host within a single gonotrophic cycle. To give a more thorough understanding of the feeding preferences and selection patterns of *Anopheles* vector species, future research could take these factors into account.

Conclusions

An. culicifacies mosquitoes in the northern districts of Sri Lanka have a greater preference for human

blood. While *An. subpictus* mosquitoes also feed on humans, they show a higher tendency to feed on animal blood. Therefore, the findings of this study suggest that both species have the capacity to transmit malaria to humans and shed light on their possible involvement in the spread of zoonotic malaria. Long-term planning for the development of effective malaria vector control measures will benefit greatly from this knowledge of *Anopheles* vector feeding patterns.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Author contribution

HS contributed to data collection, data analysis and writing the original draft. RJ contributed to funding acquisition, designing molecular investigation and co-supervision of the research study. JJ was involved in planning the study and sampling. GR contributed to conceptualization, funding acquisition, investigation, and co-supervision of the research study. All authors reviewed and edited the manuscript.

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