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MORPHOLOGICAL, SEROLOGICAL AND MOLECULAR BASED STUDIES ON PLASMODIUM SPECIES IN DIFFERENT TERTIARY CARE HOSPITALS OF QUETTA, BALOCHISTAN

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Abstract

Malaria remains a major public health challenge in many regions of the world, including Pakistan. This study aimed to determine the prevalence of malaria and identify Plasmodium species in Quetta City, Balochistan, using samples collected from various tertiary care hospitals during summer 2023. A total of 135 malaria-suspected patients were enrolled and categorized into three age groups: G1 (1–15 years), G2 (16–40 years), and G3 (above 40 years). The sample size remained limited due to the high cost of PCR primers, CBC tests, RDT kits, and the short malaria transmission season. Overall, 32 patients (23.7%) were confirmed positive for malaria. The prevalence rates were 11.11% in G1, 46.66% in G2, and 13.33% in G3. Species distribution showed Plasmodium vivax as the most prevalent (62.5%, n=20), followed by Plasmodium falciparum (25%, n=8), and mixed infections (12.5%, n=4). Three diagnostic methods were used: PCR, RDT, and microscopy with staining. All 32 positive samples were detected by PCR (100% accuracy), 27 by microscopy (84.37%), and 30 by RDT (93.75%). The findings highlight the persistent malaria burden in Quetta and emphasize the need for precise diagnostic tools to determine species-specific malaria prevalence. These results may support improved malaria control and prevention strategies in Balochistan.

Key Words: *Accuracy, Malaria, Microscopy, PCR, Plasmodium*

INTRODUCTION

Malaria continues to pose a significant threat to global health, with ongoing obstacles hindering efforts to control and eliminate the disease. According to one research work conducted in 2003, there were 198 million cases of malaria in humans and that the malarial parasite was responsible for roughly 584000 deaths, the majority of which occurred in African nations (1). The World Malaria Report 2023 by the World Health Organization (WHO) indicates that in 2022, approximately 249 million individuals worldwide contracted malaria, leading to 608,000 deaths (2). *Plasmodium* species are the major cause of malaria, a serious worldwide health concern (3). There are several *Plasmodium* species that can cause malaria in humans, known as *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Other two species, *Plasmodium ovale wallikeri* and *Plasmodium ovale curtisi*, have also been discovered via recent study. Female infected anopheles mosquito transmits *Plasmodium* species, causing malaria in humans during feeding on the blood. Mosquito lives in humidity, high temperature, and stagnant water where they can breed and spread the disease (4). When a human contacts an infected mosquito, it causes malaria resulting in headache, fever, sometimes coma and even death if not treated. malaria causes hematological changes in the patient which are Leukocytosis, infected RBC with change in shape, anemia, cytokine induction and a low level of platelets (5). Ronal Ross in 1987 discovered some cells in the mid-gut of mosquitoes and they were not aware about these cells at that

time. Even now, it seems to be considered common knowledge. It is tough to suppose Ross could have performed this accomplishment in the 1800s, especially, in July 1897 with such basic techniques and tools. The fact that Ross meticulously researched almost a "thousand brindled, grey and white mosquitoes" for more than two years prior to his final achievement. His study was fortunate to be shared with colleagues and academics throughout the world when it was accepted for publication in the prestigious British Medical Journal (5). Malaria traits are found within the population's gene pool, occasionally leading to the development of resistance. Genetic adaptations to malaria should be considered in all historical studies of the disease within specific human populations, as these adaptations are clearly present (7). The Plasmodium has a sophisticated genome that allows it to alternate between two hosts. Resistance in the Plasmodium occurs through various Instinctive mutations which make the strain to less sensitive against the antimalarial drugs. When these drugs are applied on large population of Plasmodium, the resistance rate significantly increases as the parasites lose their sensitivity. Consequently, the resistant strains survive and proliferate. Most resistance-related mutations in Plasmodium arise during its erythrocytic stage, impacting the metabolism and effectiveness of the medications (8). Rapid diagnostic tests (RDTs), light microscopy, and nucleic acid detection techniques are currently used to diagnose malaria (9). Rapid Diagnostic Tests (RDTs) also known as Immuno-chromatographic techniques are used to identify parasites in human blood. Since they are rapid and simple to use, these methods are highly used for the diagnosing malaria especially in areas with low resources, and do not call for skilled staff or specialized equipment (10). The molecular identification of human malarial parasites frequently makes use of the nuclear small subunit (SSU) RNA genes. These genes range in copy number from 4 to 8 and it is known that they have highly preserved areas. The use of these genes in phylogenetic analyses and molecular exposure of Plasmodium species is appropriate (11).

MATERIALS AND METHODS

SAMPLE COLLECTION

A total of one hundred and thirty (135) blood samples from patients suspected of malaria were collected from different tertiary care hospitals at Quetta, Balochistan. Three age groups were selected for the study to observe the immune tolerance status of the patients against malaria; each group with equal number of blood samples. Group one included children aged 1 to 15 years; the second group comprised young patients aged 16 to 40 years; and the third group consisted of older patients above 40 years of age (Table 1). Three to five ml blood was collected in anti-coagulant (EDTA) tubes and were transported in disposable biohazard labeled containers. These samples were brought to lab and stored in refrigerator at 2 – 8 °C for further process.

MORPHOLOGICAL IDENTIFICATION OF PLASMODIUM SPECIES

For microscopy thick and thin blood smears were prepared. Thick blood films were fixed in methanol and were directly stained by Giemsa's stain.

THICK AND THINBLOOD SMEAR PREPARATION

For thick blood smear preparation, a small drop of blood was placed at the center of a clean slide and spread with an applicator stick or the corner of another slide to form a thick layer. The smear was stained with Giemsa stain for 30 minutes, gently washed with clean water, air-dried, and then examined under a 100× oil-immersion objective for Plasmodium species. For thin blood smear preparation, a thin film of blood was made and fixed with 70% methanol or ethanol, followed by air drying. The fixed films were then immersed in diluted Giemsa stain for 30 minutes, rinsed with clean water, air-dried, and subsequently examined microscopically for parasite identification (17, 18).

SEROLOGICAL SCREENING OF PLASMODIUM SPECIES ICT (RAPID DIAGNOSTIC TEST MERISCREEN MALARIA KIT METHOD)

For the rapid detection of malaria from whole blood, ICT (immunochromatographic test) MERIL kit was used. Five (5ul) of blood was added in the first well and 3 drops of reagent were added in the second

well. Results were observed after 15 minutes. The kit contains three lines, one for control, second for *Plasmodium* falciparum and third was used for mixed infection in which other types of malaria (*Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium vivax*) were included (19).

PCR-BASED DETECTION OF PLASMODIUM SPECIES

DNA from blood was extracted according to the manufacturer's instruction by using highly pure PCR template preparation kit. Specie specific primer sets were used in DNA thermal cycler for amplification. For *Plasmodium falciparum* (rFAL1) or (rFAL2), *P. vivax* (rVIV1) or *(rVIV2)* were used. Separate amplification reactions were performed for the detection of specific parasite specie. PCR reagent mixture (Primer, PCR buffer, Taq DNA polymerase, nucleotides) was used as instructed by the manufacturer. The reaction was taking place about 25 cycles. In first cycle DNA denaturation was take place at 95 °C for 5 minutes, next annealing took place at 58 °C for 2 minutes, next extension was taking place at 72 °C for 2 minutes, denaturation at 94°C for 1-minute, final annealing were take place at 58°C for 2 minutes and final extension was taking place at 72 °C for 5 minutes. Positive and negative controls were used for all *Plasmodium* species (21).

AGE-WISE PREVALENCE OF MALARIA

Age-wise prevalence in malaria patients was studied in relation to the immune tolerance status of the patients at different age levels. For this purpose, equal numbers of blood samples from three age groups including children (G1 = 1-15 years, n = 45), young (G2= 16-40 years, n = 45) and old age (G3 = above 40 years, n = 45) groups was collected.

Table I. Specific primers used in PCR for detection of malarial parasites

Species	Primer	Sequence (5′-3′)	Amplicon size (bp)
Plasmodium falciparum	rFAL1	TTAAACTGGTTTGGGAAAACC AAATATATT	205
	rFAL2	ACACAATGAACTCAATCATGA CTACCCGTC	
Plasmodium vivax	rVIV1	CGCTTCTAGCTTAATCCACAT AACTGATAC	120
	rVIV2	ACTTCCAAGCCGAAGCAAAGA AAGTCCTTA	

CONSEQUENCE OF MALARIA ON HAEMATOLOGICAL PARAMETERS

A hematology analyzer Sysmex Xp100 was used to assess complete blood count (CBC) parameters in order to evaluate the impact of malaria on hematological indices. The key parameters observed were platelet count and hemoglobin concentration (20).

RESULTS

A total of one hundred thirty-five (135) blood samples from patients suspected of malaria were collected from various tertiary care hospitals in Quetta. Of these, thirty-two (n=32) samples (23.7%) showed *Plasmodium falciparum* and *Plasmodium vivax* presence in different stages, including gametocytes and trophozoites. Serological screening of *Plasmodium* species was done using the RDT Merilscreen Malaria Kit Method. Samples were run with positive and negative control. Three strips are mentioned in the kit. *Plasmodium* species was reconfirmed by PCR using specific primers, and the results were positive for *Plasmodium vivax* and *Plasmodium falciparum*. The prevalence of malaria was also studied in 3 groups. Patients below 15 years of age were included in Group 1 (G 1). Those between sixteen and forty were included in group 2 (G 2), while patients above 40 years were included in Group 3 (G 3). The prevalence in group 1 was 5/45 11.11%, in group 2 21/45 46.66% and in group 3 it was 6/45 13.33%. It was observed that platelets count was less than 200 $\times 10^3/\mu l$ in all the malaria-positive patients, indicating the effect of *Plasmodium* on platelets and Hemoglobin level was also found low due to the rupturing of RBC Red Blood Cells. Among 32 positive samples all were positive in PCR (100% accuracy), 27 were positive in microscopy (84.37 accuracy) and 30

were positive in ICT kit rapid diagnostic test (93.75% accuracy). In total of 135 samples, 32 were positive for malaria parasite. In 32 positive samples 20 were positive for *Plasmodium vivax* (62.5%), 08 samples were positive for *Plasmodium falciparum* (25%) and 04 were positive for mix infection (12.5%) in PCR including both *Plasmodium vivax* and *Plasmodium falciparum* (Fig. 2).

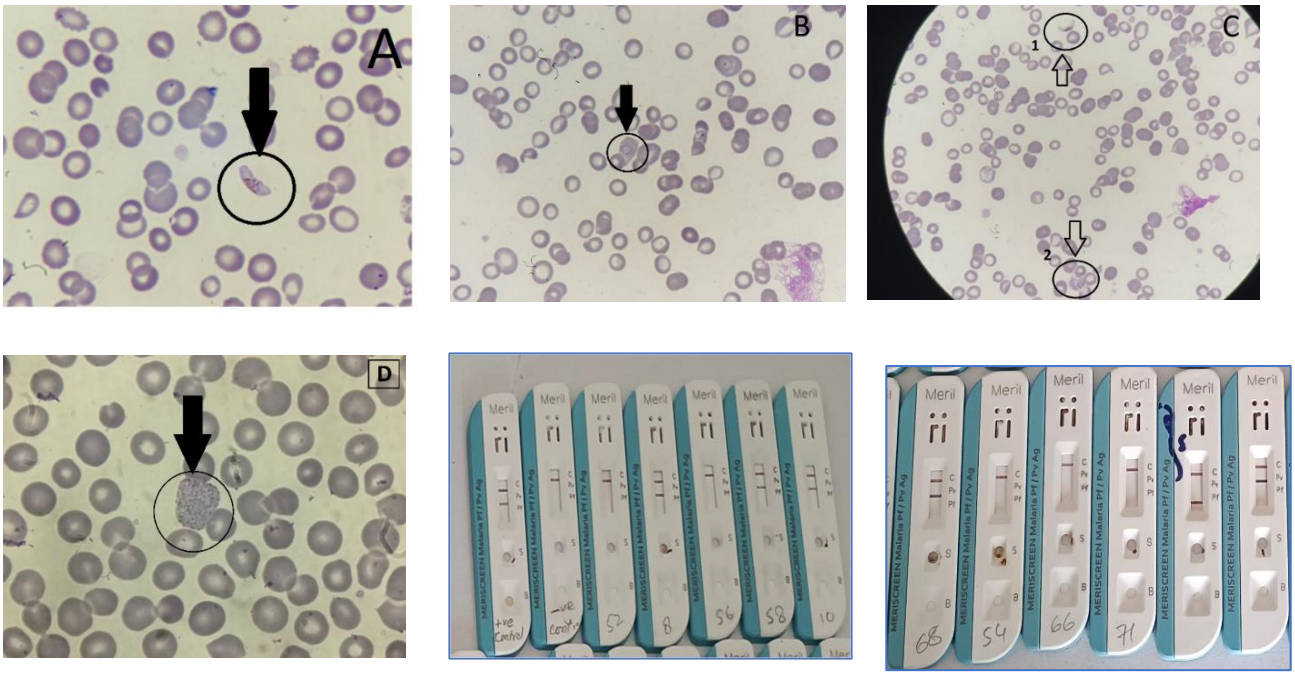

Fig. 1(a). *Plasmodium falciparum* gametocyte; **(b).** *Plasmodium falciparum* trophozoite; **(c).** Mix Infection i. *Plasmodium falciparum,* ii. *Plasmodium vivax;* **(d).** *Plasmodium vivax* gametocyte; **(e).** RDT kit result with positive and negative control; **(f).** Positive and negative results for *Plasmodium vivax* and *Plasmodium falciparum*

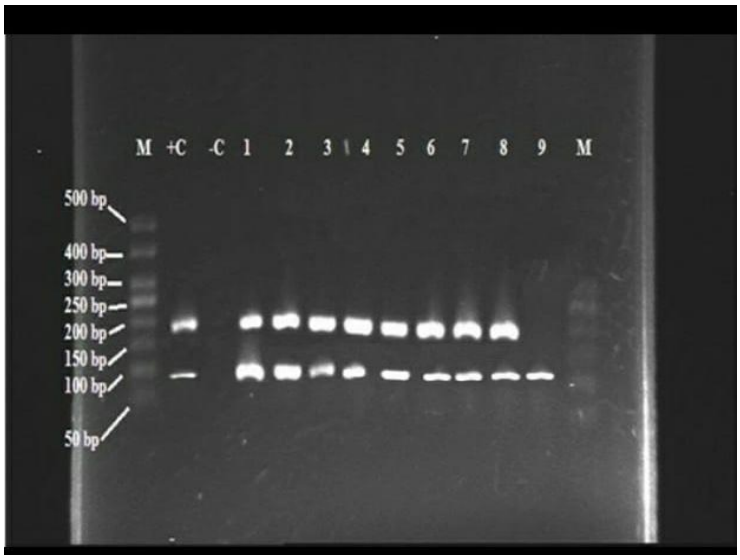

Fig. 2. Results of PCR for *Plasmodium falciparum (rFAL1 205bp), Plasmodium vivax* (rVIV1 120bp)

Table II. Age-wise prevalence of malaria

Group ID	Age range (years)	Number of samples	Number of positive cases
G1	Children (Below 15)	45	5/45 (11.11%)
G2	Young (16-40)	45	21/45 (46.66%)
G3	Old age (above 40)	45	6/45 (13.33%)
Total		135	135/32 (23.7%)

DISCUSSION

The research on malarial species (*Plasmodium vivax* and *Plasmodium falciparum*) was conducted in Quetta, Balochistan, Pakistan. Samples were collected from different tertiary care hospitals in the same city. Different

methods were used to compare the validity of the results, including CBC peripheral smears, both thick and thin for microscopy, RDT (Rapid Diagnostic Test) and PCR (Polymerase Chain Reaction). In this research, 135 samples were included in the research with 03 age groups (Group 1, Group 2, and Group 3), 45 samples in each group. In "Group 1," children were included from age 01-15 years. In "Group 2," people aged 16-40 years were included. In "Group 3," people ages above 40 years were included. In total of 135 samples, 32 samples were positive for malaria (23.7%) in which 20 samples were positive for *Plasmodium vivax* (62.5%) in PCR, 08 were positive for *Plasmodium falciparum* (25%) in PCR and 04 were positive for mix infection which include both the *Plasmodium vivax* and *Plasmodium falciparum* (12.5%) in PCR. The RDT (Rapid Diagnostic Test) kit method was used for fast and rapid detection which shows either the sample is positive or negative for *Plasmodium vivax, Plasmodium falciparum* or both with control test. In 32 positive PCR test, RDT (Rapid Diagnostic Test) percentage was 93.75%. In which 30/32 samples were positive for *Plasmodium vivax, Plasmodium falciparum* or mix infection. Staining and microscopy were used for visual confirmation of trophozoites and gametocytes of malarial species. Both thick and thin slides were observed during the procedure. In 32 positive PCR test, the percentage was 84.37% in microscopic examination, in which 27/32 samples were positive for malaria parasite. PCR (Polymerase Chain Reaction) method was performed to identify and confirm the positive and negative samples. All of 135 samples were performed on PCR in which 32 samples were positive with 100% high accuracy. This study supports the following research based on malaria prevalence across various regions.

Table III. Comparison of different diagnostic techniques in malaria

n= 32	Microscopy	RDT (rapid diagnostic test)	PCR
False Negative	5/32	2/32	0/32
Positive	27/32 (84.37%)	30/32 (93.75%)	32/32 (100%)
Plasmodium falciparum	7/27 (25.92%)	7/30 (23.33%)	08/32 (25%)
Plasmodium vivax	17/27 (62.96%)	20/30 (66.66%)	20/32 (62.50%)
Mix Infection (*Plasmodium falciparum* & *Plasmodium vivax*)	03/27 (11.11%)	3/30 (10%)	4/32 (12.50%)

Among the hematological parameters, patients who tested positive for malaria had low platelet counts (thrombocytopenia) and reduced hemoglobin concentrations (anemia) (20). However, study was performed on the prevalence of malaria in Pakistan by Khan., et al. (2023), in which they described the prevalence of malaria with 23.3 %, with specific rate of *Plasmodium vivax* at 79.13%, *Plasmodium falciparum* at 16.29%, and mixed infections at 3.98%. The results may vary a bit due to location as their research was performed in Khyber Pakhtunkhwa and data sample was collected over the five years (12).

The study was conducted in Ziarat District, Balochistan, Pakistan, and their data show a malaria prevalence of 26.8%, with Plasmodium vivax accounting for 69.5% and Plasmodium falciparum for 30.2%. The results closely resemble those of our own study (13). Another study was performed on recent Malaria reports (2023). Which concluded that a significant portion of malaria cases in Pakistan are attributed to *Plasmodium vivax*, with over 170,000 laboratory-confirmed cases, 77% of which are *Plasmodium vivax* infections? This suggests a shift in species dominance and calls for updated assessments in endemic regions (14).

Study was performed by Qureshi *et al.* (2020), which was a cross-sectional survey across three malaria-endemic districts: Dera Ismail Khan, Lakki Marwat and Bannu. Data were collected during malaria transmission seasons using a combination of rapid diagnostic tests (RDTs) and polymerase chain reaction (PCR) to confirm malaria infections. They gathered demographic and household data, including insecticide-treated net (ITN) ownership and healthcare access. The use of antimalarial drugs and the availability of malaria services were also assessed. In their study, 13.8% of participants tested positive for malaria. *Plasmodium vivax* positive patients were 92.4%, followed by *Plasmodium falciparum* 4.7% and mixed infections 2.9% (15).

The predominance of *Plasmodium vivax* as the leading cause of malaria in Pakistan, accounting for over 75% of cases, has been highlighted in multiple studies (WHO, 2022) (16). The most common malaria parasite in Pakistan is *Plasmodium vivax* responsible for about 75-80% of cases, while *Plasmodium falciparum* is about 20-25%. The study suggested the trend is consistent across most regions in Pakistan, but Balochistan report a slightly higher proportion of *Plasmodium falciparum* cases. Seasonal outbreaks are considered common especially in and after the monsoon season when mosquito breeding sites proliferate. In 2010, the Eastern Mediterranean region reported over one million malaria cases confirmed by microscopy, with Pakistan accounting for 22% of these cases. Malaria continues to be a serious public health problem in Pakistan. In 2008, 2.6 million malaria cases were reported nationwide with a mortality rate of 50,000 per year (14).

CONCLUSION

Malaria transmission is strongly seasonal, with mosquito populations and disease incidence rising during the warmer summer months. Among diagnostic tools, PCR offers the highest accuracy but is often inaccessible to many laboratories due to its high cost. Rapid diagnostic test (RDT) kits provide quick results, though their reliability may be compromised by false negatives, particularly when antimalarial drugs are used prior to testing. Microscopy remains the gold standard for malaria detection, offering both diagnostic confirmation and an estimate of peripheral parasite burden, but it requires trained personnel. Although this study did not assess antimalarial drug resistance, this remains an important area for future research.

Conflict of interest:

The authors declare no conflict of interest.

Authors' contribution:

SM Conducted the study, manuscript writing; MR & NR Data interpretation and critical analysis; FA, MY & NA Data analysis; SUJ Supervised and conceptualized the study.

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