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Original Article

Feeding Behavior and *Plasmodium* Detection in *Anopheles stephensi*, a Malaria Vector in District Khyber, Khyber Pakhtunkhwa, Pakistan

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Abstract

Background: *Anopheles stephensi* is a significant malaria vector in Pakistan, and understanding its feeding behavior is necessary to control the spread of malaria. However, limited information is available on the host preferences of *A. stephensi* in Pakistan. Therefore, we aimed to explore the feeding behavior of *A. stephensi*, a malaria vector, in the District Khyber, Khyber Pakhtunkhwa, Pakistan.

Methods: A total of 7462 mosquitoes were collected between March and September 2021, with 1674 (22.4%) identified as *A. stephensi* (952 female and 722 male). Among the female *A. stephensi*, 495 (52%) were blood-fed. DNA was extracted from the blood-fed female *A. stephensi* mosquitoes using the Ammonium Acetate Precipitation Method followed by PCR analysis, blood meal sources were identified. Nested PCR on 191 pooled samples was used to detect *Plasmodium falciparum* and *Plasmodium vivax*.

Results: Cattle blood meals were predominant (73%), followed by human (20%) and chicken (7%), with no dog blood meals detected. All individual mosquito samples were negative for *Plasmodium falciparum*, while two pooled samples (out of 191) tested positive for *P. vivax*.

Conclusion: *A. stephensi* in Khyber District primarily displayed anthropophagic feeding behavior, with a small portion of the population infected with *P. vivax*. The results underscore the importance of targeted vector control strategies, environmental management, community engagement and continuous monitoring to suppress malaria transmission.

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Introduction

Mosquitoes, belonging to the family Culicidae, are known to transmit various diseases, including malaria, dengue, and filariasis, which significantly impact human health and socio-economic development. These vectors require important nutrients for egg production, obtained through the acquisition and digestion of protein-rich blood (1). The concept of blood feeding in insects emerged when plant-sucking insects accidentally bit vertebrates, leading to the development of a digestive physiology that allows for metabolic absorption. During the ovarian cycle, female mosquitoes also transmit pathogens such as dengue and malaria to animals through blood feeding (2) .

The evolution of blood feeding in insects led to a mutually influential parasitic development between the vertebrate host and the insect, particularly when blood became the primary source of nutrients for egg production. During the ovarian cycle, female mosquitoes also transmit pathogens such as dengue and malaria to animals through blood feeding (3,4). During blood feeding, insects rely on host-specific cues to accurately identify their preferred host in a heterogeneous environment. Host preference is a critical feature that influences the reproductive success of blood-feeding insects, including mosquitoes that transmit pathogens such as dengue and malaria to animals during the ovarian cycle (5,6).

In Pakistan, malaria is a major health issue, with *Plasmodium vivax* and *P. falciparum* being the primary transmitted species. *Anopheles stephensi*, a species of mosquito, has been identified as one of the main vectors for malaria transmission in the country. The feeding behavior of *A. stephensi* is intricately linked to the transmission of malaria (7,8). When an infected mosquito bites a human, it unwittingly injects malaria parasites, known as *Plasmodium*, into the bloodstream. These parasites multiply within the liver before invading red blood cells, causing the debilitating symptoms of malaria. The parasite then completes its life cycle by returning to the mosquito during its next blood meal, potentially perpetuating the transmission (9). Understanding the feeding preferences of *A. stephensi* is necessary in this complex cycle. It helps determine whether they primarily feed on humans, perpetuating the malaria cycle, or prefer other animal hosts, acting as potential dead ends for transmission (4,6). Unraveling their blood meal choices provides critical information about the human-mosquito interface and the vulnerability of communities to malaria outbreaks (10).

We investigated the feeding behavior and host preference of *A. stephensi*, analyzes factors influencing host preference. Besides, we examined the role of *A.* stephensi in malaria transmission in District Khyber, Pakistan. Directly detecting *Plasmodium* within the mosquito population provides a snapshot of the current malaria landscape, informing control efforts and improving public health outcome

Methods

Study Area

The study area was District Khyber, in the Northern region of Pakistan, bordering Afghanistan, Peshawar City, Orakzai, and Kurram districts. With a total area of 2,576 square kilometers and only 8.22% forest cover, Khyber features a rugged and barren mountainous landscape with narrow valleys. Summers are hot (May to August), while winters are cold (November to January) (Fig. 1).

Data collection

From Mar to Sep 2021, 7,462 mosquitoes were such as *A. stephensi* (1674), *Armigeres* (2,100), *Culex pipiens* (1,565), *Aedes agyptii* (1,468) and *A. culicifacies* (565) during daytime hours using the flit method. Collections occurred within a closed room draped in white sheets. After sealing the room to restrict air movement, a fast-acting volatile insecticide

was applied, and the room remained closed for 5 minutes. Collected mosquitoes were subsequently aspirated from the sheets and placed in 3 ml blood collection tubes containing silica gel. The mosquitoes were identified on the basis of morphological characters using species identification keys given in Fauna of British India by Barraud (1934) and Christopher (1935) (11–13). Only *A. stephensi* (22.43%) was used for studying the blood feeding behavior and malaria detection.

Fig. 1: Map of Khyber district, Khyber Pakhtunkhwa (https://cmdo.org.pk/khyber-agency/).

DNA Extraction

DNA extraction from the collected mosquito samples involved pooling the blood-fed *A. stephensi* mosquitoes into groups of five, followed by DNA extraction from the head, thorax, and abdomen of the female mosquitoes using the standard ammonium acetate precipitation method (14).

PCR Amplification

To detect host blood in mosquitoes from extracted DNA, four different host specific primers (Table 1) were used for PCR amplification (15,16).

Table 1: Host specific primers used for the detection DNA from the blood of *A. stephensi*

Host Specie	*Primer sequence F: forward 5'–3' and R: reverse 5'–3'*		*Size of amplified product (bp)*
Human	5′-TTCGGCGCATGAGCTGGAGTCC-3′	F	
	5′-TATGCGGGGAAACGCCATATCG-3′	R	228
Bovine	5′-GCCATATACTCTCCTTGGTGACA-3′	F	
	5′-GTAGGCTTGGGAATAGTACGA-3	R	271
Dog	5′-GAACTAGGTCAGCCCGGTACTT-3′	F	
	5′-CGGAGCACCAATTATTAACGGC-3′	R	153
Chicken	5′GGGACACCCTCCCCCTTAATGACA-3′	F	
	5′GGAGGGCTGGAAGAAGGAGTG-3′	R	266

To identify the blood meal source, a PCR reaction mixture of 20 µl was prepared, consisting of DNTPs (2 µl), Taq Buffer (2 µl), *Taq Polymerase* (0.3 µl), MgCl2 (1.2 µl), host-specific

Primers (1 µl each for forward and reversed primer), DNA (1 µl), and distilled water (11.5 µl).

Detection of malarial parasites

To detect malarial parasites, a nested PCR technique was used with outer and inner primers specific for the *Plasmodium* genus, *P. falciparum* and *P. vivax*. Two rounds of PCR were performed, first using outer primers specific for the *Plasmodium* genus (rPLU_6 and rPLU_5), followed by inner primers specific for *P. falciparum* (rFAL_1 and rFAL_2) or *P. vivax* (rVIV_1 and rVIV_2) (Table 2).

Table 2: These primers used for the detection of malarial parasites

Primer name	*Sequence*	*Size (bp)*
rPLU_6	5′-TTAAAATTGTTGCAGTTAAAACG-3′	1200
rPLU_5	5′-CTTGTTGTTGCCTTAAACTTC-3′	1200
rFAL_1	5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′	205
rFAL_2	5′-ACACAATGAACTCAATCATGACTACCCGTC-3′	205
rVIV_1	5′-CGCTTCTAGCTTAATCCACATAACTGATAC-3′	120
rVIV_2	5′-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3′	120

The thermal conditions for the primers are as follows: 95°C for 3 min, 40 cycles of 58°C for 30 seconds (for human), 51°C for 30 seconds (for bovine), 55°C for 30 seconds (for dog), and 59.5°C for 30 seconds (for chicken), followed by 72°C for 30 seconds and 72°C for 3 min (Table 2).

Gel Electrophoresis

The PCR products were visualized on a 1% ethidium bromide-stained agarose gel using a 100 bp DNA ladder marker in a 5X Tris-borate-EDTA running buffer. The gel was subjected to electrophoresis at 90 volts for 45 min, and the agarose gel was visualized under a UV Transilluminator. The image was then photographed.

Ethical Declaration

This study adhered to ethical guidelines for field mosquito collection. Mosquitoes were captured using light traps, a non-invasive method. No anesthetic or surgical procedures were employed on the mosquitoes. The research protocol was approved by Ethical committee of Institute of Zoological sciences, University of Peshawar, Pakistan

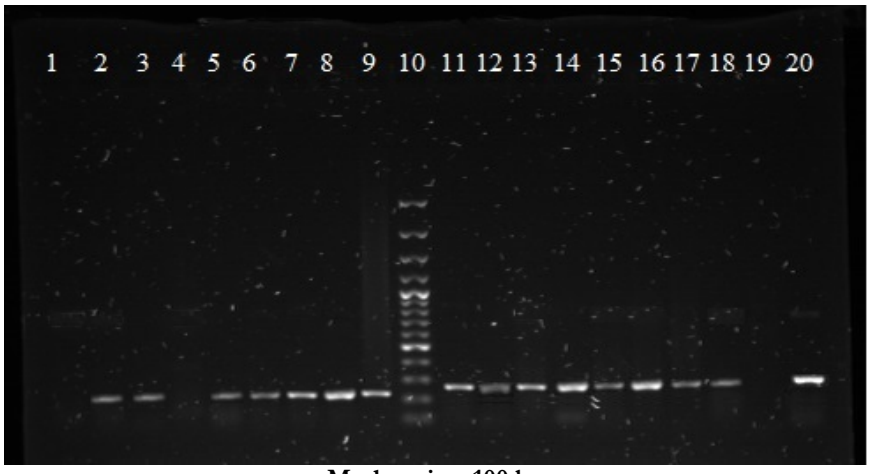
Results

Blood Meal Analysis of A. stephensi Mosquitoes

From March 2021 to September 2021 a total of 7462 mosquitoes were collected of which 1674 (22.43%) were *A. stephensi*. Among the collected mosquitoes, 722 (43.13%) were males, and 952 (56.86%) were females. Out of the 952 females, 495 (51.99%) were blood-fed, while 457 (48.00%) were unfed. Upon further analysis, out of the 495 blood-fed *A. stephensi*, host blood was detected in 490 specimens, with 5 showing no amplification product. Subsequently, all the blood samples underwent PCR analysis, revealing that 73% (357/490) of the specimens contained cattle blood, characterized by a band size of 271 bp, while 20% (98) were found to have human blood, and 7% (34) had chicken blood. Notably, none of the specimens were found to have dog blood (Table 3 and Fig. 2-4).

Table 3: Blood Meal Analysis of *Anopheles stephensi* Mosquitoes. N=495

Host category	*No. of blood fed Anopheles stephensi n(%)*
Cattle	357 (73)
Human	98 (20)
Chicken	34 (7)
Dog	0 (0)
Total known	490 (100)



Marker size; 100 bp

Fig. 2: Gel showing 228 bp band size for human blood (row 2, 3, 5-8 test samples, row 1 and 4 negative samples, row 9 positive control from human blood, row 10 100 bp ladder marker) and 271 bp band size for cattle (row 11- 18 positive samples, row 19 negative sample, row 20 positive control detected in mosquitoes from cow blood) detected in mosquitoes

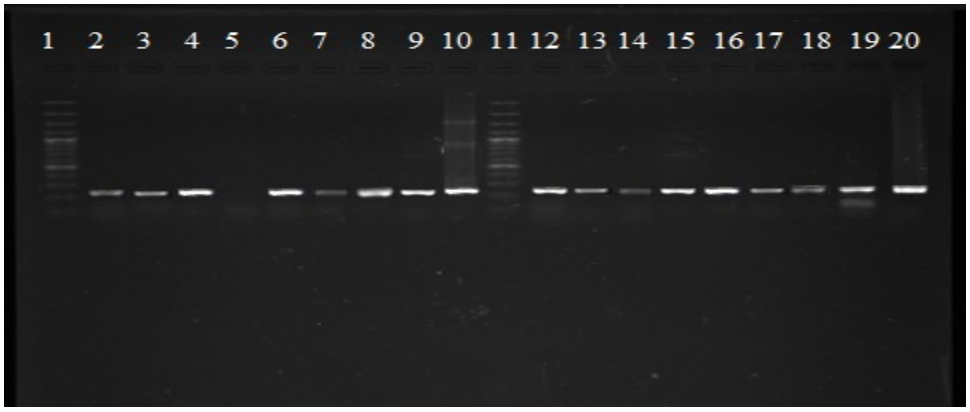


Fig. 3: Gel showing 266 bp band size for chicken blood (row 5-8 negative samples and row 20 positive control from chicken blood, row 1 and 11 100 bp ladder marker) detected in mosquitoes

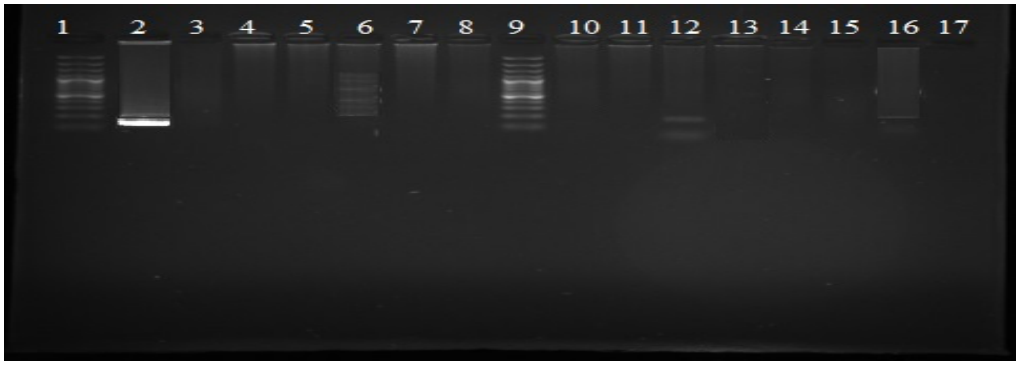


Fig. 4: Gel showing no detection of 153 bp band size for dog blood (row 2 positive control from dog blood, row 1 and 9 100 bp ladder marker) in mosquitoes

Molecular identification of Plasmodium species from blood meals sources

All *A. stephensi* pools were negative for *P. falciparum*, as none of the samples showed an amplified DNA band size of 205 bp. However, two pools were found positive for *P. vivax*, showing an amplified band size of 120 bp (Fig. 5).

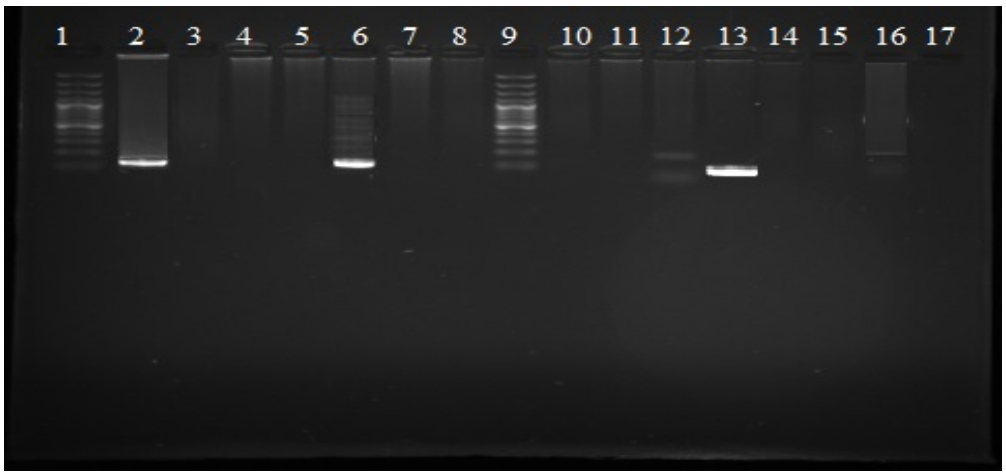


Fig. 5: Gel showing detection of P. vivax (120 bp) in *Anopheles stephensi* collected from District Khyber (row 6, 13 positive samples, row 2 positive control of P. vivax DNA sampled from human beings row 1 and 9 100 bp ladder marker

Discussion

Feeding behavior is the act of acquiring nutrients from their host, and insects, such as mosquitoes, transfer disease-causing agents to their hosts during the act of feeding. Present study, focused on the feeding behavior of *A. stephensi*, a malaria vector in the merged tribal district of Khyber in Khyber Pakhtunkhwa, Pakistan. The observed feeding pattern of *A. stephensi*, with a preference for cattle but also feeding on humans and chickens, aligns with findings from other studies. A study on *A. stephensi* in Peninsular Malaysia also found that the mosquitoes fed on various blood sources, including humans, cattle, and pigs (17). In Peninsular Malaysia, *Anopheles* mosquitoes were found to have a preference for certain blood sources, such as pigs and cattle (18).

In present study detection of human blood in *A. stephensi* indicating that this mosquito species is both zoophagic and anthropophagic in nature. In comparison to other studies, the present study has confirmed the findings of Mehravaran et al in Jiroft, southeast Iran, who reported high anthropophagy in *A. stephensi* (19), and Kumari et al in Angul district, Orissa, India, found that *A. stephensi* and *A. culicifacies* were both zoophagic and anthropophagic (20).

Present study revealed that *A. stephensi* was a significant vector for *P. vivax* in the study area, with 20% of the specimen's positive for the presence of the parasite. This highlights the importance of controlling the population of this mosquito species in the affected area to reduce the spread of malaria. In Africa Among engorged mosquitoes, cattle and goats emerged as the primary blood source for *A. stephensi* (98%) and *A. gambiae* (80%). although only *A. stephensi* showed minimal human feeding (2%). However, nearly half of the engorged *A. stephensi* mosquitoes (48%) yielded undetermined bloodmeal sources. Notably, only 0.5% of *A. stephensi* carried *P. falciparum* sporozoites, emphasizing their potential but

limited role in natural malaria transmission (21). Similarly another study conducted in Goa, India, found two *A. stephensi* mosquitoes positive for *P. falciparum* sporozoites out of 831 female *A. stephensi* examined (22). In Kenya *P. falciparum* and *P. vivax* were detected in *A. stephensi* mosquitoes (23).

Our study also reports *A. stephensi* positive for the presence of *P. vivax* through PCR. Present study employed PCR for detection of blood meal in *A. stephensi* while Mehravaran et al. (2012) used ELISA kit for host blood detection. PCR is a much more sensitive technique than ELISA but both have shown anthropophagic behavior in *A. stephensi* (19). Present study emphasizes the importance of *A. stephensi* as a malaria vector in the merged tribal district of Khyber. Vector surveillance and control strategies can help decrease the mosquito vector population in the affected area, resulting in a decrease in human-mosquito interaction and, consequently, a decrease in the disease burden. Vector control is the most suitable method to decrease malaria morbidity and mortality in the local population, thus improving the life standard of the affected population in district Khyber.

Conclusion

This study provides valuable insights into the feeding behavior and *Plasmodium* detection in *A. stephensi*, highlighting its importance as a malaria vector in Khyber. The results reveal that *A. stephensi* is both zoophagic and anthropophagic, with one-fifth of blood-fed females having human blood meals (20%). Notably, *P. vivax* is a prevalent malaria-causing agent in the area, with 20% of the specimens testing positive for the parasite. To safeguard Khyber's residents, implementing targeted vector control strategies to decrease *A. stephensi* populations, reducing breeding sites through environmental management and community engagement, and continuously monitoring mosquito population dynamics and *P. vivax* prevalence are crucial in effectively suppressing malaria transmission.

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Conflict of Interest

The authors have declared that there is no conflict of interest regarding the publication of this article

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