A systematic review and meta-analysis on sub-microscopic *Plasmodium* infections in India: Different perspectives and global challenges

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Summary

Background The long-term maintenance of parasite biomass below the detection threshold of microscopy may stymie malaria elimination. Variation in microscopists' competencies to detect and correctly identify parasite species reflect in microscopy sensitivity, resulting in incorrect species-specific burden.

Methods The study estimated *Plasmodium* SMI pooled burden from published reports using a random effect model & identifies their hotspots in India. The study applied a prediction model for the first time on Indian data, emphasizing the importance of such models that can predict PCR-prevalence from slide- prevalence.

Findings A total of 17,449 samples from 39 districts were examined for *Plasmodium* by microscopy and PCR. The overall heterogeneity in clinic-based and community-based studies was 91% and 96%, respectively, with the pooled prevalence of 3.63%. The SMI prevalence in individual studies ranged from 38.4% to 0.4%. Sensitivity of microscopy for mono-*P. vivax* (91%) was found to be better than mono-*P. falciparum* (82 %). But surprisingly, it was much lower for mixed PfPv (45%).

Interpretation Primary regional data in the form of SMIs hot spots should be generated from countries on the verge of malaria elimination, and genetic monitoring should be integrated into national programs, particularly in key areas for successful malaria elimination.

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Introduction

The ability to comprehend real *Plasmodium* burden during routine epidemiological surveys is important for successful malaria surveillance, control and elimination. Delayed or inaccurate *Plasmodium* species diagnosis is detrimental, because it can prolong parasite clearance time and contribute to recrudescence and antimalarial drug resistance.The infectious reservoir constantly bears the potential of generating new clinical cases, putting successful elimination at constant risk. Regions harbouring such reservoirs may thus enhance the overall "malaria receptivity" of that region/s.[1–5] Therefore, irrespective of clinical symptoms, all individuals with demonstrable parasitaemia are advised to be considered as "malaria cases".[6]

Microscopy (MS) of peripheral blood smears still remains the 'gold standard' malaria diagnosis for malaria control programs primarily because of its technical simplicity and relative inexpensiveness as compared to much more sensitive and specific parasite nucleic acid based tests like PCRs. However, the use of MS is limited by its sensitivity and specificity for human infecting *Plasmodium* parasites and are largely dependent on the skills of microscopist and the technology *per se*. Since the limit of detection (LOD) of MS is between 10 and 100 parasites/μL blood,[7–9] the differential skills of microscopists impart "subjectivity" in malaria diagnosis. This not only generates a range of sensitivities in detection of *Plasmodium* in the blood smear but also compromises the specificity of microscopy in terms of identifying the parasite species correctly. The drawbacks with a range of sensitivities further jeopardizes the comparability of microscopy-based malaria burden across a region and also generates the possibilities of detecting varying levels of sub-microscopic *Plasmodium* infections (SMIs), when compared with much more sensitive Nucleic Acid Amplification Test (NAAT). The outcomes of a compromised specificity manifest as

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Research in context

Evidence before this study

Evidence before this study: No exhaustive and targeted study has been reported that synthesized the data for generating robust granular evidence for sub-microscopic infections for all 5 *Plasmodium* species in India. Although individual studies have been reported in scanty, they were either restricted to certain specific Indian region and/or *Plasmodium* species.

Added value of this study

Apart from in-depth compilation of quality evidence on SMI in India for the first time, this study brings forth two critical issues for wider interest with respect to SMI: validation of a predictive model to estimate PCR prevalence of *P. falciparum* and *P. vivax* infections on the basis of their slide prevalence and identification of SMI hotspots in the country that are of value for microplanning regional malaria elimination efforts.

Implications of all evidence available

The spatiotemporal estimates for *Plasmodium* species-specific SMI raise cautionary flag for the malaria control programs to delve deeper into pan-species malaria epidemiology with respect to certain critical issues. These include regional SMI burden and hotspots, quality of microscopy, reporting and analysis of SMIs, use of predictive model and possibilities of including molecular surveillance for malaria in selected priority areas.

misidentification of the parasite species infecting a person that might result in sub-optimal representation of certain parasite species in that area thus undermining the species-specific regional *Plasmodium* burden. The LOD for PCR-based methods range from 0.022 (ultrasensitive PCR) to 5 (standard PCR) parasites/μL blood.[7–12]

Because microscopy is widely used in epidemiological surveys and routine passive case detection in India, the contribution of sub-microscopic and low-density *Plasmodium* infections (< 100 parasites/μL of whole blood) to the total burden of malaria is underestimated and remains poorly understood in terms of total and species-specific burden of the known human infecting *Plasmodium* species.

In context of global malaria elimination, India is considered as an important country due to its high contribution (83% cases in the South-East Asia Region) to the global malaria burden.[13] The targeted malaria elimination (by 2030) in India might be difficult to achieve until the true burden of *Plasmodium* is known. The success of malaria elimination program would ultimately rely on the ability to find and manage all the *Plasmodium* reservoirs.

The hidden burden of *Plasmodium* SMI had been revealed by some studies but they are either restricted to either a particular *Plasmodium* species or a certain geographical area. Therefore, the current study aims to uncover the burden *Plasmodium* SMI mixed infections from published reports and determine if a certain combination contributes more to the SMI burden. The present study was done on published reports that could estimate the pooled burden of *Plasmodium* species SMIs (either as mono- or mixed-infections) in different geographical areas within India. The study also compares the sensitivity and other diagnostic characteristics of MS and identifies challenges related to SMIs.

The outcomes of the study are crucial for understanding and remodelling of the current diagnostic and therapeutic measures under malaria elimination programs.

Methods

A. Search strategy and data screening

Published reports were screened from a previously generated primary database for mixed *Plasmodium* infection (manuscript in preparation) using the following search terms in title and/or abstract in advanced PubMed® search engine (in April 2020): "Mixed AND malaria", "Mixed AND *Plasmodium*", "Mixed AND malaria parasite", "Malaria AND co-infection", "*Plasmodium* AND co-infection", "Malaria parasite AND co-infection", "*Plasmodium* AND co-existence", "Malaria parasite AND co-existence", "Malaria AND super-infection", "*Plasmodium* AND super-infection", "Malaria parasite AND super-infection", "Malaria AND multiple infection", "*Plasmodium* AND multiple infection", "Malaria parasite AND multiple infection", "*Plasmodium* AND multi-species infection", "Malaria parasite AND multi-species infection", "Mixed AND malaria AND clinical trial", and "Mixed AND malaria AND therapeutic efficacy". To supplement, advanced Google Scholar® was also searched using the search terms "Mixed Malaria" and "Mixed *Plasmodium*" in title.

All the studies from the primary database that included data from India and allowed calculation of either proportion or prevalence of mixed *Plasmodium* species by any diagnostic method/s formed the secondary database for the current study. Following inclusion criteria were applied to select the eligible studies:

1. Studies where microscopy and PCR both were applied for diagnosis of species
2. Studies where microscopy and PCR both were performed on the same set of samples and the species-specific data for microscopy and PCR were available
3. Studies where total number of samples screened for malaria were available

Additionally, relevant studies were also included from cross-references of the included studies and from other sources.

The eligible studies were supplemented with additional search specific for *Plasmodium* mixed SMIs (November 2020) from advanced PubMed® using the following search terms in "all fields": "Sub-microscopic *Plasmodium* Mixed Infection AND India" and "Sub-microscopic *Plasmodium* Infection AND India". The protocol for this systematic review was registered on the International prospective register of systematic reviews (PROSPERO; CRD42021234278).

B. Study selection and data extraction

Two reviewers (ND & AS) independently screened the titles and abstracts of all the eligible studies and all discordant cases were resolved after discussing with third reviewer (VP). Though the study was designed to capture *Plasmodium* mixed infections, mono infections were also recorded. Data from a particular time-point (year) and a particular location (States / UTs) was treated as a single data point, so that if one particular State reported SMI in two different years, it was treated as two data points.

In case of any clarification, the corresponding authors of respective studies were contacted by email/phone call and their responses were used to rectify the data accordingly, so that comparability is ensured. Data that remained unclear despite email/phone confirmation were excluded.

C. Categorization of studies

Included studies were grouped into clinic-based (passive data from symptomatic individuals) and community-based (individuals actively screened for malaria) studies depending on the source of data collection.

D. Risk of bias assessment

All studies were assessed using risk of bias tool specifically developed for quality of prevalence studies.[14] The tool consists of ten questions designed to assess the internal and external validity of the included studies. The overall risk of bias was classified as low (0-3), moderate (4-6), or high (7-9) based on the responses. In addition to that, Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool was also used to evaluate the risk of bias and applicability of the included studies[15] wherein the bias is interpreted as low, high and unclear.

E. Data outcome measures

Prevalence

Plasmodium SMI prevalence data across different studies (for individual data-points) were extracted as proportion of the number of sub-microscopic *Plasmodium* infected individuals to the total number of samples screened for *Plasmodium* and expressed as percentage wherever needed.

Relative prevalence of *Plasmodium* infection by microscopy and PCR

In order to assess the relationship between microscopy and PCR prevalence, scatter plots were constructed using prevalence by each of the methods. To estimate the prevalence of *Plasmodium spp*. detected by PCR and microscopy independently, the number of *Plasmodium* infected persons detected by each method (microscopy & PCR) was divided by the total number of individuals screened for malaria. Further, the prevalence ratio was calculated as a ratio of SMI prevalence by microscopy to that by PCR, and the results were depicted as box and whiskers plots.

Prediction of PCR prevalence from microscopy prevalence

Using a regression model (developed by Okell et al.),[16] the data was used to predict PCR prevalence from microscopy prevalence and the results were plotted on a scatter plot. The correlation coefficient (r) and coefficient of determination (r^2) between observed and predicted PCR prevalence values were calculated using 'Pearson Correlation Coefficient Calculator' (www.socscistatistics.com/tests/pearson).[17]

Diagnostic performance of MS

The Sensitivity (SEN), Specificity (SP), Positive Predictive Value (PPV), and Negative Predictive Value (NPV) of MS as compared to PCR were estimated as below and organized state-wise for each *Plasmodium* species (separately for mono and mixed). Although MS is considered gold standard for malaria diagnosis but for evaluation of two diagnostic platform (MS & PCR) in particular detection of SMIs, PCR has relatively lower LOD and hence, considered as gold standard.

SEN=CP/(CP+FN) *100
SP=CN/(CN+FP) *100
PPV=CP/(CP+FP) *100
NPV=CN/(CN+FN) *100
CP=Concordant Positive (Samples tested positive by both MS & PCR for that particular species)
CN=Concordant Negative (Samples tested negative by both MS & PCR for that particular species)
FP=False Positive (Samples tested positive by MS but negative by PCR for that particular species)
FN=False Negative (Samples tested negative by MS but positive by PCR for that particular species)

Meta-analysis

All statistical analyses and visualization were carried out using "tidyverse", "meta", "metafor" and base R packages using the 'R' programing language and free software version 3.5.2.[18] Random effect and fixed effect models were used as per heterogeneity level. Assessment of heterogeneity was based on the I^2 statistics, when I^2 was more than 50% (substantial heterogeneity), Inverse variance random effect model was used for the pooled estimates. A forest plot was created with a 95 % confidence interval to depict the individual and pooled SMI prevalence.

Results

Search criteria are shown in Figure 1 where 9 studies were finally included. All the nine studies had low bias and 5 studies were multi-centric, hence the total number of data points is more than 9. Overall, 17,449 samples from 39 districts (13 states and UTs) were screened (during 2007-2018) for the presence of *Plasmodium* by microscopy and PCR both generating 29 data points for SMI prevalence (Figure 2A-B & Table 1) and 13 data points for relative prevalence of SMI by microscopy and PCR (Table 1, Figures. 3–6).

From Figure 2A-B and Table 1, it becomes obvious that the distribution of geographical areas from which data were collected is not uniform. Single data point was available from the following states with only 1 study for each state was found: Andhra Pradesh (AP), Bihar (BR), Punjab (PB), Uttar Pradesh (UP), Delhi (DEL) and Karnataka (KA). Two data points in 2 studies were observed for Assam (AS), Chhattisgarh (CG) and Maharashtra (MH) whereas 3 data points from 2 studies (Gujarat and Madhya Pradesh) and 4 data points from 3 studies (Tamil Nadu) were also observed. Maximum 7 data points from 5 studies were observed for Odisha (OD) which reflects the focus of research on high malaria burden states. However, other high burden states like Jharkhand (JK), West Bengal (WB) and other

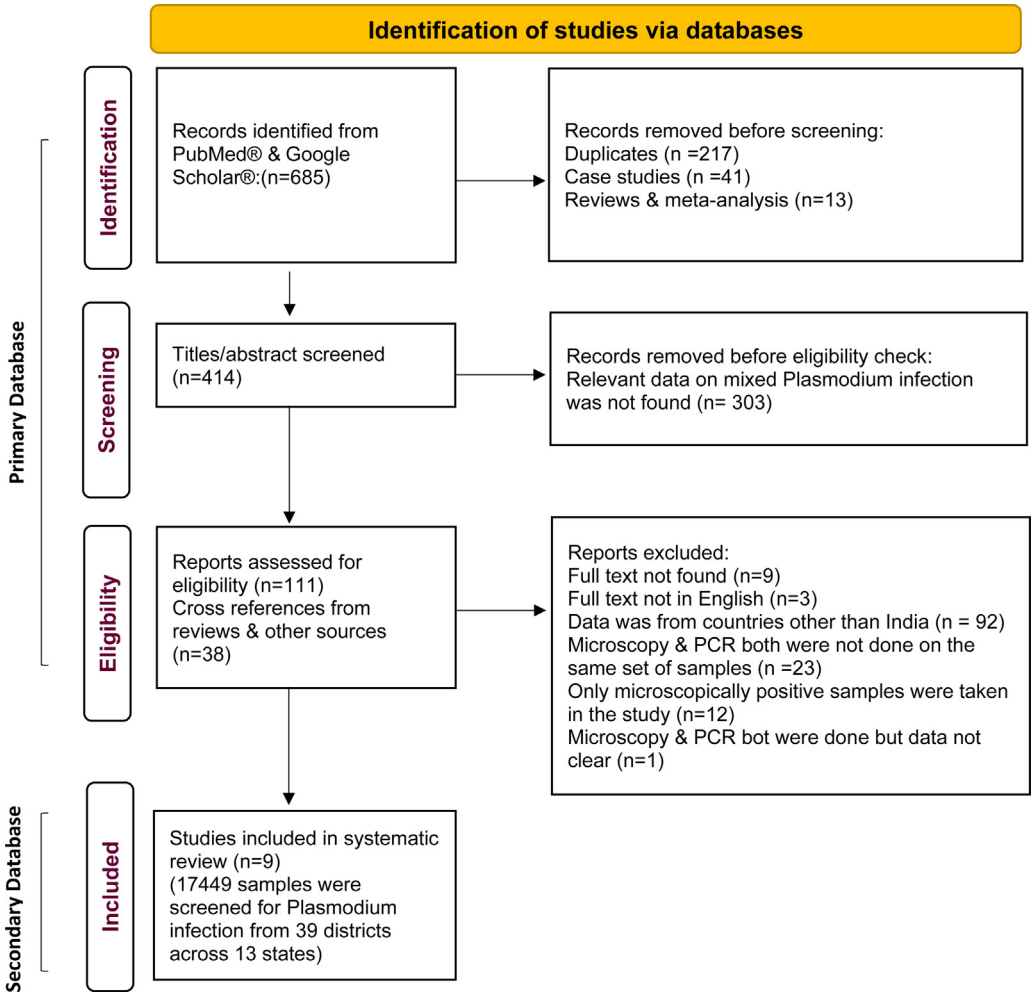

Figure 1. Selection process for systematic review on sub-microscopic *Plasmodium* mixed-infections.

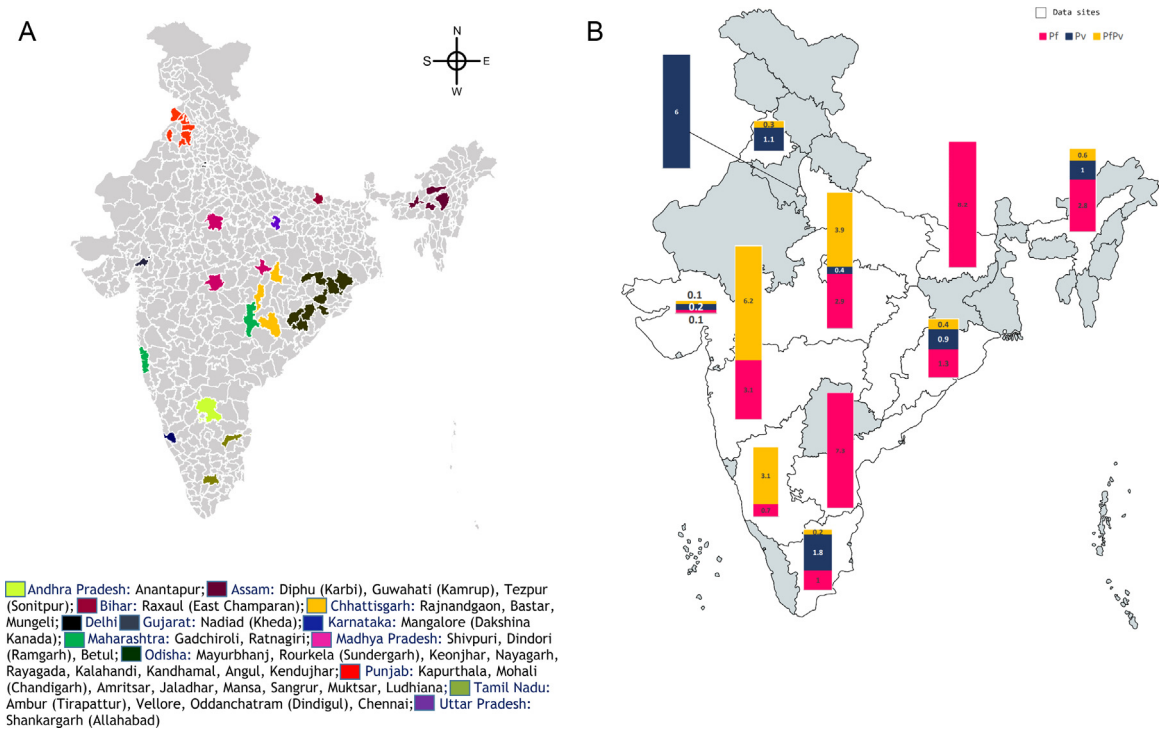

Figure 2. A: The figure depicts data-collection from 39 districts of 13 states including union territories**.** Different colours represent different administrative units or states. Different areas of one particular colour represent different districts (sub-state administrative units) of that particular state. The colour coding bears the name of the state followed by the exact site/s of data collection where available and the corresponding district in brackets. B: The figure depicts %prevalence of Pf mono, Pv mono and PfPv mixed SMIs among different states & UTs. White coloured area represents different administrative units or states from where SMIs have been reported. The bottom of the bar graphs was placed over the respective states/UTs from where data have been collected. The number over graphs shows %prevalence of that particular species.

NES were inconspicuous and states like Madhya Pradesh and Chhattisgarh, which have been high malaria burden areas were under-represented. In terms of number of districts covered per state, Odisha and Punjab had the highest representation (8 each), followed by Tamil Nadu (4), Assam (3), Chhattisgarh (3), and Madhya Pradesh (3).

Figure 3 shows that the pooled prevalence of SMI comes out to be 3.63% (95% CI 3-6%) for India (n = 17,449) with no difference between clinic (n = 10,143) 4% (95% CI 2-4%) or community-based (n = 7306) 4% (95% CI 2-7%) surveys. However, inter-state variations were observed with ~3 times (Madhya Pradesh) and 2 times (Andhra Pradesh, Assam, and Bihar) the pooled SMI prevalence for clinic-based studies and as high as 10-fold (Odisha), 4-fold (Maharashtra) and more than 2-fold (Madhya Pradesh and Odisha) pooled SMI prevalence (Odisha) in community-based studies. The overall heterogeneity in clinic-based and community-based studies was 91 and 96%, respectively which justifies the use of random effects model for estimation of the pooled prevalence.

The prevalence of sub-microscopic *Plasmodium* infections by PCR ranged from 10.1 in Maharashtra to 0.4% in Gujarat (Table 1, Figure 4). However, individual studies have quoted much higher SMI prevalence (Table 1) by Haanshuus et al., in 2011-12 in Andhra Pradesh (8.2%), Assam (7.6%), Bihar (8.2%); Singh et al., in 2009 in Madhya Pradesh (11%); Siwal et al., in 2012 in Madhya Pradesh (9.3%), Maharashtra (15.7%), Odisha (8%); Dhangadmajhi et al., in 2008 in Odisha (38.4%) and Kumari et al., in 2017 in Odisha (8%).

When the species-specific contribution to the burden of SMI was analysed (Figure 5), it was found that *P. falciparum* (Pf) contributed >50% of the SMI in Bihar (100%), Andhra Pradesh (89%), Assam (56%) and Uttar Pradesh (54%). On the contrary, *P. vivax* (Pv) contributed ≥50% of the SMI in Delhi (100%), Punjab (80%), Tamil Nadu (56%), Chhattisgarh (50%) and Gujarat (50%) whereas mixed *P. falciparum-vivax* (PfPv) predominantly contributed to SMI in Karnataka (82%), Maharashtra (63%), and Madhya Pradesh (54%). It is to be noted that, the sample size was too small in Delhi (n = 16) and no other species was tested in that particular area.

Apart from these two species, the contribution of other *Plasmodium* species infections to the SMI included *P. malariae* mono-infection (Pm) in Odisha

States	Sites	Year	N	Number of sub-microscopic infections								Prev (%)
				Pf	Pv	PfPv	Pm	PfPm	PfPvPm	PvPm	Total	
Andhra Pradesh[19]	Anantapur	2011-2012	109	8	0	0	1	0	0	0	**9**	**8.26**
Assam[19]	Tezpur (Sonitpur)	2011-2012	273	13	2	3	3	0	0	0	**21**	**7.69**
Bihar[19]	Raxaul (East Champaran)	2011-2012	85	7	0	0	0	0	0	0	**7**	**8.24**
Chhattisgarh[20]	Rajnandgaon, Bastar	2007-2008	3425	37	41	3	1	0	0	0	**82**	**2.39**
Chhattisgarh[19]	Mungeli	2011-2012	6	0	0	0	0	0	0	0	**0**	**0.00**
Gujarat[21]	Kheda	2012-2015	685	1	0	0	0	0	0	0	**1**	**0.15**
Madhya Pradesh[22]	Shivpuri, Dindori (Ramgarh)	2009	372	19	3	19	0	0	0	0	**41**	**11.02**
Maharashtra[19]	Ratnagiri	2011-2012	236	12	0	0	0	1	0	1	**14**	**5.93**
Odisha[21]	Sundargarh	2012-2015	1875	32	6	0	0	4	0	0	**42**	**2.24**
Odisha[23]	Keonjhar, Mayurbhanj	2014	1589	0	15	5	9	4	5	3	**41**	**2.58**
Tamil Nadu[19]	Ambur (Tirapattur), Vellore, Oddanchatram (Dindigul)	2011-2012	434	6	3	1	4	1	0	0	**15**	**3.46**
Tamil Nadu[21]	Chennai	2012-2015	1054	13	26	3	0	0	0	0	**42**	**3.98**
Assam[24]	Diphu (Karbi), Guwahati (Kamrup)	2014	234	1	3	0	0	0	0	0	**4**	**1.71**
Delhi[24]	Delhi	2014	16	0	1	0	0	0	0	0	**1**	**6.25**
Gujarat[21]	Kheda	2012-2015	796	1	1	0	0	0	0	0	**2**	**0.25**
Gujarat[24]	Kheda	2015	28	0	2	1	0	0	0	0	**3**	**10.71**
Karnataka[24]	Mangalore (Dakshina Kanada)	2014	289	2	0	9	0	0	0	0	**11**	**3.81**
Madhya Pradesh[24]	Betul	2012	64	0	0	6	0	0	0	0	**6**	**9.38**
Madhya Pradesh[24]	Betul	2013	355	4	0	6	0	0	0	0	**10**	**2.82**
Maharashtra[24]	Gadchiroli	2012	152	0	0	24	0	0	0	0	**24**	**15.79**
Odisha[25]	Mayurbhanj, Sundergarh, Keonjhar, Nayagarh, Rayagada, Kalahandi, Kandhamal and Angul	2008	242	2	0	2	29	49	4	7	**93**	**38.43**
Odisha[24]	Kendujhar, Rourkela	2012	140	7	0	5	0	0	0	0	**12**	**8.57**
Odisha[21]	Sundargarh	2012-2015	1307	22	8	1	0	0	0	0	**31**	**2.37**
Odisha[24]	Kendujhar, Rourkela	2013	100	0	2	1	0	0	0	0	**3**	**3.00**
Odisha[26]	Kandhamal	2017	586	13	23	11	0	0	0	0	**47**	**8.02**
Punjab[27]	Kapurthala, Mohali (chandigarh), Amritsar, Jaladhar, Mansa, Barnala or Sangrur, Muktsar, Ludhiana	2017-2018	1114	0	12	3	0	0	0	0	**15**	**1.35**
Tamil Nadu[21]	Chennai	2012-2015	928	5	11	1	0	0	0	0	**17**	**1.83**
Tamil Nadu[24]	Chennai	2014	41	0	3	0	0	0	0	0	**3**	**7.32**
Uttar Pradesh[24]	Shankargarh (Allahabad)	2015	914	20	6	11	0	0	0	0	**37**	**4.05**
Total			**17449**	**225**	**168**	**115**	**47**	**59**	**9**	**11**	**634**	**3.63**

***Table 1*: Description of the 9 included studies bearing reference numbers 19–27 shown in superscript just after the States' names. Individual states were organised alphabetically based on clinic-based (pink) and community-based (white) studies. Sites denote mentioned place of data collection. Year means the year/s of data collection. 'N' denotes number of individuals screened.**

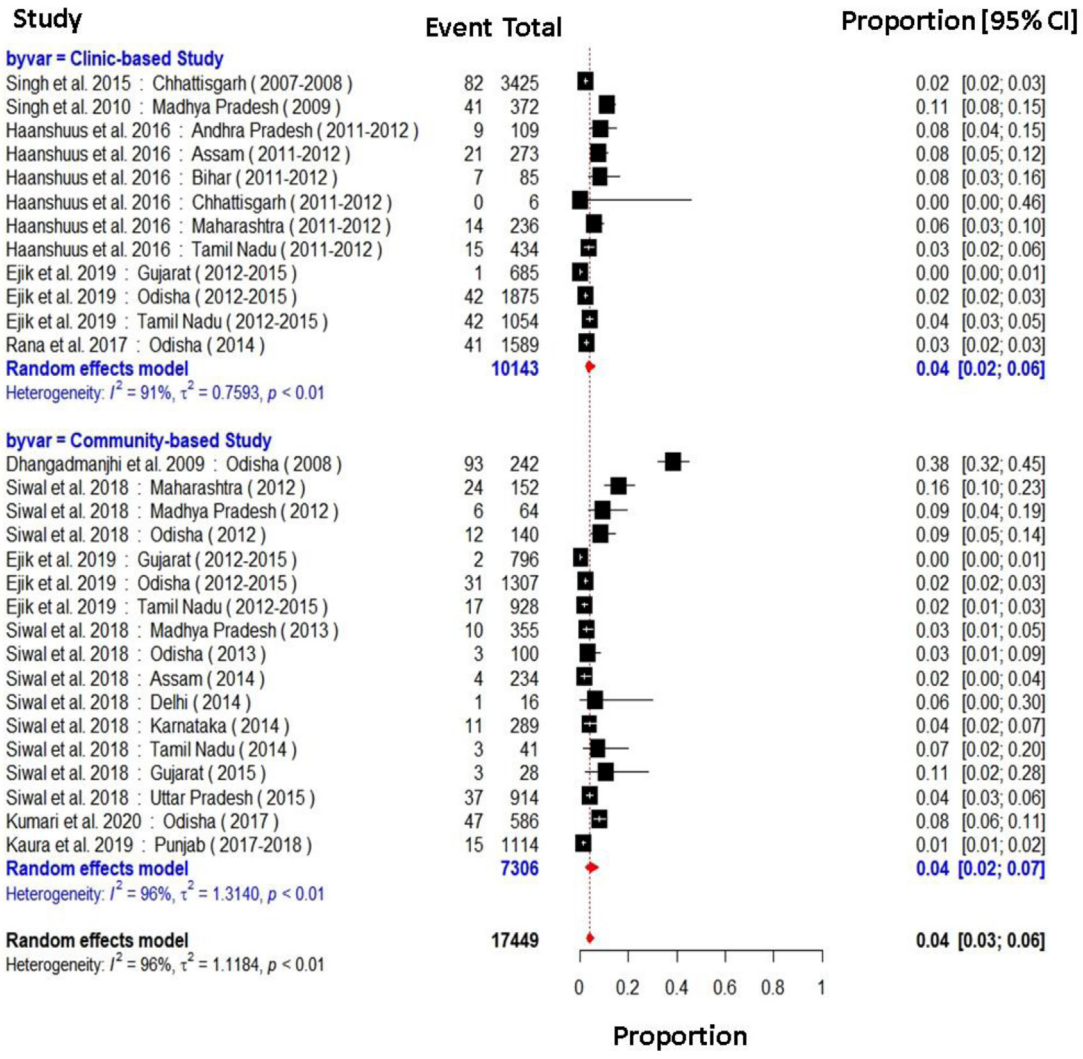

Figure 3. Pooled prevalence along with 95% CI of sub-microscopic *Plasmodium* infections in clinic-and community-based studies. Here, "Events" and "Total" represent the number of sub-microscopic *Plasmodium* infections detected and number of samples screened for *Plasmodium* infection, respectively. Within each category (clinic-and community-based), the studies are arranged in ascending order of start year of data-collection.

(14%), Assam (12%) and Andhra Pradesh (11%) and mixed *P. falciparum-malariae* in Odisha (21%). Odisha seems to be only reservoir of sub-microscopic infections which had variety of *Plasmodium* species combinations {mono *P. malariae*, mixed *P. falciparum-malariae*, mixed *P. vivax-malariae*, mixed *P. falciparum-vivax-malariae* other than Pf and Pv (Figure 5). On the other hand, Bihar (Pf) and Delhi (Pv) were the only states where only single *Plasmodium* species had been reported (Figure 5). If we investigate a little further to the most common species, Punjab was the only state that had not reported any sub-microscopic Pf mono infection, while reporting mixed infections with Pf. Similarly, despite having mixed species combinations with Pv, Karnataka and Maharashtra did not have any sub-microscopic Pv mono infection reported.

It is thus clear from the Figure 6A-C & Table 2 that all studied States reported SMI except Punjab for Pf, Andhra Pradesh, Bihar, Karnataka and Maharashtra for Pv and Andhra Pradesh for mixed PfPv. Further the magnitude (in terms of number of PCR positive samples per MS positive sample) for Pf SMI was much higher for Bihar and Andhra Pradesh whereas for Pv SMI, all States were more or less slightly below the diagonal line. In striking contrast, for mixed PfPv SMI, the magnitude was much higher as compared to Pf SMI as reflected by the relatively much higher PCR prevalence over the MS prevalence in Karnataka, Madhya Pradesh,

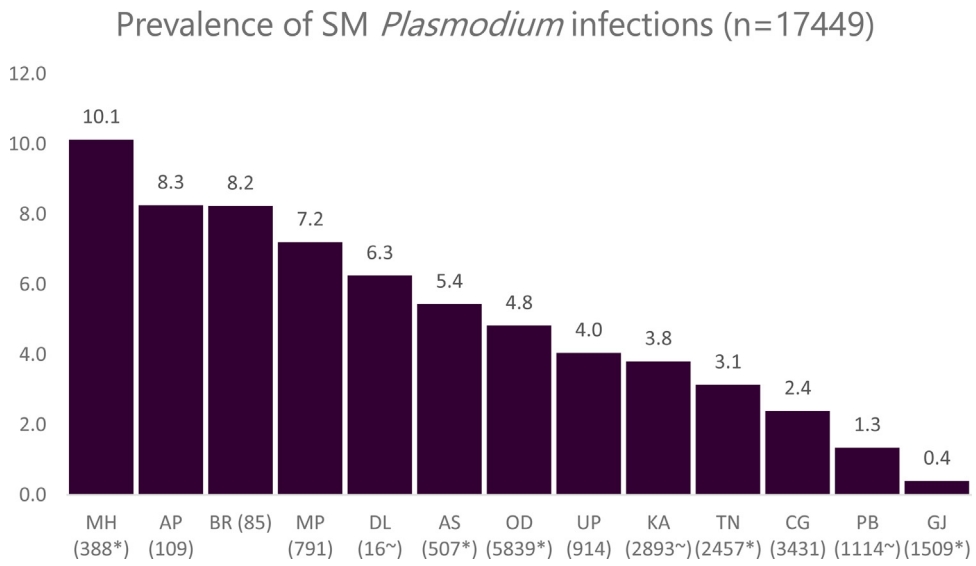

Figure 4. Prevalence (%) of sub-microscopic *Plasmodium* infections either as mono or mixed infection in different states. Studies are clubbed state-wise irrespective of year of the data-collection. The states are arranged in descending order of prevalence of sub-microscopic infections and total number of screened individuals for *Plasmodium* is mentioned in parenthesis. '~' denotes states where only Pf and Pv were screened from the suspected individuals; '*' denotes states where the number of screened samples for Pm was variable (MH = 236, AS = 273, OD = 5253 & TN = 2416) and hence, the prevalence of Pm and its mixed infections was calculated accordingly. MH = Maharashtra, AP = Andhra Pradesh, BR = Bihar, MP = Madhya Pradesh, DL = Delhi, AS = Assam, OD = Odisha, UP = Uttar Pradesh, KA = Karnataka, TN = Tamil Nadu, CG = Chhattisgarh, PB = Punjab & GJ = Gujarat.

Maharashtra and Uttar Pradesh. It is important to record that Maharashtra was the only state to report a higher MS prevalence for Pv as compared to the PCR and this might indicate towards false-positivity of MS and/or misidentification of species by the microscopist (this is elaborated in discussion section). The relative magnitudes of the SMI burden between Pf, Pv and mixed PfPv is better appreciated in Figure 6D. It is very evident that the mixed PfPv infections have the highest magnitude (lowest median) of SMI followed by that for Pf and Pv, respectively. Pv infections are more likely to be falsely identified or misidentified.

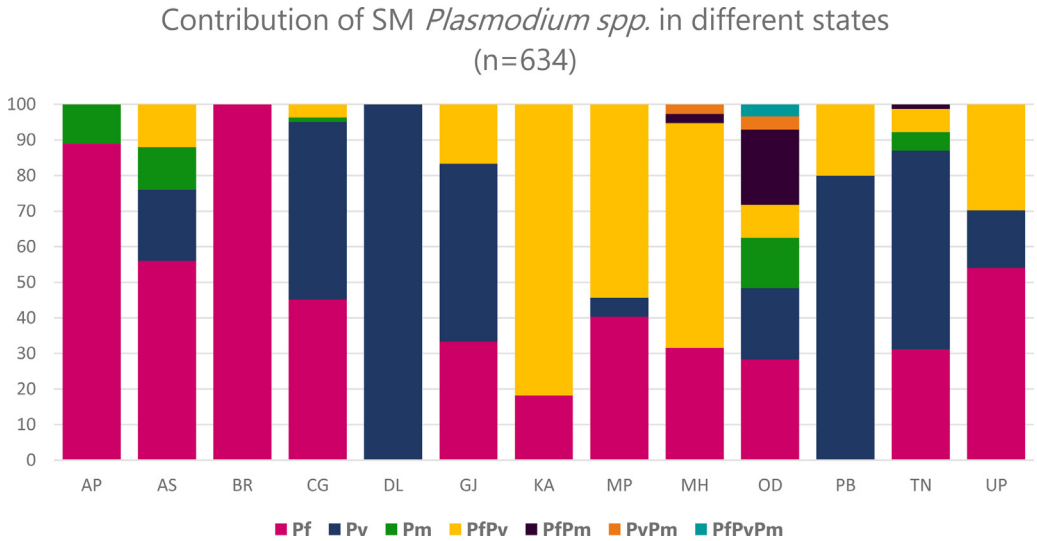

Figure 5. Proportional contribution (%) of different *Plasmodium* species either as mono- or mixed-species infection to the total burden of sub-microscopic infections (n=634) in different states. The states are arranged in alphabetical order. AP = Andhra Pradesh, AS = Assam, BR = Bihar, CG = Chhattisgarh, DL = Delhi, GJ = Gujarat, KA = Karnataka, MP = Madhya Pradesh, MH = Maharashtra, OD = Odisha, PB = Punjab, TN = Tamil Nadu & UP = Uttar Pradesh.

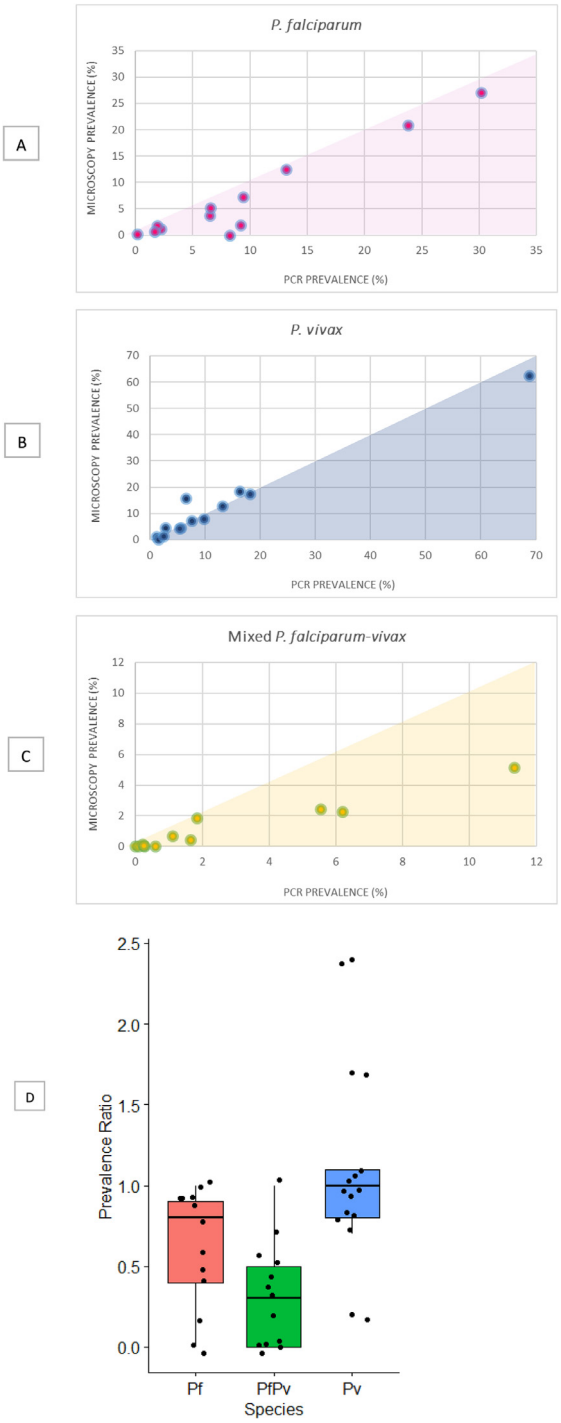

Figure 6. (A-D): Inter-relation of *Plasmodium* infection prevalence as scatter plot (A-C) and ratio (D) of the prevalence determined by microscopy and PCR on the same set of samples. 6A-C: PCR prevalence (X-axis) and microscopy prevalence (Y-axis) of Pf (6A), Pv (6B) and mixed PfPv (6C) infections. Diagonal line = identical PCR & microscopy prevalence; colored circle = single data pair representing single State; shaded area = SMI; unshaded area = false positive or species misidentified *Plasmodium* infections by microscopy. 6D: Ratio of the *Plasmodium spp*. prevalence by microscopy over that by PCR as box and whisker plots. Horizontal line = identical PCR & microscopy prevalence; black dot = paired data on microscopy and PCR for each species; black solid line inside the boxes = median value; area with prevalence ratio 1 = false positive or species misidentified *Plasmodium* infections by microscopy. The magnitude of SMI is directly proportional to the distance of the individual dot below the concordance line and inversely proportional to the median values of the prevalence ratio. Here, the studies are clubbed state-wise irrespective of the time of data-collection for the sake of clarity.

What is more important to note here is the number of SMIs per microscopic *Plasmodium* infection being detected across the study areas (Supplementary Table). It is evident that 3 states (Andhra Pradesh, Chhattisgarh and Tamil Nadu) reported approximately twice or more SMI for Pf for each Pf case detected by MS. Andhra Pradesh reported 5 Pf SMI for each and all Pf reported from Bihar was SMI (none detected by MS). Similarly, for Pv, there were 6 SMI reported per microscopically confirmed case in Chhattisgarh. None of the states reported lesser PCR burden as compared to MS burden, indicating false positive detection by MS and/or misidentification of species by MS, except for Pv in Andhra Pradesh, Karnataka and Maharashtra. The data for mixed PfPv infection reveals that, on an average, for these infections there are 2.2 SMI for each microscopically confirmed case. The number of SMI for each MS was as high as 6 in Tamil Nadu and between 3 and 4 in Uttar Pradesh, Assam, and Chhattisgarh whereas all were SMI in Punjab with zero MS mixed PfPv infection. The sub-microscopic pool is even larger for other neglected *Plasmodium* species and their mixed infections as reflected in >12 times SMI for PfPm, 10 times for PfPvPm, ~5 times for Pm and >4 times for PvPm with Odisha appearing as hotspot. In the absence of PCR-based data due to resource constraints, use of a previously developed and validated regression model to predict Pf PCR prevalence from microscopy prevalence (Okell et al. 2012) reflected significant correlation on data from India and on Pf, Pv and mixed PfPv infections (Figure 7A-C & Table 3). When tested for Pf, the correlation coefficient (r) and coefficient of determination (r^2) between observed and predicted PCR prevalence were 0.92 and 0.86, respectively which indicate a fairly strong positive correlation and goodness of fit. Similar values were obtained for Pv (r = 0.90; r^2 = 0.82) with much higher correlation and fit for mixed PfPv (r = 0.96; r^2 = 0.92) infections indicating that the model could be used to predict the PCR prevalence from their slide prevalence data.

The overall observed sensitivity of microscopy was 82% [95% CI 64%-92%] for the detection of Pf (Figure 8) which is less than expected for a high sensitive surveillance system (New perspectives: malaria

States	*Plasmodium falciparum* prevalence (%)		*Plasmodium vivax* prevalence (%)		*Plasmodium falciparum* and *Plasmodium vivax* prevalence (%)	
	PCR	MS	PCR	MS	PCR	MS
Andhra Pradesh	9.17	1.83	2.75	4.58	1.83	1.83
Assam	6.50	3.74	5.52	4.53	0.59	0
Bihar	8.23	0	1.17	1.17	0	0
Chhattisgarh	2.24	1.16	1.42	0.23	0.08	0
Delhi	N/A	N/A	68.75	62.5	N/A	N/A
Gujarat	1.92	1.78	7.55	7.35	0.19	0.13
Karnataka	13.14	12.45	16.26	18.33	5.53	2.42
Madhya Pradesh	23.76	20.85	13.14	12.76	6.19	2.27
Maharashtra	30.15	27.06	6.44	15.72	11.34	5.15
Odisha	6.52	5.22	2.44	1.52	1.09	0.66
Punjab	0.17	0.17	5.29	4.21	0.26	0
Tamil Nadu	1.66	0.69	9.72	7.97	0.24	0.04
Uttar Pradesh	9.40	7.22	18.16	17.50	1.64	0.43

***Table 2*: *Plasmodium* infection prevalence from microscopy and PCR as reported from various states/UTs. Here, the studies are clubbed state-wise irrespective of the time of data-collection for the sake of clarity.**

diagnosis 1999). However not all states had similar sensitivities, majority of states had average or above average sensitivities in community-based studies except Tamil Nadu (17%) and Odisha (72%), although the sample size was low in Tamil Nadu (n = 6). On the contrary most of the states had sub-average sensitivity in clinic-based studies (ranging from 0-74%). Three states Gujarat, Madhya Pradesh & one study in Odisha reported above average sensitivity of microscopy for Pf. Odisha reported both below & above average sensitivity during the time period (2012–15) in two different clinic-based studies.

The sensitivity of microscopy for Pv (Figure 8) appears to be slightly better as compared to that for Pf i.e. 91% [95% CI 82%-96%] with slightly better sensitivity in community-based studies 93% [95% CI 82%-98%] as compared to clinic-based studies which is 87% [95% CI 64%–96%]. In clinic-based studies, some states had lower than average sensitivity, such as Odisha with 57% [95% CI 39%–74%] and Chhattisgarh with 16% [95% CI 7%–30%]. On the other hand, community-based studies reported similar/better than average sensitivity for most states except Odisha with 23% [95% CI 10%–42%] and Tamil Nadu with 35% [95% CI 14%–62%].

When it comes to detecting mixed PfPv infection, the average sensitivity was far lower 45% [95% CI 38%-52%] with no significant difference between clinic-based/community-based studies (Figure 8). Because the number of samples reported and analysed was low, inter-study comparison could not be made.

The specificity of identifying Pf as either mono-& mixed was perfect 100% with no or minimal deviation among states.

Discussion

Although MS suffers from many drawbacks but due to its ease of operability, it still occupies a frontline place for malaria diagnosis at peripheries. The proportion of SMIs out of all *Plasmodium* infections tend to be particularly more in areas of low malaria transmission with low slide prevalence as repeated infections in a high transmission setting tends to maintain the parasite load above the LOD for MS.[16,28] Since India has witnessed a tremendous success in declining the slide-positive malaria burden in recent times, there is a high likelihood that parasite reservoir is sustained in the form of SMIs.[29] Moreover, since India is committed for malaria elimination by 2030, detecting the SM burden of *Plasmodium* becomes critical as these parasites form a potential reservoir of infections to mosquitoes and thus contributing to disease transmission.[30,31] There are at least 2 factors contributing to the SMI burden: limit of detection (LOD) and sensitivity of MS. Looking at the entire gamut of SMI, we observed that the SMI burden across the country has never been systematically evaluated thus far. Also, it is evident that the LOD of MS is a non-modifiable determinant of SMI but the sensitivity and specificity of MS in detecting malaria parasites can be improved. Therefore, we investigated the pooled prevalence of SMI in India through a systematic review of published literature and also analysed the sensitivity and specificity of gold standard microscopy in order to determine if they vary across different settings within India. Further, we also analysed the *Plasmodium* species-specific & mixed species SMI burden and diagnostic performance of MS as compared to PCR. This is the

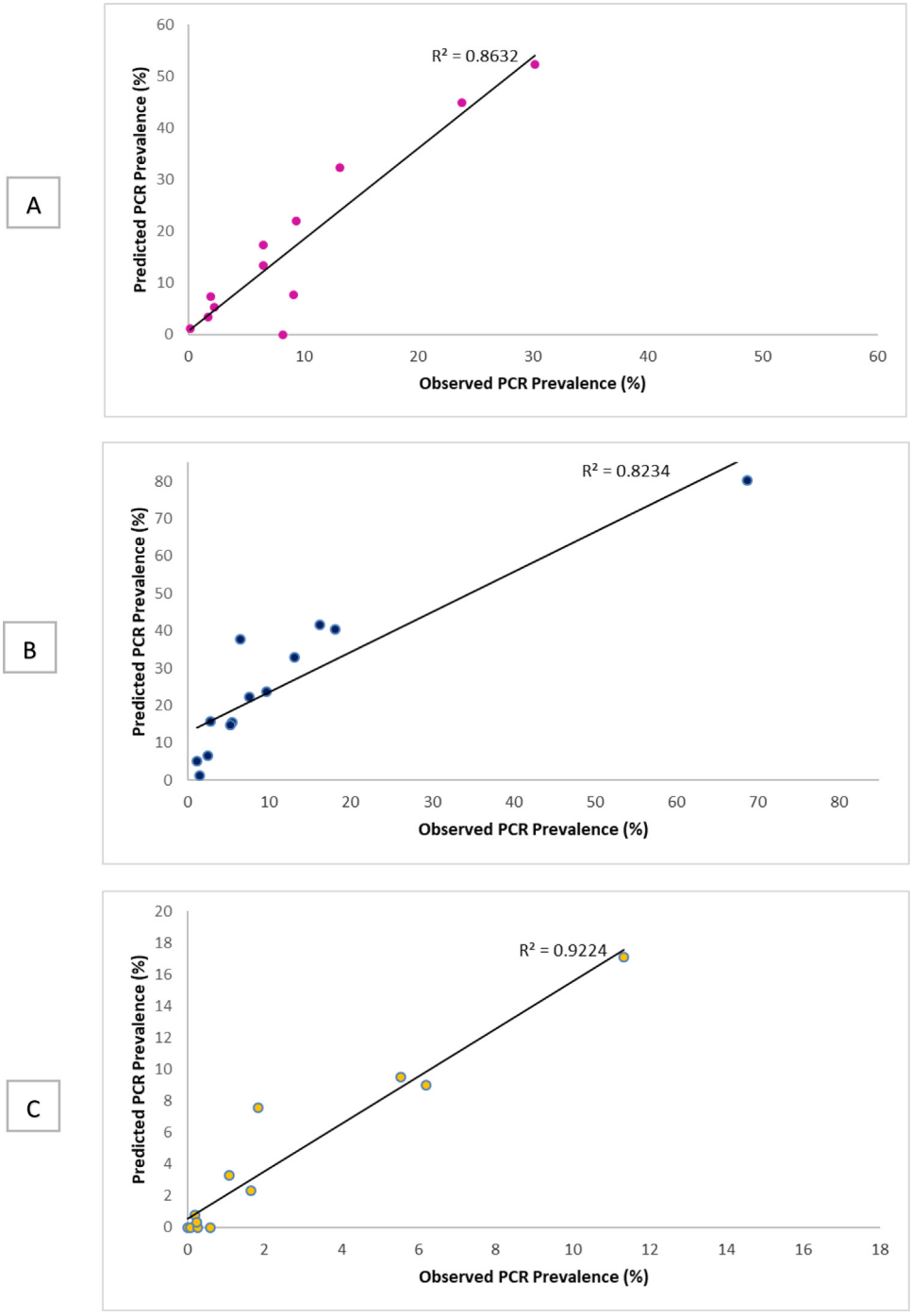

Figure 7. (A-C): The correlation between observed (x-axis) and model-predicted PCR prevalence (y-axis) for Pf, Pv and mixed PfPv infections, respectively in different Indian states. The predicted PCR prevalence was calculated using a tool developed by Okell et al., in 2012 for calculating predicted PCR or microscopy prevalence in areas where only PCR or microscopy data are available. The correlation coefficient between observed and predicted PCR prevalence values was calculated using 'Pearson Correlation Coefficient Calculator'.

first of its kind systematic review and meta-analysis on different *Plasmodium* species & mixed species SMI in India.

We noticed significant species-specific SMI burden (4% overall and as high as 38% in Odisha in 2008) and variations in the sensitivity for the most prevalent species

			Plasmodium falciparum			
States/UT	**Total samples screened**	**Positive by MS**	**Positive by PCR**	**Actual PCR Prevalence**	**Predicted PCR Prevalence**	**95% credible interval of predicted PCR prevalence**
Andhra Pradesh	109	2	10	9.2	7.6	2.4-21.7
Assam	507	19	33	6.5	13.4	9.1-19.3
Bihar	85	0	7	8.2	0	0
Chhattisgarh	3431	40	77	2.2	5.2	3.6-7.4
Gujarat	1509	27	29	1.9	7.4	5.1-10.7
Karnataka	289	36	38	13.1	32.3	25.7-39.8
Madhya Pradesh	791	165	188	23.8	44.9	40.4-49.5
Maharashtra	388	105	117	30.2	52.3	46.7-57.9
Odisha	5839	305	381	6.5	17.3	14.8-20.2
Punjab	1114	2	2	0.2	1.1	0.3-3.6
Tamil Nadu	2457	17	41	1.7	3.4	2.1-5.5
Uttar Pradesh	914	66	86	9.4	22	17.9-26.8
Plasmodium vivax						
Andhra Pradesh	109	5	3	2.75	15.7	7.8-29.2
Assam	507	23	28	5.52	15.6	11-21.5
Bihar	85	1	1	1.18	5.2	1-22.7
Chhattisgarh	3431	8	49	1.43	1.3	0.7-2.7
Delhi	16	10	11	68.75	80.2	62.2-90.9
Gujarat	1509	111	114	7.55	22.3	18.8-26.3
Karnataka	289	53	47	16.26	41.5	34.9-48.5
Madhya Pradesh	791	101	104	13.15	32.9	28.3-37.8
Maharashtra	388	61	25	6.44	37.7	31.7-44
Odisha	5839	89	143	2.45	6.5	4.9-8.6
Punjab	1114	47	59	5.30	14.7	11.3-19
Tamil Nadu	2457	196	239	9.73	23.7	20.5-27.2
Uttar Pradesh	914	160	166	18.16	40.3	36-44.8
Plasmodium falciparum* and *Plasmodium vivax						
Andhra Pradesh	109	2	2	1.83	7.6	2.4-21.7
Assam	507	0	3	0.59	0	0
Bihar	85	0	0	0.00	0	0
Chhattisgarh	3431	0	3	0.09	0	0
Gujarat	1509	2	3	0.20	0.8	0.2-2.8
Karnataka	289	7	16	5.54	9.5	5-17.2
Madhya Pradesh	791	18	49	6.19	9	5.9-13.5
Maharashtra	388	20	44	11.34	17.1	12.0-24
Odisha	5839	39	64	1.10	3.3	2.2-4.8
Punjab	1114	0	3	0.27	0	0
Tamil Nadu	2457	1	6	0.24	0.3	0.1-1.6
Uttar Pradesh	914	4	15	1.64	2.3	0.9-5.5

***Table 3*: Predicted v/s observed PCR prevalence for Pf, Pv and mixed PfPv infections in different Indian states. The predicted PCR prevalence was calculated using a tool developed by Okell et al., in 2012 for calculating predicted PCR or microscopy prevalence in areas where only PCR or microscopy data are available.**

Pf ranging from 0 to 100% (pooled estimate 82%) which might be attributable to inexperienced microscopists apart from the presence of low-density infections. However, the sample size was too low in few states[18,20,23,26] which stresses on the need of generating primary data on SMI in India. Strikingly contrasting sensitivities for Pf was observed between clinic-based (pooled estimate 57%; 95% CI 26–83%) and community-based (pooled estimate 91%; 95% CI 81–96%) studies. Because the clinic-based studies (as compared to the community-based studies) tend to involve better (high sensitive and specific) microscopy-based diagnosis owing to an "expected" presence of trained and qualified microscopists in these settings, it was anticipated that such clinic-based studies would report lesser SMI prevalence than the community-based counterpart. However, the reverse was observed. Before we discuss the possible reasons behind this contrasting observation, few things need to

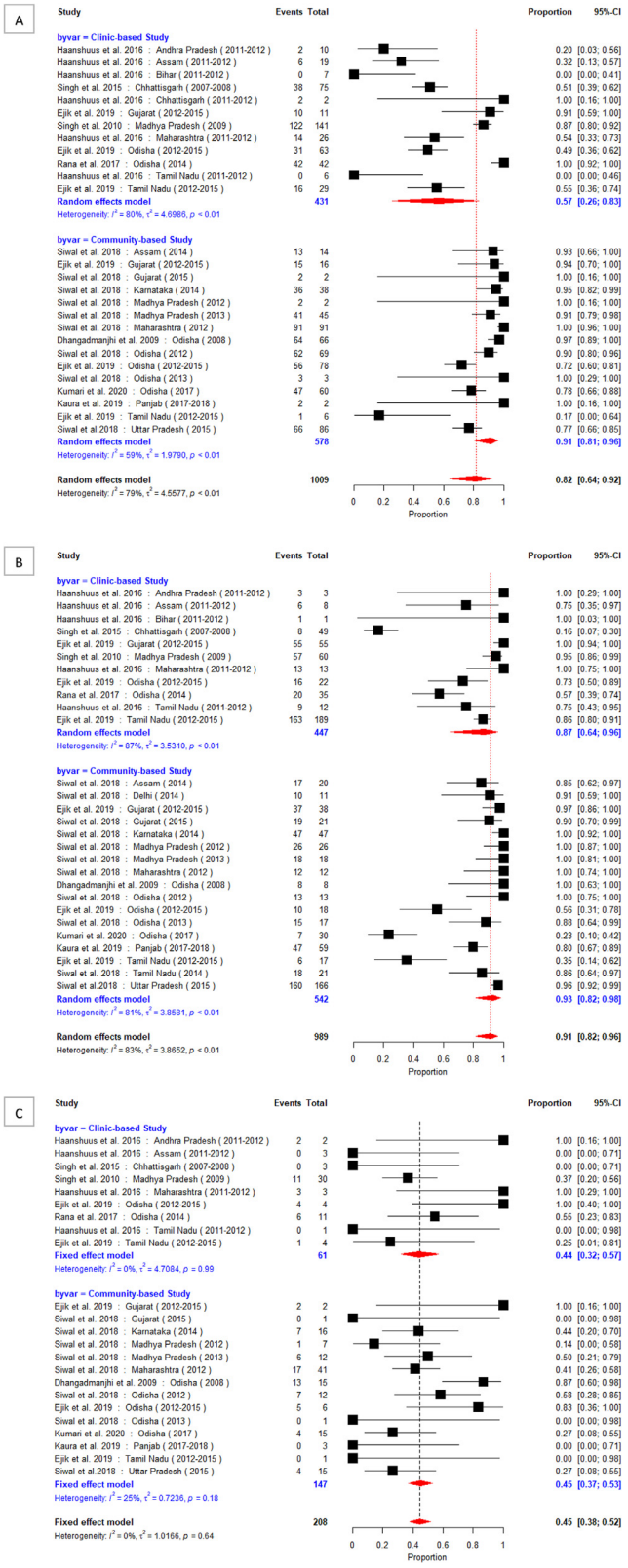

Figure 8. (A-C): Forest plots for sensitivity for *P. falciparum* (A) and *P. vivax* (B) either as mono- or mixed infections (C)**.** The figures for other *Plasmodium* species with no significant outcome are not shown. Pooled estimates for subgroups (clinic- and community-

be noted. It is to be understood here that an SMI event (false negative MS-based diagnosis) could be a true finding implying that the parasite load was, in fact, below the LOD of MS ('true' false negative). On the contrary, such false-negatives could be 'false' false negative which means that actually the parasite load was above the MS LOD but somehow, MS could not detect it. Since the basis of this classification is routed in the determination of the parasite load in the samples, which is not discernible without a qPCR, and considering that qPCR was not reportedly done on these samples, either of the two possibilities may occur, singly or in combination.

'True' false negative MS results in clinic-based studies could be contributed by the following factors: patterns of healthcare seeking in rural India, initial indications of start of treatment failure (due to inadequate/suboptimal treatment or emerging resistance to standard antimalarials), presence of low-density persistent parasite reservoir in presence of non-malaria fever, etc. Healthcare seeking, particularly for tropical and episodic or acute diseases such as malaria in rural India does not always lead the patients directly to the formal "classical facility-based" healthcare system. Rather, almost 50% of the rural and tribal patients seeking malaria care first consult an informal health care provider who is often the first and the last contact of the patients. If the patients do not recover from this first line of health care providers, they often contact one or more of such informal healthcare providers before finally ending with the formal facility-based formal healthcare.[32–35] In addition, delayed treatment seeking, self-medication, and preference given to traditional healers are common among uneducated and poor people. [36,37] During their journey from their first contact (informal healthcare provider) and the last (formal healthcare provider), these rural/tribal patients are often treated, mostly inadequately and sub-optimally using a variety of "anti-malaria" medicines that may bring down the parasite load to below the LOD of microscopy by the time they reach the "clinic".[38–42] Incomplete and sub-optimal treatment might also kill the parasites and hence not counted by microscopists (as they count healthy parasites) whereas the PCR tends to detect all parasite DNA present in a sample, thus exaggerating the count. This is one of the main reasons why PCR based parasite load estimation is not recommended in therapeutic efficacy studies of anti-malaria drugs.

Possible reasons of 'false' false negative result in clinic-based studies may include poor quality microscopy and erroneous identification and reporting of SMIs. Sub-optimal sensitivity and specificity of MS are driven by the skills of the microscopists and the technology per se. The differential skills of microscopists impart "subjectivity" in malaria diagnosis and compromises the specificity of microscopy in terms of identifying the parasite species correctly. In addition, poor slide preparation, inadequate staining, inferior microscope, inadequate volume of blood examined also affect MS quality adversely.[16,43] SMIs may erroneously be reported from the paired diagnoses of all MS+PCR samples taken together and not by independent sample paired analysis (Deora and Sinha; manuscript under consideration), which might present a flawed picture. However, when analysed for the differential training of microscopists, as reported by the studies, it was found that all microscopy was performed by "experienced" microscopists irrespective of the location of the studies irrespective of the setup (clinic- or community-based) of the included studies. It is to be noted that training is different from experience in the sense that an untrained microscopist may have a long experience and also that repeated training is needed for being a trained microscopist for a high sensitive MS diagnosis.[44] Thus, repeated training, that too from a WHO certified (level-1) trainer, and experience both are needed to maintain the sensitivity of MS. Examining all the available information in the included studies, it apparently rules out the microscopy-based concerns leading to the 'false' false negative MS. Whether the SMI reported for the clinic-based studies were specifically subjected to factual analysis error, cannot be confirmed from the granularity of data available in the reports examined for the current study and hence the possibility of such errors cannot be ruled out. On the other hand, there are some reported studies that were conducted by the same authors and that had both the setup component (clinic- and community-based) and it is not expected that the investigators may have followed differential analysis plans for clinic-based (erroneous) and community-based (factual) studies or vice versa. Therefore, in the absence of convincing evidence for 'false' false negative MS, the reasons leading to 'true' false negative MS (as discussed above) in clinic-based as compared to community-based studies cannot be ruled out. No significant variations were observed for sensitivity of MS for Pv. However, and in contrast, the sensitivity of MS for mixed PfPv infections was very low (pooled 45%; CI 38–52%) with no significant difference between the states. These findings show that microscopy sensitivity differs not just across different geographical locations, but also between different species within the same geographical area, which is a matter of concern. This raises the issue of training as the same diagnostic technique should not have varied sensitivities for different *Plasmodium* species. It is quite surprising to note that despite high sensitivity for Pf and Pv independently, 82% and 91%, respectively, the

based studies) and overall are represented as red diamonds and proportions with 95% CI. Individual study estimates of each parameter are shown as black squares and proportions with 95% CI. The events and total values represent the numerator and denominator for estimating the parameters (as described in the methods).

sensitivity for mixed PfPv infections is drastically low (45%). This brings out some more issues, apart from training for correct identification of species, and that relate to the possibilities that either one or both species were present at a level lower than the LOD of MS or to the possible tendency of microscopists to conclude the result of the blood smear as soon as s/he detects one of the species, putting the other species at risk of being detected as SMI despite being present at detectable level by MS. This finding was supported by other studies as observed in South-eastern Iran, wherein the MS was reported to have 16.6% sensitivity for the detection of mixed *Plasmodium* species.[45] Similar findings were observed in another study that collected samples from Pakistan, Iran, and Afghanistan.[46] Light microscopy could not detect a single case of mixed *Plasmodium* infection from Afghanistan and Pakistan. However, they performed PCR only on MS-confirmed mono *P. vivax* infected cases. There might be other variables related to the way microscopy and PCR are carried out across different studies included in the analysis that could affect the diagnostic performances of these methods and affect their sensitivity as mentioned in challenges. Various other studies have also found a much lower detection rate of microscopy in India's neighbouring countries. Microscopy revealed significantly less *Plasmodium* infected individuals than detected by PCR in these reports.[47–50] SMIs appear to be a serious impediment to malaria elimination operations in such malaria-endemic countries like Myanmar, Bangladesh, and Nepal. These results demonstrated the importance of molecular diagnostic methods in epidemiological surveys, as microscopy's sensitivity did not even detect half of the infected individuals.[47–50]

This *Plasmodium* species-wise analysis also raised another critical concern related to the relative higher prevalence of a particular *Plasmodium* species by MS over PCR, thus generating a sense of false-positive species diagnosis by MS. However, this apparently false-positive result could be due to the wrong identification of species by the microscopy (Figure 6), again raising the importance of training in correct identification of species.

It is estimated that nested PCR (nPCR) detects approximately two times more Pf infections in comparison to microscopy depending upon endemicity and population at risk.[16,28] However, species-wise data on this is lacking. This study reports, on an average, 1.1 (Pf) to 2.2 (PfPv) times more prevalence by PCR over microscopy. This data would be helpful for the programs to gauge the SMI burden (reservoir) according to *Plasmodium* species being detected by the microscopy in a particular area. The use of predictive model developed by Okell et al., was applied and tested for the first time for Indian data and showed fairly good correlation and fit for Pf, Pv and PfPv in India. Since the tool was not originally developed for Indian data and non-Pf species, more data need to be put into the model to validate it further for all regions across India and for all *Plasmodium* species in presenting as mono- or mixed infections. What this means is that such tools could be used to estimate the SMI burden in any area once it is validated with the data available for both MS and PCR.

Challenges for SMI:

(a) Species-specific SMI and mixed species infections: mixed *Plasmodium* species infections provide distinct challenges for microscopy-based diagnosis due to differential burden of each species, preferential acquaintance of the microscopist towards Pf and Pv, reporting guidelines for mixed-species infections as some countries prefer to report PfPv mixed infections as mono-Pf infections, etc. The species-specific SMI data is critical for the malaria control programs not only to identify the precise training needs but also to know if any particular *Plasmodium* species diagnosis is getting neglected thus leading to a building up of a reservoir with a tendency to emerge as a sudden outbreak. Majority of mixed infection cases are diagnosed as mono-infections according to the findings of several studies.[51–53] The question of whether the coexistence of more than one *Plasmodium* species in a single host causes one to go below the microscopy detection threshold, attracts little attention. The disease pattern of mixed *Plasmodium* infections is an emerging interest not only in elimination settings but in areas where malaria has already been eliminated.

(b) Transmission potential of SMI: The ability of *Plasmodium* SMI to get transmitted makes them even more important, particularly in a malaria elimination setting.[54] Despite evidence from multiple studies that SMIs are able to transmit the parasite to the mosquitoes,[55–65] although at 6-50% lower success as compared to their microscopic counterpart, more research is needed to better characterise the transmission potential of SMI reservoirs in low or very low transmission settings where elimination is being targeted.[16,28] It is important to note that mosquito infectivity determined in standard feeding assays and that in natural environments tend to differ and mere mosquito infectivity doesn't guarantee malaria transmission and sustenance thereof and this remains one of the difficult to answer questions as exemplified in Figure 9. In an ideal scenario, the answers to all the challenge points in the figure would help us better understand the transmission potential of SMIs. Attempts to address these issues reveal that SMIs have been capable to re-start and maintain malaria transmission in presence of sufficient vectorial capacity.[25,66,67]

(c) Quality of MS and PCR: It is evident that variations in the quality of diagnostic methods affect their performance and hence comparability across studies

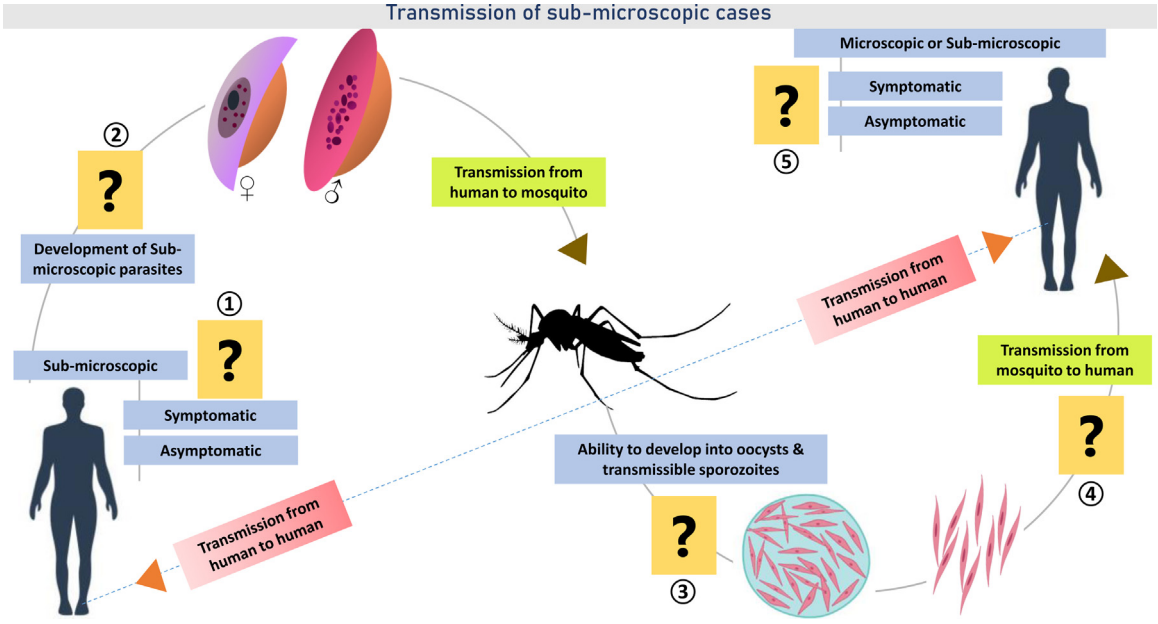

Figure 9. The figure shows, unexploited challenges associated with the sub-microscopic infections' transmission (Human-to-mosquito, mosquito-to-human and most important human-to-human). In terms of sub-microscopic infections, the figure highlights five important challenging steps that must be explored.

and geographical areas.[16] For a diverse country like India, this stands out as a big challenge in estimating and comparing the true burden of *Plasmodium* species-specific SMI. Various parameters contributing to the quality of MS[16,68] include poor slide preparation (dirt, grease, inadequate thin smear, drying and fixation), inadequate staining (dilution, duration), quality of microscope (lighting, lens), volume of blood examined (number of microscopic fields examined; number of leucocytes counted). For PCR[16,43,69–71] such parameters include source of starting material (fresh blood versus dried blood), volume of blood used for DNA extraction, variations in DNA extraction protocols, amount of extracted DNA used as template, variations in PCR protocols used, use of negative control in PCR, type of PCR performed (nPCR or qPCR), etc.

(d) Host and parasite factors affecting SMIs: a myriad of other hitherto unexplored factors could help in determining the burden of SMIs and can open new areas of research. These include factors related to providing protection to the human host from malaria / severe malaria[16,72] like hemoglobinopathies (HbAS, thallasemias, HbE, HbC), red blood cell variants (Ovalocytosis, Duffy negative, A+ blood group), G6PD enzyme deficiency variants, and human Renin Angiotensin System polymorphisms (ACE I/D, ACE2 C/T, AGT). Parasite associated factors that could result in sustained low parasitemia leading to SMIs include infection with strains having lower erythrocyte invasion efficiency,[73] lower number of merozoites per schizonts,[74] lower multiplication rates and virulence,[75,76] low genetic diversity,[77] etc.

Conclusive remarks and the way forward

Parasites successful in sustaining an SMI for longer term are less likely to be detected by routine passive microscopy under the National Malaria Control Programmes as the parasite biomass tends to be below the clinical threshold. Such parasite strains might have a relative evolutionary advantage over the microscopically detectable strains thus favouring their positive selection as this creates a win-win situation for the parasite and human host.[16,31,78] Coupled with a transmission and sufficient vectorial capacity, this sustained maintenance of parasite biomass below the LOD of MS may thwart the malaria elimination progress in long term. Thus, integration of NAAT-based detection methods in National Programs, in the form of genetic surveillance of malaria, at some point of time needs to be gradually done as a "genetic intervention".[28,79,80] Although there are many countries that have successfully eliminated malaria without the need of genetic surveillance, it might be reasonable to think that this could have been possible much sooner with the integration of molecular diagnosis.[28] Until then, the development and use of species-specific regional

predictive models[16] (Okell et al. and as discussed above) that can predict PCR prevalence from slide prevalence with sufficient confidence must be used to determine the SMI hot spots within the country. The current analysis reveals such hot spots (districts with SMI prevalence >10%) within Odisha SMI prevalence 3–38% (Mayurbhanj, Sundargarh, Keonjhar, Nayagarh, Rayagada, Kalahandi, Kandahmal, Angul), Maharashtra SMI prevalence ~16% (Gadchiroli), MP SMI prevalence ~11% (Shivpuri, Dindori), and Gujarat SMI prevalence ~11% (Kheda). These are the areas where genetic surveillance may be integrated on a priority. Based on the prevalence of SMI across different regions, it is also recommended that at least 10% to 50% of the smear-negative samples, depending on the regional prevalence of SMIs, may be sent for molecular diagnosis for different *Plasmodium* species to more accurately estimate the *Plasmodium* species-specific SMIs. Actionable guidelines may be simultaneously developed based on the regional SMI burden that may include Focussed or Mass Screening And Treatment (FSAT/MSAT)[81–86] if the SMI prevalence is above a certain level, for example, twice that of the slide prevalence. Further, use of qPCR over nPCR is recommended for research targeted for detection of SMIs not only for its higher sensitivity but because qPCR can also estimate the parasite burden which can then be used to classify the SMIs based on the LOD of microscopy. Such actionable SMI classification into those above or below the MS LOD will help the program managers to identify the region- and species-specific LOD of microscopy and design targeted interventions, including improving microscopy techniques, sensitivity and training, in areas where the SMI is above the MS LOD.

This is the first comprehensively synthesised evidence on all human malaria causing *Plasmodium* species SMIs across India, as per the authors best knowledge. No research came across the authors that was targeted to detect species-specific SMIs and/or *Plasmodium* mixed-species infection SMIs in India and therefore we recommend that such primary regional data should be generated in all countries nearing elimination of malaria.

Contributors

ND: Data collection & acquisition, analysis and interpretation, drafting the manuscript, illustrations and editing the manuscript critically; CPY: Data analysis and interpretation, editing the manuscript critically; VP: Data analysis and interpretation, editing the manuscript critically; AS: Conceptually designing the work, data interpretation, drafting the manuscript and revising it critically

Data availability

All manuscript related data is available in the tables provided and in supplementary material. If any further information is needed, the corresponding author may be contacted for the same.

Editor note

The Lancet Group takes a neutral position with respect to territorial claims in published maps and institutional affiliations.

Declaration of interests

The authors declare no conflict of interest for the current study

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.lansea.2022.05.001.

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