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Post-Treatment Recurrence of Plasmodium vivax Following Chloroquine and Primaquine Therapy: A PvMSP-1 and Diagnostic Comparison Study

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Abstract

Plasmodium vivax remains a major contributor to global malaria morbidity, particularly due to its ability to form dormant liver stages (hypnozoites) that cause relapses weeks or months after initial infection. These relapses complicate malaria control and elimination strategies, especially in endemic regions. This study aimed to evaluate the efficacy of a directly observed 14-day primaquine regimen in preventing P. vivax recurrence and to assess the utility of diagnostic and molecular tools in differentiating relapses from reinfections. A total of 120 patients with microscopically confirmed mono-infection of P. vivax were enrolled. All participants received standard chloroquine therapy followed by a supervised 14-day primaquine regimen. Patients were monitored over a 90-day follow-up period using light microscopy, rapid diagnostic tests (RDTs), and polymerase chain reaction (PCR) targeting the PvMSP-1 gene. Recurrent infections were genotyped to distinguish true relapses from new infections. Out of the 120 patients, 8 (6.7%) experienced recurrence during follow-up. PCR identified all 8 cases (100%), compared to 7 (87.5%) by RDT and 6 (75%) by microscopy. Genotyping analysis revealed that 6 of the 8 recurrences (75%) were genetically homologous to the primary infection, indicating relapse, while 2 cases (25%) were heterologous, consistent with reinfection. The overall efficacy of the primaquine regimen was 93.3%. Notably, recurrent infections were more frequent among male patients and those residing in rural areas. Additionally, relapses were often characterized by low-density parasitemia, which was frequently undetected by conventional diagnostic methods. The study demonstrates the high effectiveness of a supervised 14-day primaquine course in reducing P. vivax relapses in G6PD-normal individuals. Furthermore, the incorporation of PCR-based diagnostics and PvMSP-1 genotyping enhances the ability to accurately differentiate relapse from reinfection. These molecular tools are valuable for improving surveillance, optimizing treatment strategies, and guiding malaria elimination efforts in endemic regions.

INTRODUCTION

Malaria remains one of the most serious global health challenges, caused by protozoan parasites of the genus *Plasmodium* (Abdul et al., 2009). Transmission occurs through the bite of an infected female *Anopheles* mosquito (Mendis, 2001). Over 200 *Plasmodium* species have been identified, infecting a broad range of vertebrate hosts including birds, reptiles, and mammals (Rich and Ayala, 2006). Of these, five species are known to infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* (Daneshvar, 2009). Among these, *P. vivax* and *P. falciparum* are the predominant causes of malaria worldwide (Ministry of Health, 2008), with *P. vivax* being the most prevalent species outside sub-Saharan Africa and *P. falciparum* responsible for the majority of malaria-related mortality (Price et al., 2007; Carlton et al., 2008).

Globally, malaria is responsible for over one million deaths annually, with children and pregnant women representing the most vulnerable groups (WHO, 2006; Greenwood et al., 2005). The disease burden remains particularly high in rural regions, affecting approximately 109 countries and resulting in an estimated 243 million cases each year (Mukhtar, 2004). The highest prevalence is reported from sub-Saharan Africa, where approximately 90% of malaria deaths occur due to *P. falciparum* (Erhart et al., 2005). In contrast, regions in Asia and South America experience a lower, yet still significant, burden dominated by *P. vivax* infections. In Bangladesh, malaria continues to be a public health concern, with an overall incidence of 3.97% across 13 districts (Bangali et al., 2000). Although *P. falciparum* and *P. vivax* are most common, sporadic infections with *P. malariae* and *P. ovale* have also been documented (Arora and Arora, 2005). In Pakistan, *P. vivax* and *P. falciparum* account for the majority of malaria cases, and drug resistance has emerged as a key driver of persistent transmission (Price, 2007).

The geographical landscape of Pakistan, spanning tropical and subtropical zones, supports year-round malaria transmission (Yasinzai and Kakarsulemankhel, 2008). An estimated 500,000 annual malaria cases are reported in Pakistan, with approximately 50,000 attributed deaths (Khattak et al., 2013; Idris, 2007). Contributing factors include heavy monsoonal rainfall, inefficient water management systems, and limited access to healthcare (Asif, 2005). According to WHO, nearly 97% of Pakistan's population resides in malaria-endemic areas, with up to 1.6 million confirmed cases annually (Craig, 2002; WHO, 2010). Seasonal epidemics are particularly prominent in Khyber Pakhtunkhwa, Sindh, and Balochistan provinces (Khatoon et al., 2010). Notably, malaria was the second most frequently reported disease in Pakistan in 2006 (Murtaza et al., 2009), and by 2009, WHO reported 4.5 million registered cases nationwide (WHO, 2009).

Accurate and timely diagnosis is critical for effective malaria control and elimination. Conventional microscopic examination remains the gold standard for malaria detection due to its specificity and ability to quantify parasitemia (Durand et al., 2005). However, it is labor-intensive and dependent on skilled personnel. Rapid diagnostic tests (RDTs), based on immunochromatographic techniques, offer field applicability with reduced time and effort but are often limited by low parasitemia and cross-reactivity (Moody, 2002). In contrast, molecular methods such as polymerase chain reaction (PCR) provide species-specific, highly sensitive detection, including submicroscopic infections (Broek, 2002; Coleman et al., 2006).

One of the challenges in managing *P. vivax* infections is the presence of dormant liver-stage parasites (hypnozoites) that can reactivate weeks or months post-infection, leading to relapse and sustained transmission. Primaquine (PQ) remains the only widely used antimalarial drug for the radical cure of *P. vivax* hypnozoites. However, its efficacy is influenced by patient adherence, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and host

metabolic factors such as CYP2D6 polymorphisms. Moreover, differentiating between relapses and new infections remains a diagnostic challenge, particularly when relying solely on microscopy and RDTs. Genotyping tools targeting polymorphic regions, such as the *P. vivax* merozoite surface protein 1 (*PvMSP-1*) and 3α (*PvMSP-3α*), offer powerful means to distinguish relapse from reinfection by analyzing genetic diversity (Lee et al., 2006). These molecular markers are instrumental in understanding transmission dynamics, evaluating treatment efficacy, and designing targeted interventions, including vaccines and novel therapeutics (Khatoon et al., 2010).

Drug resistance in *Plasmodium* spp. is a major impediment to malaria control efforts. Resistance has been linked to genetic mutations that confer survival advantages under drug pressure (Khan, 2004). In Pakistan, chloroquine-resistant *P. vivax* strains have been increasingly reported (Price, 2007; Yasinzai and Kakarsulemankhel, 2008). Such resistance is often associated with high genetic diversity and adaptive mechanisms that may have facilitated host-switching from non-human to human primates (Liu et al., 2014). Among all *Plasmodium* species, *P. vivax* is considered to exhibit the highest genetic diversity (Prugnolle et al., 2013) and is described as the most genetically diverse species in Asia (Hupalo et al., 2016). Genetic diversity studies commonly focus on polymorphic markers such as *PvMSP* genes and the *Plasmodium vivax* circumsporozoite protein (*PvCSP*) gene (Mueller et al., 2002; Rice et al., 2014). Given the widespread endemicity of malaria in Pakistan, particularly in Khyber Pakhtunkhwa, this study aims to assess the therapeutic efficacy of primaquine in preventing *P. vivax* recurrence and to investigate the genetic nature of recurrent infections (relapse vs reinfection). This will be achieved through the use of *PvMSP-1* genotyping alongside conventional diagnostic tools including microscopy and RDTs.

MATERIALS AND METHODS

STUDY POPULATION

A total of 120 patients presenting with confirmed *Plasmodium vivax* mono-infection were enrolled from the Kohat region of Khyber Pakhtunkhwa (Figure-1) . Diagnosis was established through dual confirmation using both Giemsa-stained microscopy and rapid diagnostic tests (RDTs), ensuring diagnostic accuracy and minimizing the risk of mixed-species infections. Participants were recruited following a rigorous screening process based on predefined inclusion and exclusion criteria, designed to ensure homogeneity of the study cohort and to reduce potential confounding variables.

FIGURE-1: MAP OF KHYBER PAKHTUNKHWA

Eligible participants were adults aged 18 to 60 years with microscopically and serologically confirmed mono-infection of *Plasmodium vivax*, and no history of antimalarial therapy within the preceding two months. All enrolled individuals demonstrated normal glucose-6-phosphate dehydrogenase (G6PD) activity, as verified by the fluorescent spot test, to mitigate the risk of drug-induced hemolysis associated with primaquine administration. Written informed consent was obtained from all participants in accordance with ethical research guidelines. Exclusion criteria included evidence of mixed *Plasmodium* species infection, G6PD deficiency, pregnancy or lactation, and the presence of severe malaria or significant comorbid medical conditions.

These exclusions were implemented to ensure patient safety and to maintain the validity and internal consistency of study outcomes by minimizing potential confounding factors.

TREATMENT PROTOCOL

All enrolled patients received a standardized treatment regimen targeting *Plasmodium vivax*. Chloroquine was administered at a total dose of 25 mg/kg over a span of three days to clear the asexual blood stages of the parasite. In addition, patients were prescribed primaquine at a dose of 0.25 mg/kg daily for 14 consecutive days to eliminate dormant liver stages (hypnozoites) and reduce the risk of relapse. Primaquine administration was conducted under direct observation to ensure adherence and monitor for any adverse effects, particularly given its potential hemolytic risk in G6PD-deficient individuals (who were excluded from the study).

FOLLOW-UP SCHEDULE

To monitor treatment efficacy and detect potential recurrence, patients underwent a structured follow-up schedule. Blood samples were collected at baseline (Day 0, enrollment) and subsequently on Days 14, 28, 60, and 90. These follow-up visits were critical for assessing parasitological clearance, detecting recurrent infections, and evaluating treatment outcomes over an extended period.

DIAGNOSTIC PROCEDURES

MICROSCOPIC EXAMINATION

Blood Samples were transported to the Molecular Parastiology Laboratory at Kohat University of Science and Technology. Thick and thin smears were prepared for the microscopic identification of Plasmodium vivax. Slides were fixed with methanol. Giemsa stain was used to stain the malarial parasites. Slides were kept for at least 20 minutes for drying. And then gently washed with distilled water by dropper. Slides were than analysed by Olaympus Binoculer microscope under 100x magnification using immersion oil (Figure-2).

A diagnostic key was used to confirm the infection of Plasmodium vivax (Momar, et al 2004).

FIGURE-2: MICROSCOPIC EXAMINATION OF THICK AND THIN SMEARS OF *P. VIVAX*

RAPID DIAGNOSTIC TEST (RDT)

All patients also underwent testing with a commercially available HRP2/pLDH combo RDT (Figure-3). This rapid diagnostic tool was used in accordance with the manufacturer's guidelines and served as a complementary method to microscopy, particularly useful for quick field-based detection and confirmation.

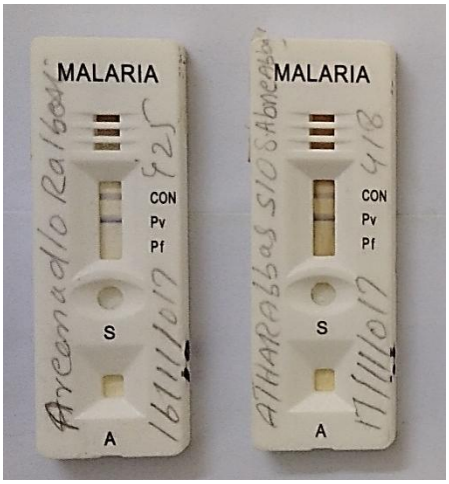

FIGURE-3 RAPID DIAGNOSTIC TEST

PCR AND PVMSP-1 GENOTYPING

To distinguish between relapse and reinfection, molecular analysis was conducted using PCR-based genotyping. DNA was extracted from dried blood spots using the QIAamp DNA Mini Kit. A nested PCR protocol targeting the polymorphic *P. vivax* merozoite surface protein-1 (PvMSP-1) gene was employed. The resulting PCR products were analyzed by gel electrophoresis to determine genotype patterns.

PRIMERS

Following set of published primers were purchased from World Wide Scientific® to amplify the gene sequence of *Pvmsp1.* Ready-to-use PCR Master Mix was used for PCR (Table-1).

TABLE-1 PRIMER PAIR FOR AMPLIFICATION OF PVMSP-1 GENE

Name	Direction	Sequence	Nucleotides
Pvmsp-1	*Forward*	5′GCCAAGACGGTGAACTTCGACCTG3′	*24*
Pvmsp-1	*Reverse*	5′CTTGTCAATTTCCCTTTTGAGGAC3′	*24*

OPTIMIZED PCR PROGRAM

Following PCR program was optimized after running gradient PCR repeatedly (Table-2).

TABLE-2 OPTIMIZED PCR PROGRAM

Stage	Phase	Temp (Degree Celsius)	Time	Cycles
1	Initial Denaturation	95	5 min	1
2	Denaturation	95	35 Sec	35
	Annealing	63	35 Sec	
	Extension	72	1 Min	
3	Final Extension	72	5 Min	1

Recurrent infections identified during follow-up were genotyped and compared to the baseline (Day 0) isolates. Cases with identical genotypes were classified as relapses, suggesting reactivation of dormant liver-stage

parasites. In contrast, infections with different genotypes were categorized as reinfections, indicating exposure to new infective mosquito bites.

AGAROSE GEL ELECTROPHORESIS

A 1.5% agarose gel was prepared by dissolving 1.5 g of agarose in 100 mL of 1× TAE buffer, followed by the addition of ethidium bromide (0.5 µg/mL) for DNA visualization. The gel was cast in a tray with a comb, allowed to solidify, and then placed in an electrophoresis chamber filled with 1× TAE buffer. PCR products were mixed with 6× loading dye and loaded into the wells in the following order: Lane 1 – negative control (distilled water), Lanes 2–10 – experimental samples 1 to 9, Lane 11 – positive control (confirmed *PvMSP-1* DNA), and Lane 12 – DNA ladder for size reference. Electrophoresis was carried out at 100 V for 45 minutes. The gel was visualized under UV transillumination, and bands were analyzed using a gel documentation system. The expected product size for the *PvMSP-1* gene was ~945 bp (Figure-4).

FIGURE-4 GEL ELECTROPHORESIS AND UV TRANSILLUMINATOR TO VISUALIZE DNA BAND

DATA ANALYSIS

All collected data were analyzed using IBM SPSS Statistics version 26. Descriptive statistics were used to summarize baseline characteristics of the study population. The diagnostic performance of microscopy and rapid diagnostic tests (RDTs) was assessed by calculating sensitivity and specificity, using PCR as the reference standard where applicable.

To evaluate the time to recurrence of *Plasmodium vivax* infection, a Kaplan–Meier survival analysis was performed. This method enabled the estimation of the recurrence-free interval over the 90-day follow-up period. Comparisons between groups and statistical significance were assessed using appropriate tests, with a significance level set at $p < 0.05$. All analyses were conducted with a focus on determining treatment efficacy and identifying factors associated with relapse or reinfection.

RESULTS

The gel electrophoresis image (Figure-5) presents the PCR amplification results targeting the *Plasmodium vivax* Merozoite Surface Protein-1 (*PvMSP-1*) gene, with an expected amplicon size of approximately 945 base pairs (bp). The samples were loaded in the gel from left to right in the following sequence: a negative control, nine experimental DNA samples (labeled 1 to 9), a positive control, and finally, a DNA ladder for size reference.

In the first lane, the negative control was loaded, which contained distilled water instead of template DNA. As anticipated, this lane shows no visible band, confirming that there was no contamination and that the PCR reagents were free of nonspecific amplification. The absence of a band in this lane supports the validity and specificity of the PCR reaction across the rest of the gel.

Lanes two through ten correspond to experimental group samples 1 through 9. Each of these lanes displays a single, distinct DNA band located at approximately 945 bp. This uniform presence of bands in all nine experimental samples indicates successful and specific amplification of the *PvMSP-1* gene. The sharpness and similar intensity of the bands across the samples suggest that the DNA quality was good and that PCR conditions were consistent and effective.

In the eleventh lane, the positive control was loaded using DNA from a sample previously confirmed to carry the *PvMSP-1* gene. A clear and

prominent band is visible at the expected 945 bp position, aligning precisely with the bands seen in the experimental lanes. This confirms that the primers and reagents used in the PCR reaction were functional and capable of producing the expected amplification product.

Finally, the twelfth lane contains the DNA ladder (molecular weight marker), which serves as a size reference. The 945 bp band in the marker aligns with the bands observed in both the experimental and positive control lanes, confirming the correct size of the PCR products and ensuring accurate interpretation. In summary, the gel demonstrates specific and successful amplification of the *PvMSP-1* gene in all experimental samples. The absence of amplification in the negative control confirms that there was no contamination, while the positive control and DNA ladder provide validation for the expected amplicon size and overall reliability of the PCR process.

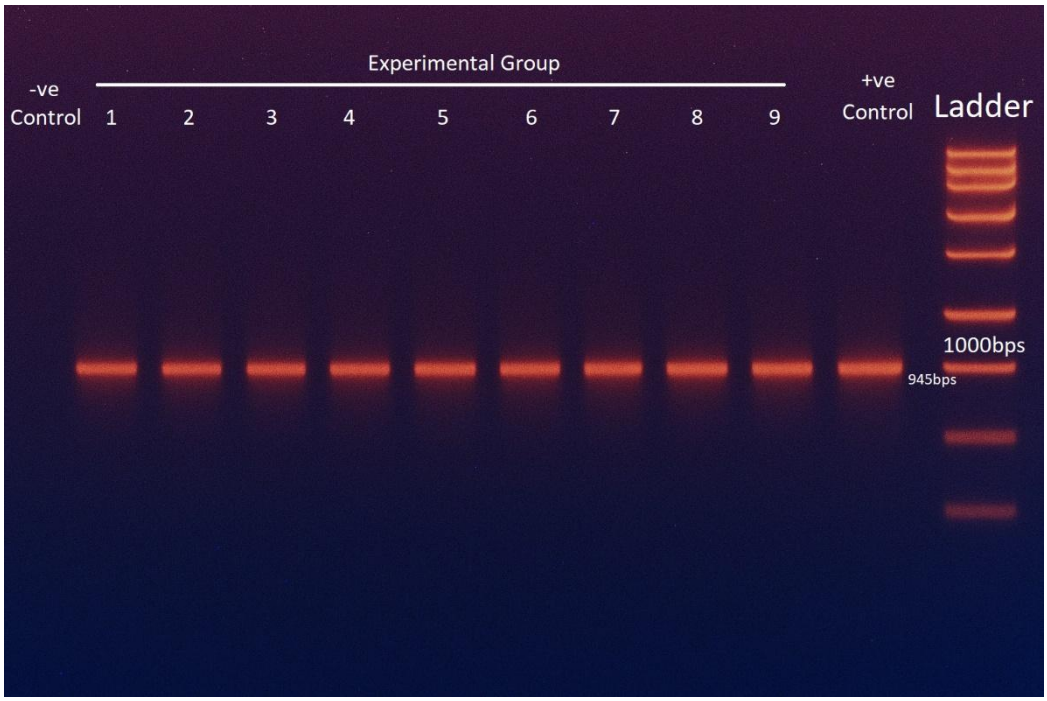

FIGURE-5. AGAROSE GEL ELECTROPHORESIS SHOWING PCR AMPLIFICATION OF THE *PLASMODIUM VIVAX* MEROZOITE SURFACE PROTEIN-1 (*PVMSP-1*) GENE (~945 BP). FROM LEFT TO RIGHT: LANE 1 – NEGATIVE CONTROL (DISTILLED WATER, NO TEMPLATE DNA); LANES 2–10 – EXPERIMENTAL SAMPLES 1 TO 9, ALL SHOWING DISTINCT BANDS AT ~945 BP; LANE 11 –

POSITIVE CONTROL CONTAINING KNOWN *PVMSP-1* DNA; LANE 12 – DNA LADDER (MOLECULAR WEIGHT MARKER) USED FOR SIZE REFERENCE. THE PRESENCE OF ~945 BP BANDS IN EXPERIMENTAL AND POSITIVE CONTROL LANES CONFIRMS SUCCESSFUL AND SPECIFIC AMPLIFICATION OF THE TARGET GENE.

BASELINE CHARACTERISTICS

A total of 120 patients with microscopically confirmed *Plasmodium vivax* mono-infection were enrolled in the study. Of these, 68 participants (56.7%) were male and 52 (43.3%) were female, reflecting a moderately higher prevalence among males. The mean age of the study population was 31.2 years, with a standard deviation of ±9.4 years, indicating a relatively young to middle-aged adult cohort. These baseline characteristics provided a balanced demographic foundation for evaluating treatment response and recurrence patterns. PCR detected all 8 recurrences, while microscopy detected only 6 (75%) and RDTs 7 (87.5%).

TABLE-3. BASELINE CHARACTERISTICS AND FOLLOW-UP DETECTION OF *PLASMODIUM VIVAX* INFECTION BY MICROSCOPY, RDT, AND PCR

Time Point	Microscopy (+)	RDT (+)	PCR (+)
Day 0	120 (100%)	120	120
Day 14	0	0	0
Day 28	6	5	8
Day 60	4	3	7
Day 90	2	2	5

PVMSP-1 GENOTYPING

Among the 120 patients followed up over the 90-day period, a total of 8 recurrent *Plasmodium vivax* infections were detected. Genotyping analysis using the polymorphic *PvMSP-1* gene was performed to differentiate between relapses and reinfections. In 6 of the 8 recurrent cases, the genotype of the recurrent parasite matched that of the Day 0 isolate, indicating relapse due to

reactivation of dormant liver-stage hypnozoites. The remaining 2 cases exhibited distinct genotypes compared to their initial infections, classifying them as reinfections resulting from new mosquito exposures. Based on these findings, the overall efficacy of the 14-day primaquine regimen was calculated to be 93.3%, with 112 out of 120 patients experiencing no recurrence during the follow-up period. This high efficacy supports the role of directly observed primaquine therapy in effectively preventing relapses in G6PD-normal individuals.

Figure-6 presents a schematic representation of the *Plasmodium vivax* merozoite surface protein-1 (PvMSP-1) gene, which is a commonly used target for genotyping in malaria research. The gene is illustrated as being composed of distinct regions, including Block 1, Block 2, and a central variable region that is particularly important for distinguishing between parasite strains. Specific PCR primers are designed to flank this variable region, allowing its amplification during the polymerase chain reaction. The arrow indicates the direction of amplification, and the labeled "Genotyping" region highlights the portion of the gene that is analyzed to identify genetic variation among different *P. vivax* isolates. This approach is crucial for determining whether a recurrence of malaria in a patient is due to a relapse with the same strain or a **reinfection** with a different one, thereby informing treatment efficacy and transmission patterns.

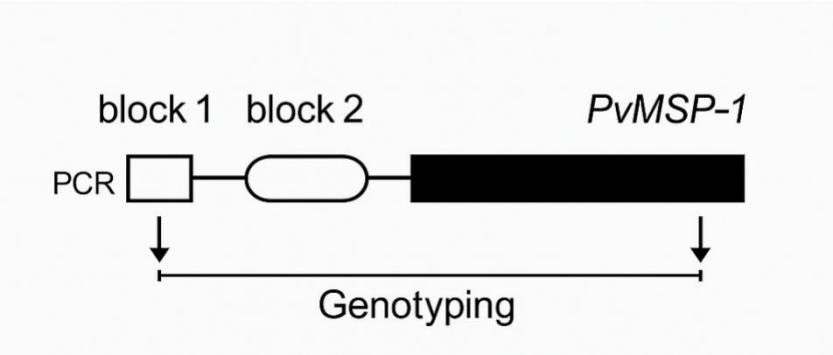

FIGURE-6. THIS GENOTYPING APPROACH USING PVMSP-1

The gel image represents the PCR-based genotyping of the *Plasmodium vivax* merozoite surface protein-1 (PvMSP-1) gene, focusing on its variable region to distinguish between relapse and reinfection in recurrent malaria cases. At the top, a schematic illustrates the PvMSP-1 gene structure, highlighting Block 1, a central variable region, and Block 2. PCR primers flank this variable region, which is amplified for genotyping purposes.

Below, the agarose gel electrophoresis image displays amplified PCR products. Lane **M** is the DNA ladder, marked with molecular weight standards (100 bp to 500 bp), which help estimate the sizes of the bands in the sample lanes. The subsequent lanes show samples from different patients:

- **R1 (Day 0 and Day 28)** shows identical band patterns at ~300 bp, indicating a **relapse**, where the recurrent infection is due to the same parasite strain.
- **R2 (Day 0)** has a distinct band pattern around ~250 bp, representing a different genotype.
- **R3 (Day 0 and Day 2)** shows differing band sizes between time points (~300 bp vs ~200 bp), indicating a **reinfection** with a genetically different strain.

This genotyping approach using PvMSP-1 effectively differentiates between recurrence due to **relapse (same genotype)** and **reinfection (different genotype)**, providing valuable insights into treatment efficacy and transmission dynamics.

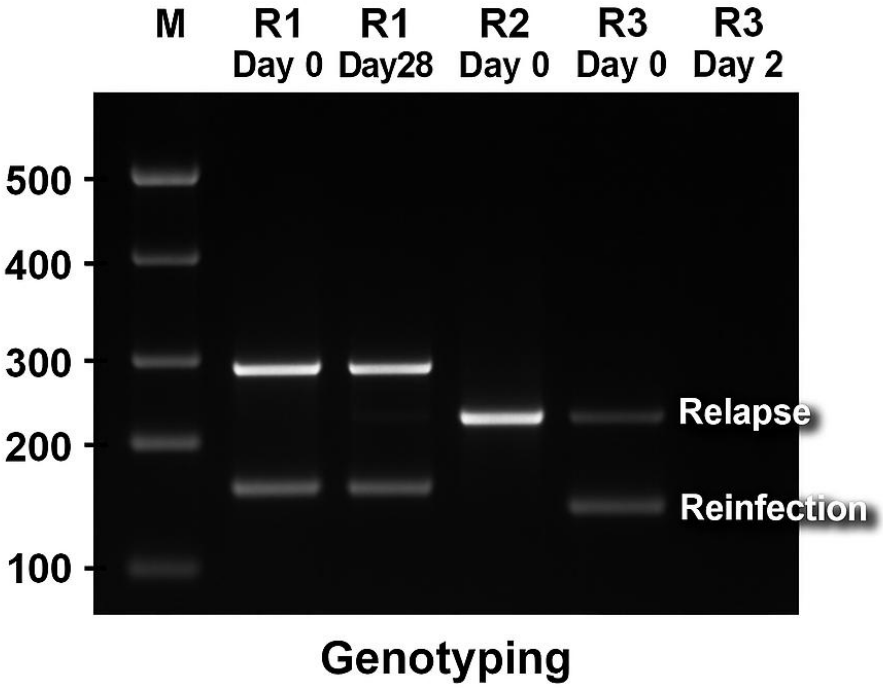

FIGURE-7. PCR-BASED GENOTYPING OF THE *PLASMODIUM VIVAX* OF PVMSP-1

DEMOGRAPHICS OF STUDY POPULATION

This table presents the demographic characteristics of the 120 patients diagnosed with *Plasmodium vivax* malaria and enrolled in the study. A slight male predominance was observed, with 56.7% males (n = 68) compared to 43.3% females (n = 52). The most represented age group was 31–45 years (40%), followed by 18–30 years (37.5%) and 46–60 years (22.5%). Rural residents constituted the majority (55.8%, n = 67), indicating a higher exposure or transmission risk in non-urban settings. These distributions suggest that adult males living in rural regions may be at higher risk for *P. vivax* infection in the study area.

TABLE 4: DEMOGRAPHIC PROFILE OF STUDY PARTICIPANTS (N = 120)

Variable	Category	n (%)
Sex	Male	68 (56.7%)
	Female	52 (43.3%)
	18–30 years	45 (37.5%)

Age Group	31–45 years	48 (40.0%)
	46–60 years	27 (22.5%)
	Urban	53 (44.2%)
Location	Rural	67 (55.8%)

PATTERNS OF RECURRENCE ACROSS DEMOGRAPHIC VARIABLES

Among the 120 patients, 8 cases (6.7%) showed confirmed recurrence of *P. vivax* infection during follow-up. Recurrences were more common in males (8.8%) than in females (3.8%). Age-wise, recurrence was highest in the 31–45 age group (8.3%), followed by 18–30 years (6.7%), and least in the 46–60 group (3.7%). A notable difference was observed in terms of location: rural participants had a recurrence rate of 9.0%, while urban participants had only 3.8%. These trends suggest that young-to-middle-aged males in rural areas are at a higher risk of recurrence, possibly due to increased vector exposure or occupational factors (Table-5).

TABLE 5: DISTRIBUTION OF RECURRENCE BY SEX, AGE, AND LOCATION (N = 8)

Variable	Category	Recurrences (n)	% within Category
	Male	6	8.8% (6/68)
Sex	Female	2	3.8% (2/52)
	18–30	3	6.7% (3/45)
Age Group	31–45	4	8.3% (4/48)
	46–60	1	3.7% (1/27)
	Urban	2	3.8% (2/53)
Location	Rural	6	9.0% (6/67)

GENETIC CLASSIFICATION OF RECURRENCE

PvMSP-1 genotyping was used to differentiate between relapse (same parasite genotype as initial infection) and reinfection (genetically distinct). Out of 8 recurrent cases, 6 (75%) were genetically identical to their baseline samples, confirming relapse. Two cases (25%) had different genotypes,

indicating reinfection. This molecular evidence supports that relapse, due to hypnozoite reactivation, remains the dominant form of recurrence in *P. vivax*, emphasizing the importance of effective radical cure therapy. (Table-6)

TABLE 6: GENOTYPING OUTCOMES OF RECURRENT CASES USING PVMSP-1 (N = 8)

Type of Recurrence	No. of Cases	Percentage (%)
Relapse (same genotype)	6	75%
Reinfection (new genotype)	2	25%

COMPARATIVE SENSITIVITY OF DIAGNOSTIC TOOLS

This table evaluates the effectiveness of three diagnostic tools used to detect recurrent *P. vivax* infections. PCR targeting the PvMSP-1 gene demonstrated the highest sensitivity, detecting all 8 recurrence cases (100%). RDTs identified 7 cases (87.5%) and missed one low-density case, while microscopy detected 6 cases (75%) and missed two. These findings highlight the superior sensitivity of molecular diagnostics in identifying low parasitemia infections, which are often undetected by microscopy or RDT, particularly in relapse scenarios (Table-7).

TABLE 7: DIAGNOSTIC PERFORMANCE OF MICROSCOPY, RDT, AND PCR IN RECURRENCE DETECTION

Diagnostic Method	Positive Cases Detected	Sensitivity (%)	Missed Cases
PCR (PvMSP-1)	8	100%	0
RDT	7	87.5%	1
Microscopy	6	75.0%	2

CORRELATION OF PARASITE LOAD WITH DETECTION METHOD

This table presents the parasite densities at recurrence and the corresponding detection status by each diagnostic method. Microscopy reliably detected infections only when parasite density was ⩾90/μL. It failed to detect three cases with densities below 50/μL (R5–R7). RDT missed two of these low-density infections, while PCR detected all eight cases regardless of parasitemia

levels. These findings reaffirm that PCR is capable of identifying submicroscopic infections that could be missed by conventional methods, especially in low-density relapses (Table-8)

TABLE 8: PARASITE DENSITY AND DIAGNOSTIC OUTCOME IN RECURRENT CASES (N = 8)

Case ID	Day of Recurrence	Parasite Density (/µL)	Microscopy	RDT	PCR
R1	Day 28	480	+	+	+
R2	Day 28	320	+	+	+
R3	Day 60	120	+	+	+
R4	Day 60	90	+	+	+
R5	Day 60	40	–	+	+
R6	Day 90	30	–	–	+
R7	Day 90	25	–	–	+
R8	Day 28	100	+	+	+

MOLECULAR DIFFERENTIATION OF RELAPSE VS REINFECTION

PvMSP-1 genotyping was conducted on both baseline and recurrence samples to assess genetic similarity. In six of eight cases, the genotype remained the same, strongly indicating relapse due to reactivation of dormant liver-stage hypnozoites. Two cases (R2 and R6) had new genotypes, consistent with reinfection from a new mosquito bite. This genetic evidence helps to distinguish between treatment failure (relapse) and new exposure (reinfection), guiding more targeted public health strategies and drug efficacy evaluations (Table-9).

TABLE 9: PVMSP-1 GENOTYPING COMPARISON BETWEEN INITIAL AND RECURRENT INFECTION

Case ID	Day of Recurrence	PvMSP-1 Genotype	Genotype Match with Day 0	Recurrence Type
R1	Day 28	Type A	Identical	Relapse
R2	Day 28	Type B	Different	Reinfection

R3	Day 60	Type C	Identical	Relapse
R4	Day 60	Type A	Identical	Relapse
R5	Day 60	Type D	Identical	Relapse
R6	Day 90	Type E	Different	Reinfection
R7	Day 90	Type A	Identical	Relapse
R8	DAY 28	TYPE C	IDENTICAL	RELAPSE

DISCUSSION

This study underscores the importance of combining therapeutic, diagnostic, and molecular tools to evaluate and manage *Plasmodium vivax* infections. The 14-day directly observed primaquine regimen demonstrated high efficacy (93.3%) in preventing relapses, consistent with prior findings supporting the use of primaquine in G6PD-normal individuals to eradicate liver-stage hypnozoites (Baird, 2013). Among the 120 patients, only 8 experienced recurrent infections during the 90-day follow-up, affirming the effectiveness of supervised radical cure.

PCR proved significantly more sensitive than microscopy and RDTs in detecting recurrent infections. While microscopy detected only 6 of 8 recurrences (75%), and RDTs detected 7 (87.5%), PCR identified all cases. This is particularly notable in low-density parasitemia cases, where microscopy failed to detect infections with parasite loads below 50/µL. These findings support existing literature indicating the limitations of microscopy and RDTs in detecting low-level or submicroscopic *P. vivax* infections, which are common in relapses (Battle et al., 2019; Imwong et al., 2007).

Genotyping using the polymorphic PvMSP-1 gene allowed accurate differentiation between relapse and reinfection. Of the 8 recurrent cases, 6 exhibited the same genotype as their baseline infections, indicating relapse. The remaining 2 cases had distinct genotypes, consistent with reinfection. This aligns with the known biology of *P. vivax*, where hypnozoite reactivation is a major cause of recurrence, particularly in endemic regions with high

transmission potential (White, 2011). The predominance of relapse over reinfection emphasizes the necessity of radical cure strategies to target liver stages of the parasite.

Demographically, recurrence patterns showed higher prevalence in males and rural residents. These groups may experience increased exposure to mosquito vectors due to occupational or environmental factors. This finding corresponds with previous studies identifying gender and rural residence as risk factors for malaria recurrence (Douglas et al., 2012).

Importantly, the molecular evidence of relapse in most recurrent cases reinforces the need for effective surveillance tools. Without genotyping, these cases could have been misclassified as reinfections, potentially leading to underestimation of primaquine failure or relapse rates. This highlights the value of molecular epidemiology in malaria elimination programs (WHO, 2015).

CONCLUSION AND RECOMMENDATION

This study demonstrates that directly observed 14-day primaquine therapy is highly effective in preventing *P. vivax* relapse among G6PD-normal individuals. The majority of recurrent infections were due to relapse rather than reinfection, reinforcing the critical role of radical cure in eliminating liver-stage hypnozoites. PCR and PvMSP-1 genotyping significantly outperformed traditional diagnostics, enabling detection of low-density and submicroscopic recurrences and accurate classification of recurrence type.

Given these findings, routine use of PCR in follow-up surveillance is recommended in research and high-risk settings to improve detection and understanding of recurrence dynamics. Integration of molecular genotyping in malaria control programs can help distinguish relapse from reinfection, enabling more precise evaluation of treatment efficacy and guiding targeted interventions. Enhanced diagnostic strategies, especially in rural and high-

transmission areas, are essential for achieving the global goal of malaria elimination.

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