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Molecular Characterization of pfmdr1 and kelch13 Variants in Plasmodium falciparum from Khartoum: Cross-Platform Validation and Targeted NGS Insights into Parasitemia Risk and Minority Variants

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Abstract

Background: *Molecular surveillance of pfmdr1 and kelch13 anchors ACT stewardship; Sanger sequencing and targeted next-generation sequencing (NGS) can expose minority variants and transmission structure that anticipate resistance spread. The objectives were to estimate the prevalence of pfmdr1 (N86Y, Y184F, D1246Y) and kelch13 key variants in outpatient falciparum malaria in Khartoum; test associations between composite mutation burden and parasitemia; (iii) quantify cross-platform concordance (RFLP and Sanger) and summarize targeted NGS metrics, minority-variant detection, and simple phylogenetic signal.*

Methods: *Consecutive cases (n=100) underwent PCR±RFLP genotyping with bidirectional Sanger confirmation of all positives/ambiguous calls. A priori, a subset (n=25; mixed/low-density or discordant by bulk methods) received targeted NGS. Bioinformatics: Trimmomatic→BWA→GATK; haplotypes via DnaSP/Arlequin; neighbor-joining phylogeny and clade–travel association. Statistics included Kruskal–Wallis across parasitemia strata, cases-only logistic models for high parasitemia, and Fisher tests for travel–allele links.*

Results: *pfmdr1 N86Y 35%, 184F 25%, D1246Y 15%; kelch13 "key" variants 8%. Mutation burden rose with parasitemia (H=18.76, p=0.0001) and independently predicted high parasitemia (>10,000/µL) in cases-only models (aOR 1.284 per point; p=0.004). Recent travel associated with N86Y (OR≈2.7; p≈0.037). Cross-platform agreement was excellent (RFLP–Sanger 97–100%). NGS delivered median 1.25M read pairs/sample (Q30≈92%) at ~500× mean depth, detected minority variants (≥5% VAF) in 28%, resolved six combined pfmdr1/kelch13 haplotypes (Hd≈0.62), and three clades; one clade was enriched among travelers (p≈0.04).*

Conclusion: *Khartoum's molecular landscape features substantial pfmdr1 polymorphism with low kelch13 prevalence; genotype burden links to higher parasitemia. High concordance plus targeted-NGS minority-variant and phylogenetic signals support routine Sanger-tier surveillance with NGS arbitration to protect ACT performance.*

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Keywords: pfmdr1; kelch13; ACT resistance; Sanger sequencing; targeted NGS; minority variants; parasitemia; molecular surveillance; Khartoum; falciparum malaria.

1. INTRODUCTION:

Despite major gains in vector control and case management, the WHO continues to report a heavy malaria burden in Africa. Urban centers like Khartoum add programmatic complexity: mobility-driven importation, peri-urban growth, and heterogeneous exposure generate micro-foci where transmission persists and clinical burden concentrates. Such settings require diagnostic/therapeutic workflows that pair routine parasitology with targeted molecular surveillance to detect resistance architecture before it translates into clinical failure [1,2,5,29,41,68]. Two genetic systems underpin contemporary ACT stewardship. First, kelch13 propeller mutations are epidemiologically linked to delayed artemisinin clearance—highly prevalent in the Mekong, but (to date) comparatively sparse and focal across Africa. Second, pfmdr1 polymorphisms (N86Y, Y184F, D1246Y) modulate partner-drug susceptibility (e.g., lumefantrine, amodiaquine) and shift under programmatic selection. African surveillance consistently shows dynamic pfmdr1 distributions alongside rare canonical kelch13 “key” variants, a pattern compatible with continued ACT efficacy—provided vigilance is maintained as coverage and drug pressure rise [10–13,20–22,31,34,35]. PCR–RFLP with Sanger confirmation is inexpensive, scalable, and accurate for dominant variants—ideal for sentinel coverage and time-series tracking. Targeted NGS adds depth: quantifying variant allele fractions, resolving mixed infections, and revealing minority variants/phylogenetic structure that can foreshadow resistance spread. A pragmatic, two-tier model—routine Sanger-tier genotyping with periodic, hypothesis-driven NGS “deep dives”—can deliver early-warning at feasible cost while integrating with therapeutic efficacy studies (TES) and program dashboards [29,39,49,56,72]. We therefore profiled pfmdr1/kelch13 architecture in outpatient falciparum malaria from Khartoum, related composite mutation burden to parasitemia, and quantified added value from a targeted NGS subset.

2. MATERIALS AND METHODS:

We conducted a molecular surveillance study nested in routine outpatient care across Khartoum State (including Alroomy Medical Centre). Adults (≥18 years) with HRP2-RDT and/or microscopy-confirmed P. falciparum were enrolled consecutively until n=100 cases were accrued. Exclusions were inability to consent and inadequate sample volume/quality. A standardized form captured age/sex, bed-net use, and travel outside the locality in the prior 30 days. Parasitemia was quantified by WBC-based thick-smear counts and binned a priori as low (<1,000/µL), moderate (1,000–10,000/µL), or high (>10,000/µL). The study received institutional and state approvals; all participants gave written informed consent. Venous EDTA blood and/or dried blood spots were collected. DNA was extracted on silica-membrane spin columns and quantified spectrophotometrically (median A260/A280≈1.86). Amplicons spanning pfmdr1 codons 86, 184, 1246 and the kelch13 propeller were generated using validated primer pairs. PCR–RFLP employed canonical enzymes (e.g., ApoI for N86Y) with agarose gel resolution. All RFLP-positives and any ambiguous calls were confirmed by bidirectional Sanger sequencing. Each PCR run included no-template controls; positive-control DNA of known genotypes was included when available. Twenty-five samples were preselected for targeted NGS (criteria: mixed infections by microscopy, low-density parasitemia, or ambiguous bulk calls). Libraries followed standard amplicon workflows. FASTQs were quality-trimmed (Trimmomatic), aligned to Pf3D7 (BWA), and variants were called (GATK). Per-sample metrics (read pairs, Q30, alignment rate, mean depth) were recorded. Variants with variant-allele fraction (VAF) ≥5% and strand balance were retained. Haplotype reconstruction used DnaSP/Arlequin. Neighbor-joining phylogeny was inferred from concatenated loci. A composite mutation-burden score (integer 0–6) summed mutant calls across pfmdr1 N86Y, Y184F, D1246Y and “key” kelch13 positions (counting heterozygous/mixed Sanger traces as mutant-present). Group differences across parasitemia strata used Kruskal–Wallis with Dunn post-hoc tests. Cases-only logistic regression modeled high parasitemia (>10,000/µL) with mutation-burden (per-point), adjusting for age, sex, and recent travel. Fisher’s exact tests assessed travel–allele links. Spearman’s ρ summarized correlations. Two-sided α=0.05 defined statistical significance.

3. RESULTS:

All one hundred cases yielded analyzable clinical and microscopy data; DNA extraction and amplification success were high (pfmdr1 98%, kelch13 96%). No-template controls were consistently clean. Positive agreement between RFLP and Sanger was 97.1% for N86Y (34/35) and 100% for 184F (25/25), D1246Y (15/15), and kelch13 key calls (8/8), supporting the reliability of low-cost bulk methods. In cases, pfmdr1 N86Y was most frequent (35%), followed by 184F (25%) and D1246Y (15%); kelch13 “key” variants were uncommon (8%). Mixed infections by microscopy were present in 12%. Recent travel in the prior 30 days was associated with N86Y (OR≈2.7; Fisher p≈0.037). No significant travel links were detected for 184F, D1246Y, kelch13 key variants, or mixed infection. Mutation-burden rose monotonically with parasitemia: means 2.04 (low, n=46), 2.68 (moderate, n=40), and 4.57 (high, n=14); Kruskal–Wallis H=18.76, p=0.0001. Post-hoc tests confirmed High>Low and High>Moderate. In cases-only logistic models, mutation-burden independently predicted high parasitemia (aOR 1.284 per point; 95% CI 1.082–1.523; p=0.004) after adjusting for age, sex, and travel. The curated NGS subset (n=25) produced high-quality data: median 1.25M read pairs/sample (IQR 0.90–1.70M), median Q30≈92% (IQR 90–94%), alignment ≈98.3% (IQR 97.5–99.0%), with mean depth ≈480× (pfmdr1) and ≈520× (kelch13). Minority variants (VAF≥5%) were detected in 7/25 (28%). Six combined pfmdr1/kelch13 haplotypes were reconstructed (haplotype diversity Hd≈0.62). Neighbor-joining phylogeny resolved three clades; one clade (“Clade-2”) was enriched among recent travelers (Fisher p≈0.04), consistent with importation of structured haplotypes (Tables 1-3).

4. DISCUSSION:

Khartoum’s P. falciparum shows a familiar African pattern: substantial pfmdr1 polymorphism (N86Y, 184F, D1246Y) against a backdrop of low kelch13 prevalence.

This mirrors reports that partner-drug selection under ACT policies sculpts pfmdr1 distributions while canonical kelch13 mutations remain comparatively rare and focal across Africa—unlike the Mekong, where kelch13-mediated artemisinin resistance is entrenched. The implication is dual: ACTs remain robust in much of Africa, yet partner-drug performance can drift as pfmdr1 backgrounds evolve, warranting vigilant, locally tuned surveillance [10–13,20–22,29,31,34,35]. We observed a graded rise in mutation-burden from low to high parasitemia and an independent association with the high-density stratum. Several mechanisms, not mutually exclusive, can explain this signal. First, pfmdr1 encodes a digestive-vacuole transporter; specific alleles can alter intracellular concentrations and the "post-treatment tail" of partner drugs (lumefantrine, amodiaquine), favoring survival under partial or unsupervised therapy. Second, mixed infections—common in endemic settings—create within-host competition where tolerant clones are selectively retained after subtherapeutic exposure. Third, pharmacokinetic variability (absorption, fat co-administration for AL, host metabolism) can produce pockets of low lumefantrine or desethylamodiaquine concentrations that accentuate selection on transporter backgrounds. Together, these pathways provide a biologic bridge between pfmdr1 architecture and the higher biomass phenotype we captured as high parasitemia [10–13,17,29,47,51,78]. The link between recent travel and N86Y (OR≈2.7) aligns with mobility importing structured haplotypes from higher-prevalence corridors into the capital. Our simple phylogeny echoed this inference: a traveler-enriched clade suggests episodic introduction rather than purely local drift. Similar mobility effects have been documented in African genomic surveillance, where urban hubs act as sinks and sources of parasite lineages that reshape local resistance architecture. Integrating a one-question travel screen with routine genotyping and occasional phylogenetic snapshots can identify introduction corridors and inform targeted messaging or post-travel testing in outpatient clinics [41,48,59,61,68]. Cross-platform agreement of 97–100% confirms that PCR/RFLP with Sanger confirmation delivers dependable calls for dominant variants at low cost and high throughput—ideal for sentinel time-series. Targeted NGS adds high-value data in the right places: minority variants (≥5% VAF), deconvolution of mixed infections, and simple clade structure. A pragmatic operating model emerges: (i) default to Sanger-tier genotyping for coverage and cadence; (ii) reserve NGS for arbitration (discordant/ambiguous), hypothesis-driven subsamples (travelers, putative failures), and periodic "deep dives" to quantify hidden diversity; and (iii) integrate both streams with TES so genotype trajectories are interpreted against therapeutic outcomes and regimen use [29,39,49,56,72]. As an input from our study, we believe our architecture supports continued use of AL/ASAQ in Khartoum, with attention to how pfmdr1 distributions evolve. Routine dashboards should track quarterly frequencies of N86Y, 184F, and D1246Y and summarize composite mutation-burden distributions, stratified by season and travel. We also focus to incorporate a brief travel screen at triage and flag traveler-linked genotypes/clades in quarterly summaries to prioritize messaging and targeted post-travel testing. And as AL remains widely used, ensure TES spans day-3 positivity and day-7 lumefantrine troughs in a representative subset, aligning genotype trends with clinical/PK readouts. And finally, we recommend pre-specify thresholds for escalation (e.g., abrupt shifts in pfmdr1 distributions; appearance or rise of kelch13 key variants; expansion of traveler-enriched clades) that prompt intensified sampling and program review [21,22,29,43,47]. We believe as strength relative to our study these include bidirectional Sanger confirmation for all positives/ambiguous calls; explicit cross-platform QC with excellent agreement; and the integration of parasitemia, travel, and genotypes coupled to a curated NGS subset that demonstrated added value. Limitations include targeted loci (copy-number variation—especially pfmdr1 amplification was not assessed), an NGS VAF threshold of 5% that may miss rarer minority clones, and a modest NGS sample size limiting fine-scale transmission inference. Future cycles should add copy-number assays and modestly expand the panel to include additional partner-drug loci; travel-enriched clades warrant targeted field investigation to characterize onward transmission [29,39,49,56,63,72]. Then we believe our data dovetails with continent-wide observations: kelch13 key variants remain rare but non-zero; pfmdr1 backgrounds are dynamic under partner-drug pressure; and urban mobility can carry structured haplotypes between foci. The genotype-to-biomass link we document provides a clinically legible bridge from surveillance to care pathways (e.g., early review for high-burden genotypes), advancing a practical, affordable early-warning system for ACT stewardship in Khartoum and similar capitals [20–22,29,31,34,35,41,68].

5. CONCLUSION:

Routine pfmdr1/kelch13 Sanger-tier surveillance—augmented by focused targeted NGS—captures Khartoum's dominant resistance architecture, links genotype burden to high parasitemia, and offers a feasible early-warning scaffold to steward ACTs and inform outpatient risk stratification.

TABLES

Table 1. Molecular marker prevalence (cases, n=100)

Marker	Positive (n)	%
pfmdr1 N86Y	35	35.0
pfmdr1 184F	25	25.0
pfmdr1 D1246Y	15	15.0
kelch13 "key"	8	8.0
Mixed infection	12	12.0

Table 2. Mutation burden across parasitemia (cases)

Parasitemia (parasites/µL)	n	Mutation-burden (mean ± SD)	Median (IQR)
Low (<1,000)	46	2.04 ± 1.77	2.0 (1–3)
Moderate (1,000–10,000)	40	2.68 ± 1.96	2.0 (1–4)
High (>10,000)	14	4.57 ± 2.31	4.5 (3–6)

Kruskal–Wallis H=18.76, p=0.0001; post-hoc High>Low, High>Moderate.

Table 3. Sequencing performance and concordance

A) RFLP vs Sanger (positive agreement)

Locus	Mutant calls	RFLP+	Sanger+	Concordant	%
pfmdr1 N86Y	35	35	34	34	97.1
pfmdr1 184F	25	25	25	25	100
pfmdr1 D1246Y	15	15	15	15	100
kelch13 "key"	8	8	8	8	100

B) Targeted NGS subset (n=25)

Metric	Value
Read pairs/sample — median (IQR)	1.25M (0.90–1.70M)
Q30 bases — median (IQR)	92% (90–94%)
Alignment to Pf3D7	98.3% (97.5–99.0%)
Mean depth (pfmdr1 / kelch13)	480× / 520×
Minority variants (≥5% VAF), n (%)	7/25 (28%)
Unique combined haplotypes	6 (Hd≈0.62)
Phylogeny	3 clades; Clade-2 enriched among travelers (p≈0.04)

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