

SUPPLEMENTARY MATERIAL

High burden of febrile sub-microscopic *Plasmodium* mixed-infections in central India – a cross-sectional study

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Details of molecular diagnosis by probe-based qPCR and nested PCR

Table S1: Description of probe-based qPCR

Target gene	Primers & probes	Sequence (5'-3')	Reference
18S rRNA (<i>Plasmodium</i> spp.)	Forward	GCTCTTTCTTGATTTCTTGGATG	Kamau et al 2013
	Reverse	AGCAGGTTAAGATCTCGTTTCG	
	Probe	FAM-ATGGCCGTTTTTAGTTCGTG	
18S rRNA (<i>P. falciparum</i>)	Forward	ATTGCTTTTGAGAGGTTTTGTTACTTT	Veron et al 2009
	Reverse	GCTGTAGTATTCAAACACAATGAACTCAA	
	Probe	FAM-CATAACAGACGGGTAGTCAT	
18S rRNA (<i>P. vivax</i>)	Forward	GCAACGCTTCTAGCTTAATCCAC	Kamau et al 2013
	Reverse	CAAGCCGAAGCAAAGAAAGTCC	
	Probe	VIC-ACTTTGTGCGCATTTTGCTA	
RNaseP (Human)	Forward	TGTTTGCAGA TTTGGACCTGC	Kamau et al 2013
	Reverse	AATAGCCAAGGTGAGCGGCT	
	Probe	VIC-TGCGCGGACTTGTGGA	

The reactions were set up in 96-well plate (Hard-Shell[®]; Bio-rad, US) in a final volume of 20µL, using 7.6µL template DNA, 400nM of each forward and reverse primers, 200nM of Taqman minor-groove binder or MGB probe (Applied Biosystems[™], Lithuania) and 2X TaqMan[™] fast advanced master mix (Applied Biosystems[™], Lithuania). The amplification reactions were carried out and analysed in CFX96[™] Real-Time System (Bio-rad, US).

The reaction conditions were as follow:

UNG activation*: 50°C/ 2 mins
 Polymerase activation: 95°C/ 1 min
 Denaturation: 95°C/ 5 secs
 Annealing/Elongation: 60°C/ 30 secs } 41 cycles

*UNG: Uracil-N-glycosylase

PCR: Polymerase Chain Reaction

qPCR: Quantitative Polymerase Chain Reaction

rRNA: Ribosomal RNA

RNase P: Ribonuclease P

P. falciparum: *Plasmodium falciparum*

P. vivax: *Plasmodium vivax*

Table S2: Description of nPCR

Target gene	Primers	Sequence (5'-3')	Reference
18S rRNA (<i>Plasmodium</i> spp.)	Forward	CCTGTTGTTGCCTTAAACTTC	Snounou et al 1993
	Reverse	TTAAAATTGTTGCAGTTAAAACG	
18S rRNA (<i>P. falciparum</i>)	Forward	TTAAACTGGTTTGGGAAAACCAAATATATT	& Johnston et al 2006
	Reverse	ACACAATGAACTCAATCATGACTACCCGTC	
18S rRNA (<i>P. vivax</i>)	Forward	CGCTTCTAGCTTAATCCACATAACTGATAC	
	Reverse	ACTTCCAAGCCGAAGCAAAGA AAGTCCTTA	

Step-1 (*Plasmodium* spp.) involved a 25µL reaction with 500nM of each forward and reverse primers, 2X Go Taq[®] Green master mix (Promega, US), 2µL DNA template and the remaining volume was nuclease-free molecular grade water (ThermoFisher scientific, US). In step-2 (separate reactions for *P. falciparum*/*P. vivax*), 1µL PCR product from step-1 was used as a DNA template, while concentration of the other components remained unchanged. The amplification reactions were performed in ProFlex[™] PCR System (ThermoFisher Scientific, US).

The reaction conditions were as follow:

Step-1 (*Plasmodium* spp.)

Initial Denaturation: 94°C / 5 mins
 Denaturation: 94°C / 1 min
 Annealing temperature: 60°C / 2 mins
 Elongation: 72°C / 2 mins
 Final Extension: 72°C / 7 mins

} 30 cycles

Step-2 (*P. falciparum*/*P. vivax*)

Initial Denaturation: 94°C / 5 mins
 Denaturation: 94°C / 1 min
 Annealing temperature: 55°C / 2 mins
 Elongation: 72°C / 2 mins
 Final Extension: 72°C / 7 mins

} 30 cycles

PCR: Polymerase Chain Reaction

nPCR: Nested Polymerase Chain Reaction

rRNA: Ribosomal RNA

P. falciparum: *Plasmodium falciparum*

P. vivax: *Plasmodium vivax*

Table S3: Malaria Situation in Madhya Pradesh & Bhopal (2020-2023)

Year	Site	BSE	BSE+RDT	Pf	Pv	Mixed	Total	Pf%	Pv%	Mixed%	Total% (BSE)	Total% (BSE+RDT)
2023	Bhopal	257881	406288	3	10	0	13	23	77	0	0.01	0.00
	MP	3150649	11530451	1169	2568	57	3794	31	68	2	0.12	0.03
2022	Bhopal	339884	426273	2	17	0	19	11	89	0	0.01	0.00
	MP	3752261	11031117	2376	1374	76	3826	62	36	2	0.10	0.03
2021	Bhopal	275510	394017	2	34	1	37	5	92	3	0.01	0.01
	MP	3610813	9864546	1635	1493	53	3181	51	47	2	0.09	0.03
2020	Bhopal	192759	274693	1	17	0	18	6	94	0	0.01	0.01
	MP	3990119	9056958	3914	2789	57	6760	58	41	1	0.17	0.07

The table presents the annual malaria prevalence data for Madhya Pradesh and Bhopal from 2020 to 2023. The data illustrate the temporal trends and relative species composition of malaria cases in both the state and district during the study period.

MP: Madhya Pradesh

BSE: Blood Smear Examination

RDT: Rapid Diagnostic Test

Pf: *P. falciparum*

Pv: *P. vivax*