Detection of Mixed Infections with Plasmodium spp. by PCR, India, 2014

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In 8 malaria-endemic states in India, mixed Plasmodium spp. infections were detected by PCR in 17.4% (265/1,521) of blood samples that microscopy had shown to contain only *P. falciparum*. The quality of microscopy must be improved because use of PCR for detection of malaria parasites is limited in rural areas.

Five *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*) cause human malaria. Malaria is not uniformly distributed in India; of the 35 states and union territories contain most malaria cases (1). Infections with *P. falciparum* and *P. vivax* occur at approximately equal frequencies (2–4). This finding increases the possibility of mixed infections, as reported in other countries, such as Peru (5), Papua New Guinea (6), Brazil (7), and Ethiopia (8).

In India, malaria control usually involves vector control with indoor residual spraying of insecticides and insecticide-treated bed nets, and chemotherapy with artemisinin-based combination therapy. Malaria diagnosis is based mainly on microscopic detection of parasites in peripheral blood smears from symptomatic persons. In addition, bivalent, rapid diagnostic tests (RDTs) are useful detection tools (9) but cannot differentiate *P. falciparum* monoinfections from co-infections with other *Plasmodium* species (2,3). Moreover, genetic polymorphisms in diagnostic antigens limits detection by monoclonal antibodies. Misdiagnoses might also arise from gene deletions that prevent expression of proteins by the parasite (10). We report that a high proportion of mixed infections with 4 *Plasmodium* species detected by PCR in 8 states of India to which malaria is highly endemic were not detected by bivalent RDTs and microscopy.

The Study
This study was approved by the Institutional review board of the National Institute for Research in Tribal Health (Jabalpur, India). Written informed consent was obtained from all participants or parents of children, according to Indian Council of Medical Research guidelines.

The study was conducted in 2 community health centers (CHCs), 1 in an area that had a high level of malaria endemicity and 1 that had a low level of malaria endemicity, in each of 8 states in India: Orissa, Chhattisgarh, Jharkhand, Maharashtra, Madhya Pradesh, Tripura, Gujarat, and Rajasthan (Figure 1; Table 1). Selected CHCs were located in different regions, and forest areas in these regions ranged from 13% in Jhabua (Madhya Pradesh) to 81% in Tripura. Elevation above sea level ranged from 13 m in Valsad (Gujarat) to 870 m in Koraput (Orissa). Inhabitants of most study areas were ethnic tribes (39%–87%). All areas had received 2 rounds of indoor residual spray (DDT/synthetic pyrethroid) as a vector control measure.

Blood samples were collected from persons with suspected malaria during July–December 2014 at malaria clinics in CHC hospitals at 15 sites. Microscopy and RDTs (Bioline Ag Malaria Pf/Pv Test; Standard Diagnostics Inc., Gyeonggi-do, South Korea) were performed at outpatient department clinics of CHCs, and molecular diagnosis (PCR and sequencing) was performed at the molecular parasitology laboratory at the National Institute for Research in Tribal Health.

For microscopy, thick and thin blood smears were prepared from finger prick blood samples, which were air-dried, fixed in methanol, and stained with Giemsa. A total of 100 thick blood smear fields were examined by using an oil immersion lens at 100× magnification before a sample was considered negative. Malaria parasite density was determined from thick blood smears by counting the number of parasites against 200 leukocytes (11). Microscopy was also performed on samples that had negative results by RDT. Blood smears were cross-checked by a senior laboratory technician. RDT was performed according to manufacturer’s instructions (9) and was repeated for samples in which discordant results were obtained (e.g., microscopy positive, RDT negative).

Genomic DNA was isolated from samples that microscopy showed to contain only *P. falciparum* by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Species-specific nested PCRs that targeted the 18S rRNA gene were used to detect 4 malaria parasite species (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) (12). *P. knowlesi* was detected by using a set of primers specific for the 18S rRNA gene (13), and differentiation of 2 subspecies of *P. ovale* (*P. curtisi* and *P. wallikeri*) was performed as described (4). PCR primers and conditions are shown in...
Table 2. An independent research assistant, who was unaware of microscopy or RDTs results, performed PCR on coded samples.

Of 1,521 samples determined by microscopy to be *P. falciparum* monoinfections, PCR confirmed results for 1,256 (83%). However, PCR showed mixed infections with *P. falciparum* and *P. vivax* in 239 (16%) samples; *P. falciparum* and *P. malariae* in 19 (1%) samples; *P. falciparum* and *P. ovale* in 6 (0.4%) samples; and *P. falciparum*, *P. malariae*, and *P. ovale* in 1 (0.1%) sample (Table 1). Microscopy could not identify these mixed infections (17.4% [265/1,521]). PCR amplification of DNA from 4 *Plasmodium* species is shown in Figure 2.

Secondary microscopic analysis of blood smears by a second technician showed that only 22/239 (9.2%) samples contained mixed infections with *P. falciparum* and *P. vivax*. PCR analysis showed that the highest prevalence of mixed infections with *P. falciparum* and *P. vivax* was in Jharkhand (25.5%, 55/216), followed by Madhya Pradesh (20.8%, 47/226), Rajasthan (18.6%, 26/140), Orissa (15%, 40/267), and Tripura (15%, 19/127), and Chhattisgarh (10.7%, 23/214). The lowest prevalences were in Maharashtra (9%, 21/234) and Gujarat (8.2%, 8/97).

Mixed infections with *P. falciparum* and *P. malariae* were found in all 8 states, although in small numbers. Mixed infections with *P. falciparum* and *P. ovale* were found in only 4 states, particularly at CHCs in areas to which malaria was highly endemic. Of 7 mixed infections that contained *P. ovale*, 5 contained *P. ovale curtisi* and 2 contained *P. ovale wallikeri*. *P. knowlesi* was not found in any state.

Conclusions

This study was conducted in 8 states in India that contain 80% of malaria cases (85% of which are caused by *P. falciparum*) and 70% of deaths caused by malaria in the entire country (1). Misdiagnosis by microscopy occurs because in mixed infections there is a tendency of 1 parasite to predominate and microscopy usually does not detect low numbers of other parasites (6). Thus, rare malaria parasites and mixed infections are underestimated through routine
Detection of Mixed Infections with *Plasmodium* spp.

Table 1. Characteristics of mixed infections with 4 *Plasmodium* species identified by PCR for 1,521 blood samples that were *P. falciparum*–positive by microscopy in 8 malaria-endemic states, by district, India, 2014*†

| District (state) or variable       | CHC             | Period  | No. infections | Odds ratio (95% CI), p value† | Mixed (PF + Pv) | Mixed (all pooled) |
|-----------------------------------|-----------------|---------|----------------|-------------------------------|---------------|-------------------|
| Koraput (OD)                      | Bandhugao       | Jul–Aug | 188 35 4 2 0   | –                             | 1.8 (1.0–3.1), 1.8 (0.9–2.3) | – (0.9–2.3) |
| Rayagada (OD)                     | Jagannathpur    | Jul–Aug | 31 5 2 0 0    | –                             | 0.004         | 0.0916           |
| Simdega (JH)                      | Jaldega         | Aug–Nov | 82 41 0 2 0   | –                             | 3.5 (2.0–6.0), <0.0001 | – (2.0–6.0) |
| Simdega (JH)                      | Bano            | Aug–Nov | 76 14 0 0 1   | –                             | 3.4 (2.0–5.8), <0.0001 | – (2.0–5.8) |
| Jagdalpur (CG)                    | Maharanin       | Jul–Oct | 178 23 2 1 0  | –                             | – (2.0–6.0)   | – (2.0–6.0) |
| Baikunthpur (CG)                  | District Hospital | Jul–Nov | 10 0 0 0 0    | –                             | 1.2 (0.7–2.3), 1.2 (0.7–2.3) | – (0.7–2.3) |
| Jhabua (MP)                       | Ranapur         | Sep–Oct | 92 29 3 1 0   | –                             | 2.7 (1.4–4.7), 2.7 (1.4–4.7) | – (1.4–4.7) |
| Anuppur (MP)                      | Pushprajgarh    | Sep–Nov | 82 18 1 0 0   | –                             | 2.3 (1.3–4.2), 2.3 (1.3–4.2) | – (1.3–4.2) |
| Gadchiroli (MH)                   | Malewada        | Sep–Nov | 108 6 0 0 0   | 1 (Ref)                       | 0.0068        | 0.0056           |
| Gondia (MH)                       | Darekasa        | Sep–Nov | 103 15 2 0 0  | 1 (Ref)                       | 1 (Ref)       | – (1.0–2.0) |
| Udaipur (RJ)                      | Bekaria         | Sep–Dec | 112 26 2 0 0  | –                             | – (0.5–2.3)   | – (0.5–2.3) |
| Dahod (GJ)                        | Devgadh Baria   | Sep–Dec | 77 8 2 0 0    | –                             | – (0.5–2.3)   | – (0.5–2.3) |
| Valsad (GJ)                       | Lavkar          | Sep–Nov | 10 0 0 0 0    | 0.9 (0.4–2.1)                 | 0.8316        | 0.8946           |
| South Tripura (TR)                | Manubazar       | Oct–Dec | 41 4 0 0 0    | –                             | 1.8 (0.9–3.5), 1.7 (0.9–3.3) | – (0.9–3.3) |
| South Tripura (TR)                | Santirbazar     | Oct–Dec | 66 15 1 0 0  | –                             | 0.084         | 0.0978           |

Total NA NA 1,256 239 19 6 1 NA NA

Median no. parasites/µL NA NA 1,897.3 1,600 1,273.6 1,180 4,040 NA NA

Range NA NA 35– 1,785,71 40– 380,464 31– 56,818 200– 124,118 NA NA

| p value | NA   | NA   | Ref 0.138 | 0.251 | 0.439 | NA   | NA   |
|---------|------|------|-----------|-------|-------|------|------|

*CHC, community health center; PF, *P. falciparum*; Pv, *P. vivax*; Pm, *P. malariae*; Po, *P. ovale*; OD, Odisha; JH, Jharkhand; CG, Chhattisgarh; MP, Madhya Pradesh; MH, Maharashtra; Ref, reference; RJ, Rajasthan; GJ, Gujarat; TR, Tripura; NA, not applicable.
†–, indicates that analysis was conducted after data for the 2 districts in that state were combined.

Figure 2. Identification of *Plasmodium* spp. by nested PCR at 15 community health centers in 8 states in India to which malaria is endemic. A) *Plasmodium falciparum* (205-bp fragment). Lane 1, molecular mass marker; lane 2, negative (−) control; lane 3, positive (+) control; lanes 7–27, positive samples; lanes 5 and 6, negative samples. B) *P. malariae* (144-bp fragment). Lane 25, + control; lane 26, − control; lane 27, molecular mass marker; lane 12, positive sample; lanes 1–11, 13–24, negative samples. C) *P. ovale* (800-bp fragment). Lane 1, molecular mass marker; lane 2, − control; lane 3, + control; lane 17, positive sample; lanes 4–16, 18–27, negative samples. D) *P. vivax* (120-bp fragment). Lane 1, molecular mass marker; lane 2, + control; lane 3, − control; lanes 5, 11, 16, and 25, positive samples; lanes 4, 6–10, 12–15, 17–24, 26, and 27, negative samples.
microscopy and RDTs (2–6), and misidentification of malaria parasites could prolong parasite clearance time and lead to anemia and drug resistance (14). A high proportion of mixed infections with \textit{P. vivax} and \textit{P. falciparum} have been reported in India (15). However, in that study, Gupta et al. did not look for \textit{P. malariae} or \textit{P. ovale} and their sample size was small (180 persons).

Our study had some limitations. Monoinfections or mixed infections were not verified by PCR if parasitemia levels were too low to be detected by microscopy or RDTs. Thus, mixed infections with parasitemia levels below the limit of detection of microscopy or RDTs would not have been detected. Detailed studies in different ecosystems during different transmission seasons and large sample sizes are required for a more accurate picture of mixed infections with common and uncommon parasite species, clinical epidemiology, adverse effects, relapse, and recrudescence.

Our results highlight the role of mixed infections, particularly those with \textit{P. vivax}, \textit{P. malariae}, and \textit{P. ovale}, which are not detected accurately by microscopy or RDTs. Although \textit{P. vivax} and \textit{P. ovale} are responsible for relapses (4), \textit{P. malariae} is sustained at low rates among sparse and mobile human populations for decades, thus facilitating transmission by mosquitoes (2). Our results also emphasize a major concern in the diagnosis of malaria by microscopy or RDTs and has serious repercussions for malaria epidemiology and subsequent control. These findings indicate the need to improve quality of microscopy and RDTs because PCR techniques are expensive. Until PCR becomes much less expensive and more available as a point-of-care test for field testing, its use will be limited for malaria detection.

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N.S. conceived the study; N.S., P.K.B., and MPS designed the study protocol; S.K., H.S.C., A.A., R.K., and P.P.S. conducted sample collection and molecular experiments; S.K., H.S.C., and P.K.B. analyzed sequencing data; N.S., M.P.S., and P.K.B., analyzed and interpreted data; and N.S., P.K.B., and M.P.S. drafted the manuscript. All authors read and approved the final manuscript.

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