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A Cross-Sectional Study of Malarial Patients in District Faisalabad, Pakistan: Frequency of Infection, Species Distribution, and Diagnostic Efficiency Comparison

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Abstract

BACKGROUND/AIMS: Malarial infection, caused by *Plasmodium* parasites transmitted by female Anopheles mosquito, is still a serious public health concern, especially in endemic areas such as Sub-Saharan Africa and Pakistan. The study aims to investigate the frequency of malarial infection in district Faisalabad.

MATERIALS AND METHODS: A cross-sectional study was conducted across the district Faisalabad to study the frequency of malarial infection. Blood samples were collected from 1460 suspected malaria cases between May 2023 and April 2024 at various government sector hospitals across the six administrative units (tehsil) of district, Faisalabad. For the purpose of the diagnosis of malarial patients, rapid diagnostic tests (RDTs), microscopy, and polymerase chain reaction (PCR) methods were employed.

RESULTS: RDTs detected 649 (44.4%) positive cases, while microscopy and PCR detected 452 (30.6%) and 459 (31.4%) cases, respectively. Among *Plasmodium* species, *P. vivax* was the most frequently detected, followed by *P. falciparum* and mixed infections. Significant differences were observed in diagnostic outcomes across the three methods. Notably, 55 RDT-positive cases were found to be negative by both microscopy and PCR, indicating potential false positives due to antigen persistence. The highest positivity rate was observed in the 1-15 years age group among males as well as in the months of June and July. These findings highlight the need for confirmatory diagnostics alongside RDTs to improve accuracy and support malaria surveillance and control efforts in endemic regions. Positivity rates varied across municipalities, with Faisalabad city showing the highest burden.

CONCLUSION: This study demonstrates that while RDTs offer rapid malaria detection, they are prone to false positives due to persistent parasite antigens, especially in mixed infections. PCR and microscopy provided more accurate results and revealed significant differences in diagnostic outcomes. Moreover, the study highlights a significant frequency of malaria in district Faisalabad, with *P. vivax* being the dominant species. The data indicate higher susceptibility among males and children aged 1-15 years, particularly during the peak months of June and July. These findings highlight the need for confirmatory diagnostics to accompany further investigations. RDTs to improve accuracy and support malaria surveillance and control efforts in endemic regions.

Keywords: *Plasmodium*, rapid diagnostic test, microscopy, polymerase chain reaction, Faisalabad

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INTRODUCTION

Malarial infection is a serious medical issue caused by the *Plasmodium* parasite and spread by the female Anopheles mosquito. Malaria causes widespread death in endemic places, killing 62,700 individuals in 2021, the majority of whom were less than 5 years old, in Sub-Saharan Africa.[1] Pakistan reported 74,860 cases of malaria in March 2023 and 75,185 in April 2023, as compared to 14,371 cases in March 2022 and 23,562 in April 2022.[2] *P. vivax, P. falciparum, P. malariae, P. ovale* are the most common species found worldwide, and the fifth species is *P. knowlesi*, which has been recently discovered in several countries.[3,4] The malarial fever has a severe impact on human health and causes significant decreases in hemoglobin, red blood cells, lymphocytes, basophils, monocytes, sodium, potassium, chloride ions, creatinine, cholesterol, bilirubin and increases the serum glutamic pyruvic transaminase.[5]

The diagnosis of malaria is a critical step in managing the disease and preventing its spread, and accurate diagnosis is essential for effective treatment and control.[6,7] Malarial fever is typically diagnosed using rapid diagnostic test (RDT) kits based on antigens in a blood sample, by making both thick and thin blood films for microscopy, or efficiently detected through polymerase chain reaction (PCR).[8-10] Pakistan is a tropical agricultural country where 64% of the population lives in rural areas with inadequate water infrastructure, which are suitable habitats for mosquito egg laying.[11] Malarial infection is endemic throughout Pakistan and is becoming more prevalent, particularly in Baluchistan and Khyber Pakhtunkhwa.[12] In the Multan district of Punjab, Pakistan, 905 *P. vivax*, 425 *P. falciparum*, and 148 mixed cases were recorded.[13] Previous research has found significant endemicity in regions such as Rawalpindi and Islamabad, with *P. vivax* predominating.[14] Similarly, another previous study reveals that *P. vivax* (90.6%) was more common during the summer months as compared to any other *Plasmodium* species in Khyber Pakhtunkhwa, with the highest incidence recorded in southern regions.[15]

Furthermore, a study in the Khyber Pakhtunkhwa province revealed a malarial prevalence of 13.8%, with *P. vivax* accounting for 92.4% of cases.[16] In Quetta, Baluchistan, about 18.4% of clinically suspected people tested positive for malaria, with *P. vivax* responsible for 81.6% of these cases.[17] The prevalence of malaria and species distribution might differ dramatically across districts, seasons, and age groups, with some places exhibiting higher rates and others showing lower rates than the expected prevalence.[18] The previous studies based on region, district, age, and gender, overall reveal that *P. vivax* was mostly prevalent.[19-22] Despite numerous studies on malaria prevalence in various Pakistani districts, the epidemiology of malaria infection in Faisalabad district has not been thoroughly investigated. This study is designed to analyze the frequency of malaria infection in different administrative units of the district of Faisalabad, Punjab, Pakistan.

MATERIALS AND METHODS

Types of Study

A cross-sectional study was conducted to determine the frequency of malaria infection in the district Faisalabad, Punjab, Pakistan. The present study was conducted from May 2023 to April 2024.

The study was approved by the Ethics Review Committee of Government College University Faisalabad, Punjab, Pakistan (approval number: GCUF/ERC/460, date: 15.05.2023). Verbal informed consent was obtained from all participants prior to inclusion in the study.

Study Area

Faisalabad district (73°74 E; 30°31.5N) is approximately 604 feet above sea level; during summer, the maximum (max.) and lowest temperatures are 41 °C and 27 °C respectively. In winter, the temperatures fluctuate between 21 °C to 6 °C. Faisalabad district comprises six administrative units (tehsils) shown in Figure 1 Faisalabad Saddar, Faisalabad city, Tandilianwala, Jaranwala, Samundri, and Chak Jhumra.[23] Blood samples were collected from only Government Sector Hospitals likewise Tehsil Headquarter Hospital Chak Jhumra (31°33'56.8"N73°11'17.1"E), Tehsil Headquarter Hospital Tandilianwala (31°02'04.0"N 73°07'52.6"E), Tehsil Headquarter Hospital Jaranwala (31°20'26.2"N 73°25'27.2"E), Tehsil Headquarter Hospital Samundri (31°03'41.8"N 72°57'21.9"E), Allied Hospital Faisalabad (31°26'55.3"N 73°04'48.7"E), Civil Headquarter Hospital Faisalabad (31°25'16.9"N 73°05'40.9"E), Government General Hospital Ghulam Muhammad Abad Faisalabad (31°26'34.4"N 73°02'45.9"E) Government General Hospital Samanabad Faisalabad (31°23'06.8"N 73°04'00.7"E) and Social Security Hospital Faisalabad (31°25'09.5"N 73°06'59.6"E).

Laboratory Analysis

Blood samples were taken from 1,460 suspected cases that had symptoms of malaria, such as headache, chills, fever, and nausea; and subjected them to RDT, microscopy, and PCR. For RDT commercially available test kits (Abbot **Malaria** Ag P.f/Pan kit, LOT NO: 05EDI010C) were used.

Microscopic and Polymerase Chain Reaction Analysis

The slides were prepared for the identification of *Plasmodium* species and their stages. Thick and thin films were prepared on a clean glass slides, fixed and stained with Giemsa stain. Slides were observed under the 100x objective lens according to guidelines.[24] For PCR analysis, blood samples were brought to Integrated Genomics, Cellular Developmental and Biotechnology Laboratory University of Agriculture, Faisalabad (31°43'51.9" N, 73°07'16.9" E). DNA was extracted from 3 mL of an infected person's blood using a commercially available DNA extraction kit according to the manufacturer's instructions. For the extraction of DNA from blood samples, the QIAamp DNA Mini Kit (Catalogue No. 51304, Qiagen, Hilden, Germany) was used. This kit provides efficient extraction of high-purity DNA, commonly used in molecular diagnostics, including malaria studies. A nested PCR approach, of the small subunit RNA (ssrRNA) was used for the identification of *Plasmodium* species.[25] The *ssrRNA*

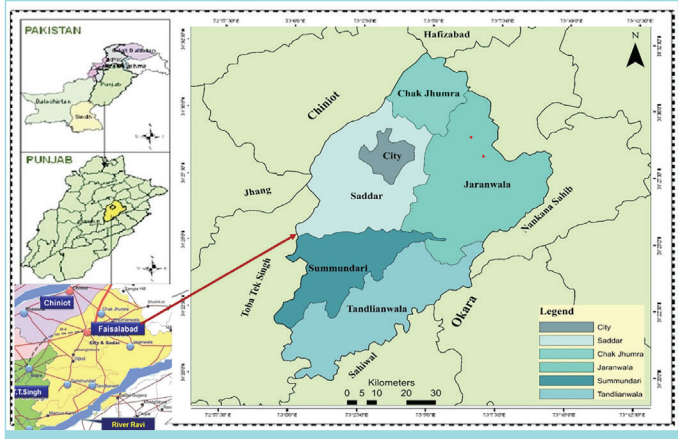

Figure 1. Map of study area.

gene specific to malaria parasites[26] was amplified using primers for *P. falciparum* (Forward primer: 5′-CTTTTGAGAGGTTTTGTTACTTTGAGTAA-3′; Reverse primer: 5′-TATTCCATGCTGTAGTATTCAAACACAA-3′) and for *P. vivax* (Forward primer: 5′-ACGCTTCTAGCTTAATCCACATAACT-3′; Reverse primer: 5′-ATTTACTCAAAGTAACAAGGACTTCCAAGC-3′), used in conjunction with the Taq PCR Core Kit (Catalogue No. 201223, Qiagen, Hilden, Germany). This kit facilitates accurate and efficient amplification of target genes and is widely used for molecular detection of *Plasmodium* species. The amplified PCR products were run alongside a 100 base pair DNA ladder on 1.5% agarose gel stained with ethidium bromide. DNA was visualized on an ultraviolet trans illuminator and photographed, as shown in Figure 2, using a gel documentation system.

Statistical Analysis

The data were analyzed using Microsoft Excel to determine the percentage infection rate. Categorical data were expressed as percentage and chi-square statistical analyses were applied. The performance of diagnostic methods was assessed by calculating sensitivity, specificity, and predictive values, with confidence intervals reported for each estimate. Formulas are used to calculate the diagnostic accuracy of RDT and microscopy compared to PCR as the reference standard.

Sensitivity (%): [true positives/(true positives + false negatives)] × 100

Specificity (%): [true negatives/(true negatives + false positives)] × 100

Positive predictive value (%): [true positives/(true positives + false positives)] × 100

Negative predictive value (%): [true negatives/(true negatives + false negatives)] × 100

RESULTS

A total of 1,460 blood samples were obtained from these nine Government Hospitals such as Tehsil Headquarter Hospital Chak Jhumra, Tehsil Headquarter Hospital Tandilianwala, Tehsil headquarter hospital Jaranwala, Tehsil Headquarter Hospital Samundri, Allied Hospital Faisalabad, Civil Headquarter Hospital Faisalabad, Government General Hospital Ghulam Muhammad Abad Faisalabad, Government General Hospital Samanabad Faisalabad, and Social Security Hospital Faisalabad. Out of 1,460 samples, 649 (44.4%) were positive, with 384 *P. vivax* (59.1%), 134 *P. falciparum* (20.6%), and 131 mixed (20.1%) cases recorded. The patients that were diagnosed with malaria show symptoms of chills, headache, weakness, and elevated temperature.

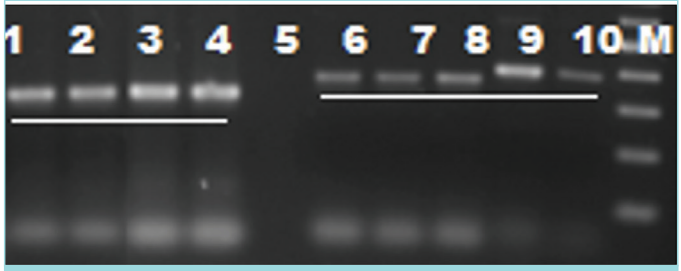

Figure 2. Gel image showing diagnosis of *Plasmodium* species. Lane M: DNA ladder (molecular marker), (Lane 1-3: Positive for *P. vivax*), (Lane 6-8: Positive for *P. falciparum)*, (Lane 10: showed Mixed infection), (Lane 5: Negative control as well as lane 4 and 9 are positive for *P. vivax* and *P. falciparum* respectively).

The month-wise frequency of malarial patients in Faisalabad city (Figure 3A) shows cases peaked in August 2023 (48), with *P. vivax* being the most frequent parasite (83.3%) in November and positivity rates highest in June 2023 (63.8%). Data from Faisalabad Saddar (Figure 3B) show that suspected cases were at their max. in September 2023 (48), with the positivity rate highest in July 2023 (68.5%) and *P. vivax* dominance (95.5%) in September. Figure 3C shows that Jaranwala city was under the highest attack in September 2023 (34 cases), with the highest positivity rate in June 2023 (85.1%) and notable *P. vivax* rates (47.8%). Samundri (Figure 3D) showed the highest case ratio in June 2023 (22 cases) with a max. positivity rate (86.3%), dominated by *P. vivax* (68.7%). Tandilian Wala (Figure 3E) showed peaks in June and July 2023, with mixed infections and *P. vivax* most prevalent, while Chak Jhumra (Figure 3F) showed moderate positivity rates, peaking at 65.3% in June, with mixed infections and *P. vivax* common. The species distribution among the positive cases highlighted *P. vivax* as the most prevalent species with 384 cases (61.1%), followed by mixed infections with 131 cases (20.64%), and *P. falciparum* with 118 cases (18.17%). The data indicate significant seasonal and regional variations in malaria prevalence, with peaks observed during the summer months, particularly in June and July. This underscores the importance of targeted public health interventions and continuous monitoring to effectively manage and control malaria transmission in the region.

Age-Based Frequency of Malarial Infection

Table 1 shows data on malaria cases categorized by age groups. In the 1-15 years age group, 111 individuals tested positive for malaria, accounting for 62.3% of suspected cases. Among these positive cases, 71.1% were attributed to *P. vivax,* 11.7% were attributed to *P. falciparum*, and 16.2% were mixed infections. In the 16-30 age group, 335 suspected cases were tested, with 104 (65.0%) being *P. vivax,* 21.2% being *P. falciparum*, and 13.7% being mixed infections. In the 31-45 age group, 204 (43.4%) tested positive, with 109 (53.4%) being for *P. vivax*, 26.0% for *P. falciparum,* and 20.5% for mixed infections. In the 46-60 age group, 111 (35.7%) tested positive, with 58 (52.2%) attributed to *P. vivax*, 28 (25.2%) to *P. falciparum*, and 25 (22.5%) attributed to mixed infections. In the 61 and above age group, 37.9% tested positive, with 52.3% due to *P. vivax*, 9.5% due to *P. falciparum*, and 38.1% as mixed infections. These findings indicate a significant variation in infection frequency and species distribution across age groups. Statistical analysis confirmed that the differences were significant across all age groups (p-values ranging from 0.01 to 0.04, with overall $p<0.01$), emphasizing age as an important risk factor in malaria epidemiology.

Gender-Based Frequency of Malarial Infection Across the Administrative Units

Table 2 showed the frequency of malarial infections among male and female patients across the various administrative units in Faisalabad district, Punjab, Pakistan. Our research results revealed a significant ($p<0.001$) variation in malarial infection among males and females in Faisalabad city, with a total of 134 (41.7%) positive cases reported, and males showing a higher rate of 80 (59.7%) compared to females with 54 (40.2%). The notable differences are that *P. vivax* is responsible for infection in 48 males (60.0%) and 34 females (62.9%), while *P. falciparum* causes infection in 14 males (17.5%) and 9 females (16.6%), out of a total of 23 (17.1%) positive cases.

Data from the administrative unit Faisalabad Saddar (Table 2) shows that in males, *P. vivax* 48 (63.1%) was the leading cause of malarial infection,

followed by *P. falciparum* 17 (22.3%) and mixed infection 11 (14.4%). In females, *P. vivax* 39 (73.5%) was also the leading cause of malaria, *P. falciparum* and mixed cases showed a similar proportion of 7 (13.2%). Overall, in Faisalabad Saddar administrative unit, 58.9% male cases and 41.1% female cases were reported. Out of 129 positive cases, *P. vivax* (67.4%) was the most prevalent, followed by *P. falciparum* (18.6%), and then both *P. vivax* and *P. falciparum* (13.9%). Likewise, in Jaranwala, the males were more susceptible than females, males accounting for 62.1% of the total cases and females accounting for 37.8%.

Moreover, Table 2 shows that in Jaranwala, Table 2 the frequency of *P. vivax* (52.2%) was highest compared to *P. falciparum* (26.1%) and mixed infection (21.6%). In three other remaining administrative units of District Faisalabad, such as Samundri, Tandilianwala, and Chak Jhumra, we identified the same pattern of *Plasmodium* frequency among males and females. In Samundri, infected males comprise 62.5% of the total positive cases in comparison to females, who account for only 37.5%. Moreover, we had identified 104 total positive cases in Samundri and out of the total, (59.6%) of these *P. vivax*, (24.0%) P. falciparum, and (16.3%) mixed cases were diagnosed.

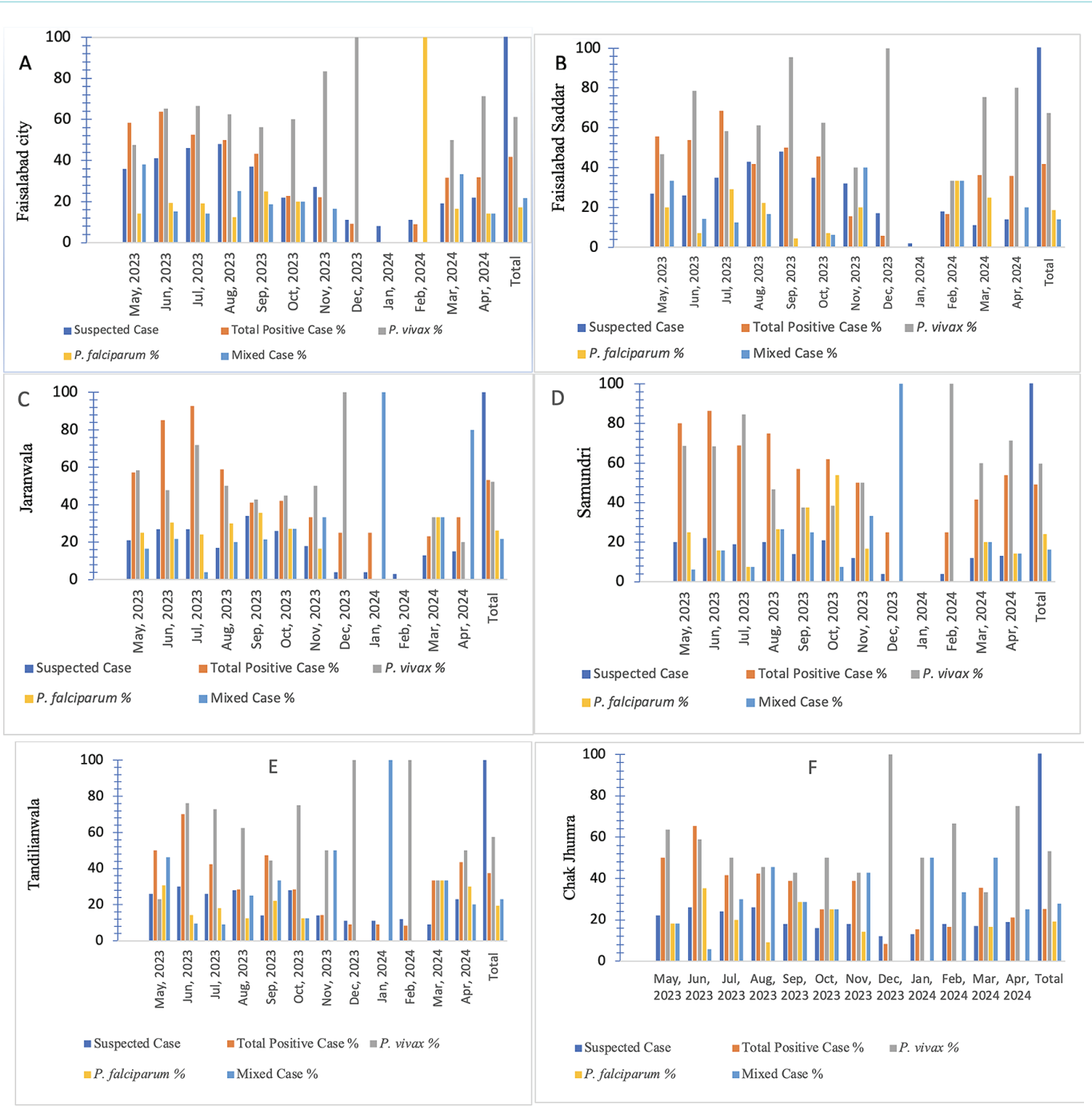

Figure 3. Monthwise frequency of malarial patients Faisalabad city (A), Faisalabad Saddar (B), Jaranwala (C), Samundri (D), Tandilianwala (E), Chak Jhumra (F).

Table 1. Age base (%) frequency of malarial infection

Age year group	Suspected case	Positive case (n, %)	*P. vivax* (n, %)	*P. falciparum* (n, %)	Mixed case (n, %)	p-value
1-15	178	111 (62.3)	80 (72.1)	13 (11.7)	18 (16.2)	0.04*
16-30	335	160 (47.7)	104 (65.0)	34 (21.2)	22 (13.7)	0.01*
31-45	470	204 (43.4)	109 (53.4)	53 (26.0)	42 (20.5)	0.01*
46-60	311	111 (35.7)	58 (52.2)	28 (25.2)	25 (22.5)	0.03*
61-above	166	63 (37.9)	33 (52.3)	6 (9.5)	24 (38.1)	0.04*
Total	1460	649 (44.4)	384 (59.1)	134 (20.6)	131 (20.1)	0.01*

*Significant

Table 2. Gender base (%) frequency of malarial infection in various administrative units

Species	Faisalabad city	Faisalabad Sadar			Jaranwala		Samundri		Tandilianwala		Chak Jhumra	
	Male (n, %)	Female (n, %)	Male (n, %)	Female (n, %)	Male (n, %)	Female (n, %)	Male (n, %)	Female (n, %)	Male (n, %)	Female (n, %)	Male (n, %)	Female (n, %)
P. vivax	48 (60.0)	34 (62.9)	48 (63.1)	39 (73.5)	37 (53.6)	21 (50.0)	42 (40.3)	20 (51.2)	39 (61.9)	12 (48.0)	30 (56.6)	14 (46.6)
P. falciparum	14 (17.5)	9 (16.6)	17 (22.3)	7 (13.2)	17 (24.6)	12 (28.5)	13 (12.5)	12 (30.7)	11 (17.4)	6 (24.0)	9 (16.9)	7 (23.3)
Mixed	18 (22.5)	11 (20.3)	11 (14.4)	7 (13.2)	15 (21.7)	9 (21.4)	10 (9.6)	7 (17.9)	13 (20.6)	7 (28.0)	14 (26.4)	9 (30.0)
Sub-total	80 (59.7)	54 (40.3)	76 (58.9)	53 (41.1)	69 (62.2)	42 (37.8)	65 (62.5)	39 (37.5)	63 (71.5)	25 (28.4)	53 (63.8)	30 (36.1)
Total	134 (41.7)	129 (41.8)			111 (53.1)		104 (64.6)		88 (37.5)		83 (25.2)	
Df	2	2			2		2		2		2	
F-value	55.135	119.031			22.031		26.375		10.798		15.047	
p-value	0.001**	0.001**			0.001**		0.002**		0.001**		0.001**	

**Highly significant

In Tandilianwala, Table 2 reveals a few disparities in the distribution of *Plasmodium* species between male and female cases. For instance, the *P. vivax* ratio (48.0%) was usually the max., but the infection rate for mixed cases was observed to be slightly higher (28.0%) compared to *P. falciparum* (24.0%) in females. The same pattern was observed in males, with *P. vivax* (61.9%), *P. falciparum* (17.4%), and mixed infection rate (20.6%). Overall, the infection rate among males and females was recorded as 71.5% and 28.4%, respectively. Moreover, across the municipality, Tandilianwala, *P. vivax* was predominant with a ratio of (57.4%), followed by mixed cases at 22.9%, and *P. falciparum* at (19.5%), out of a total of 88 positive cases.

In Chak Jhumra, the same pattern was observed as in the administrative unit Tandilianwala: the infection mixed cases ratio (27.7%) was recorded as high compared to that of *P. falciparum* (19.2%), and *P. vivax* infection rate (53.1%) was the highest out of the total 83 positive cases, as shown in Table 2. *P. vivax* infections were predominant, accounting for approximately 54.8% of all cases, followed by *P. falciparum* at 21.5%, and mixed infections at 23.5%. Males showed a higher frequency overall, comprising 62.5% of cases, compared to females who made up 37.5%. The present research results indicate that 406 (62.5%) males and 243 (37.4%) females out of 649 tested positive for malarial infection in the district of Faisalabad, Punjab, Pakistan. Statistical analysis showed this gender difference was significant ($p<0.03$), underscoring the relevance of gender-specific behavior and exposure patterns in malaria transmission.

Sensitivity and Specificity Analysis of Diagnostic Methods

Tables 3a and b summarize the diagnostic performance and species-specific detection across RDT, microscopy, and PCR methods. Of the 1,460 tested samples, RDT identified 384 (59.2%) *P. vivax* infections, 134 (20.6%) *P. falciparum* infections, and 131 (20.2%) mixed infections, whereas microscopy confirmed 281 (62.2%) *P. vivax* infections, 101 (22.3%) *P. falciparum* infections, and 70 (15.5%) mixed infections. PCR, used as the reference method, detected 266 (58.1%) *P. vivax*, 96 (21.1%) *P. falciparum*, and 97 (21.2%) mixed cases. The discrepancy was most notable in mixed infections, where microscopy underestimated them compared to PCR. Sensitivity and specificity values calculated using PCR as the reference standard revealed that RDT had 429 true positives, 55 false positives, 30 false negatives, and 946 true negatives, resulting in a sensitivity of 93.47% and specificity of 94.5%. In comparison, microscopy showed 435 true positives, 17 false positives, 24 false negatives, and 984 true negatives, yielding 94.78% sensitivity and 98.3% specificity. Statistical analysis confirmed significant differences among the diagnostic tools ($p<0.001$), validating that PCR consistently provided more accurate detection, particularly for mixed infections. These results demonstrate that while RDTs offer quick and useful preliminary diagnosis, confirmatory testing with microscopy and especially PCR is essential for accurate malaria detection and effective case management.

DISCUSSION

The current study, conducted from May 2023 to April 2024, provides a detailed analysis of the frequency of *Plasmodium* infection in various municipalities located in the district of Faisalabad, Pakistan. The present study showed that the frequency of malarial infection peaked in the months of June and July across the entire district of Faisalabad. For instance[27], reported a frequency of 21.3% in August, with a temperature of 34.2 °C, humidity 66.5%, and rainfall 6.64 mm, while the lowest frequency rate was recorded in January at 4.8% with a temperature

Table 3a. Sensitivity analysis of diagnostic methods (Percentages are calculated based on the number of positive cases for each method relative to the total tested population)

Species	Tested case	RDT %	Microscopy%	PCR%
P. vivax	384 (59.2%)	384 (59.2)	281 (62.2)	266 (58.1)
P. falciparum	134 (20.6%)	134 (20.6)	101 (22.3)	96 (21.1)
Mixed case	131 (20.2%)	131 (20.2)	70 (15.5)	97 (21.2)
Total	1,460	649 (44.4)	452 (30.6%)	459 (31.4)
Df		2	2	2
F-value		27.877	19.393	14.733
p-value		0.001**	0.001**	0.001**

**Highly significant.
RDT: Rapid diagnostic tests, PCR: Polymerase chain reaction.

Table 3b. Sensitivity and specificity of diagnostic methods (PCR as reference)

Method	Positive cases (n,%)	TP1*	FP2*	FN3*	TN4*	Sensitivity (%)	Specificity (%)
RDT	649 (44.4%)	429	55	30	946	93.47	94.5
Microscopy	452 (30.6%)	435	17	24	984	94.78	98.3
PCR (gold Std)	459 (31.4%)						

1* True positive; 2* False positive; 3* False negative; 4* True negative.
RDT: Rapid diagnostic tests, PCR: Polymerase chain reaction.

of 14 °C. During the summer months, the Faisalabad district region experienced heavy rainfall and temperatures ranging from 34 °C to 41 °C. That is why the high infection positivity ratios during the summer season were attributed to seasonal and climatic factors that increased mosquito densities in these Faisalabad district administrative units. Similar attributions were also recorded in the sub-Saharan Africa region.[28]

A total of 1,460 suspected malaria cases were tested across six administrative municipal units using three diagnostic methods: RDT, microscopy, and nested PCR. The highest positivity rate was observed with RDT (44.4%), followed by PCR (31.4%) and microscopy (30.6%), highlighting the tendency of RDTs to overestimate infection rates due to residual HRP2 antigen detection even after parasitemia clearance. Out of 1,460 samples, 649 (44.4%) were positive, with *P. vivax* 384 (59.1%), *P. falciparum* 134 (20.6%), and mixed 131 (20.1%) cases recorded. Our study reported a significantly higher frequency of *P. vivax* than *P. falciparum*. The present study results are in accordance with the findings of a previous study conducted in the Malakand district of Khyber Pakhtunkhwa province, which reported 59.57% *P. vivax* and 40.42% *P. falciparum* cases.[29] A previous study conducted in Khyber Pakhtunkhwa province, which also supports our findings, reported that the prevalence of *P. vivax* (92.4%) was highest compared to *P. falciparum* (4.7%) (18). Two previous studies conducted in India also exhibit the same pattern: *P. vivax* 55% and 41% were revealed higher ratio of infection showed higher ratios of infection at 55% and 41%, whereas *P. falciparum* 45% and 59% showed lower incidence showed lower incidences at 45% and 59%, respectively.[30,31] Another study conducted in District Umer kot, Sindh, Pakistan also reported that *P. vivax* had the highest incidence at 95% compared to *P. falciparum* at 4%, and 1% mixed cases.[32] The differences in our findings with respect to previous studies can be attributed to several factors, including regional ecological variations, differences in vector species composition, and diagnostic methodologies. Faisalabad's mixed urban-agricultural landscape, combined with higher population density and industrial activities, may influence vector ecology, favoring *P. falciparum* transmission and lowering the *P. vivax* incidence.

In two other previous studies, it had been demonstrated that researchers who used nPCR for the diagnosis of disease typically reported more accurate results as compared to those who depend commonly on RDTs and microscopy.[33,34] Microscopy, while shown to be a reliable method in malaria diagnosis, is extremely dependent on the technician's expertise and can miss low density infections.[35] RDTs had 95% sensitivity and 95.2% specificity for detecting PfHRP2, as reported by the World Health Organization in 2015. In our present study, we observed a higher rate of false positive cases with RDT compared to microscopy and PCR, underscoring the limitations of RDT in accurately identifying species-specific infections, particularly with the *P. falciparum*/*Pan-Plasmodium* RDT format. Prior comparative diagnostic research showed that nPCR outperforms other techniques, such as RDTs and microscopy, in producing accurate and sensitive diagnostic results, especially in cases of mixed infection.

The present research results indicate that 62.5% males and 37.4% females tested positive for malarial infection across the district Faisalabad, Punjab, Pakistan. Males were more prone to malarial infections due to their work in fields and exposure to mosquitoes. These reasons were also described in previous research papers that demonstrated that males were more susceptible than females due to their lifestyle factors and occupation.[36] Another previous study recorded the higher infection rate (54.7%) in males than (45.2%) in females, mainly infected by *P. vivax* (94.2%) and *P. falciparum* (5.6%), in district Kohat, which support our current results with little variations.[27]

Our current study determines the frequency across age groups and provides a comprehensive new analysis of malarial patients in district Faisalabad. We divided the data into five different age groups and found the max. frequencyies in the 1-15 age group, 16-30 age group, and 31-45 age group with infection rates of 62.3%, 47.7%, and 43.4%, respectively. As stated by Khan et al.,[16] our findings reveal that the children's age group had the highest infection rate when compared to others, owing to their lower immunity. The study also exhibited a 37% infection rate. A previous study conducted in Pakistan also

reported a high prevalence of malaria in the age group >14 years.[18] The study highlights a significant frequency of malaria in Faisalabad district, with *P. vivax* being the dominant species. The data indicate a higher susceptibility among males and children aged 1-15 years, particularly during the peak months of June and July. The nPCR method was found to be a highly efficient technique for diagnosing malarial infections, known for its superior sensitivity and specificity compared to other diagnostic methods. The findings underscore the importance of targeted malaria control and prevention strategies in high-risk groups and peak seasons.

Study Limitations

The limitations of this study include reliance on RDT-based positivity rates prone to false positives, restriction to government hospital data only, absence of multi-year trend analysis, lack of clinical and hematological correlation, and no assessment of mosquito vector dynamics.

CONCLUSION

This study demonstrates that while RDTs offer rapid malaria detection, they are prone to false positives due to persistent parasite antigens, particularly in low-transmission settings. PCR remains the most sensitive and specific method but is resource-intensive. Microscopy, despite being labor-intensive, still holds value when performed by trained personnel. A multi-method diagnostic approach tailored to local epidemiology may offer the most reliable results for accurate malaria diagnosis and control.

MAIN POINTS

- The frequency of malaria infection in Faisalabad district was 44.4%, with Plasmodium vivax identified as the dominant species.
- Rapid diagnostic tests showed higher positivity rates but were prone to false positives compared to microscopy and polymerase chain reaction (PCR).
- PCR demonstrated superior accuracy in detecting mixed infections and served as the most reliable diagnostic reference.
- Males and children aged 1-15 years were the most affected groups, with peak infection rates during June and July.
- Seasonal and regional variations highlight the need for confirmatory diagnostics and targeted malaria control strategies.

ETHICS

Ethics Committee Approval: The study was approved by the Ethics Review Committee of Government College University Faisalabad, Punjab, Pakistan (approval number: GCUF/ERC/460, date: 15.05.2023).

Informed Consent: Verbal informed consent was obtained from all participants prior to inclusion in the study.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: R.A., Concept: S.S., Design: R.A., Data Collection and/or Processing: S.S., Analysis and/or Interpretation: A.S., Literature Search: M.A., Writing: M.A.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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