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National Malaria Prevalence in Cambodia: Microscopy versus Polymerase Chain Reaction Estimates

Dysoley Lek,* Jean Popovici, Frederic Ariey, Seshu Babu Vinjamuri, Sylvia Meek, Jan Bruce, Walter R. J. Taylor, Duong Socheat, Didier Menard, and William O. Rogers

National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia; School of Public Health, National Institute of Public Health, Phnom Penh, Cambodia; Institut Pasteur in Cambodia, Phnom Penh, Cambodia; Department of Parasites and Insect Vectors, Institut Pasteur, Paris, France; Institut Cochin, Institut National de la Santé et de la Recherche Médicale U1016 (INSERM U1016), Faculté de Médecine, Université Paris Descartes Sorbonne Paris Cité, Paris, France; Laboratoire de Parasitologie-Mycologie, Hôpital Cochin, Assistance Publique–Hôpitaux de Paris (AP-HP), Paris, France; World Health Organization Lao People's Democratic Republic Office, Vientiane, Lao People's Democratic Republic; London School of Hygiene and Tropical Medicine, London, United Kingdom; Service de Médecine Tropicale et Humanitaire, Hôpitaux Universitaires de Genève, Geneva, Switzerland; Mahidol Oxford Tropical Medicine Research Unit, Bangkok, Thailand; Naval Medical Research Unit 2 Detachment, Phnom Penh, Cambodia

Abstract. Accurate information regarding malaria prevalence at national level is required to design and assess malaria control/elimination efforts. Although many comparisons of microscopy and polymerase chain reaction (PCR)–based methods have been conducted, there is little published literature covering such comparisons in southeast Asia especially at the national level. Both microscopic examination and PCR detection were performed on blood films and dried blood spots samples collected from 8,067 individuals enrolled in a nationwide, stratified, multistage, cluster sampling malaria prevalence survey conducted in Cambodia in 2007. The overall malaria prevalence and prevalence rates of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium malariae* infections estimated by microscopy ($N = 8{,}067$) were 2.74% (95% confidence interval [CI]: 2.39–3.12%), 1.81% (95% CI: 1.53–2.13%), 1.14% (95% CI: 0.92–1.40%), and 0.01% (95% CI: 0.003–0.07%), respectively. The overall malaria prevalence based on PCR detection ($N = 7{,}718$) was almost 2.5-fold higher (6.31%, 95% CI: 5.76–6.89%, $P < 0.00001$). This difference was significantly more pronounced for *P. falciparum* (4.40%, 95% CI: 3.95–4.90%, $P < 0.00001$) compared with *P. vivax* (1.89%, 95% CI: 1.60–2.22%, $P < 0.001$) and *P. malariae* infections (0.22%, 95% CI: 0.13–0.35%, $P < 0.0001$). The significant proportion of microscopy-negative but PCR-positive individuals (289/7,491, 3.85%) suggest microscopic examination frequently underestimated malaria infections and that active case detection based on microscopy may miss a significant reservoir of infection, especially in low-transmission settings.

BACKGROUND

Over the past 15 years, Cambodia has made significant strides in controlling malaria with the introduction of artemisinin-based combinations, establishing rapid diagnosis tests (RDTs), and treatment through a system of village malaria health workers (set up in 2001 and scaled up in 2004) and the recent wide distribution of long-lasting insecticide-treated bed nets (LLITNs).[1–4] In 2011, Cambodia committed itself to eliminating malaria by 2025. This goal may be challenged by the emergence of multidrug-resistant, including artemisinin-resistant *Plasmodium falciparum* (ART-R)[5–8] and, now, the demise of dihydroartemisinin–piperaquine.[9]

Accordingly, conducting accurate, large-scale assessments of malaria prevalence, including pools of asymptomatic individuals detected by the polymerase chain reaction (PCR), is crucial for planning malaria control and elimination efforts and in measuring the impact of pertinent interventions. In Cambodia, efforts are being focused on containing the spread and ultimately the elimination of ART-R; thus, identifying and quantifying reservoirs of falciparum infection are seen as essential because these pools represent areas where there is ongoing transmission of ART-R.

Conventionally, quantitative malariometric surveys are mainly based on light microscopy (LM) diagnosis and, more recently, RDTs, which are tools with limits of detection that range from 50 to 200 malaria parasites/μL.[10] In recent years, various molecular detection approaches like PCR, with lower theoretical limits of detection (0.5–5 parasites/μL), have been increasingly used for improving the detection of malaria infections. In malaria prevalence surveys, it has been shown that PCR may detect twice as many cases as microscopy,[11] and that most of the positive cases harbor asexual and sexual parasites. Such asymptomatic cases are able to transmit infection to mosquitoes without coming to the attention of the health system, and so may complicate malaria control.[12–14] Although submicroscopic parasitemias may be less infective to mosquitoes than microscopically detectable ones, they may nonetheless be an important source of transmission because they are severalfold more prevalent than microscopic parasitemias.[15] These undetected reservoirs maintain parasite populations, especially multidrug-resistant parasites, and may contribute to the spread of ART-R within Cambodia through the movement of migrant and mobile populations.

Although studies have compared the performances of PCR and microscopy, few published studies have compared parasite prevalence rates estimated by these two methods at the national level.[16,17] Nationwide malaria prevalence surveys have been conducted in Cambodia in 2004, 2007, 2010, and 2013; PCR detection was first used in 2007. Herein, we report the microscopy and PCR results of the 2007 national survey.

MATERIALS AND METHODS

Survey design and study population. The 2007 Cambodian National Malaria Survey (CNMS) was a stratified, multistage cluster sampling design study. The country was stratified into

*Address correspondence to Dysoley Lek, National Center for Parasitology, Entomology and Malaria Control, No. 477 Corner Street 92, Trapeng Svay Village, Sangkat Phnom Penh Thmei, Khan Sen Sok, Phnom Penh, Cambodia. E-mail: soleyl@cnm.gov.kh

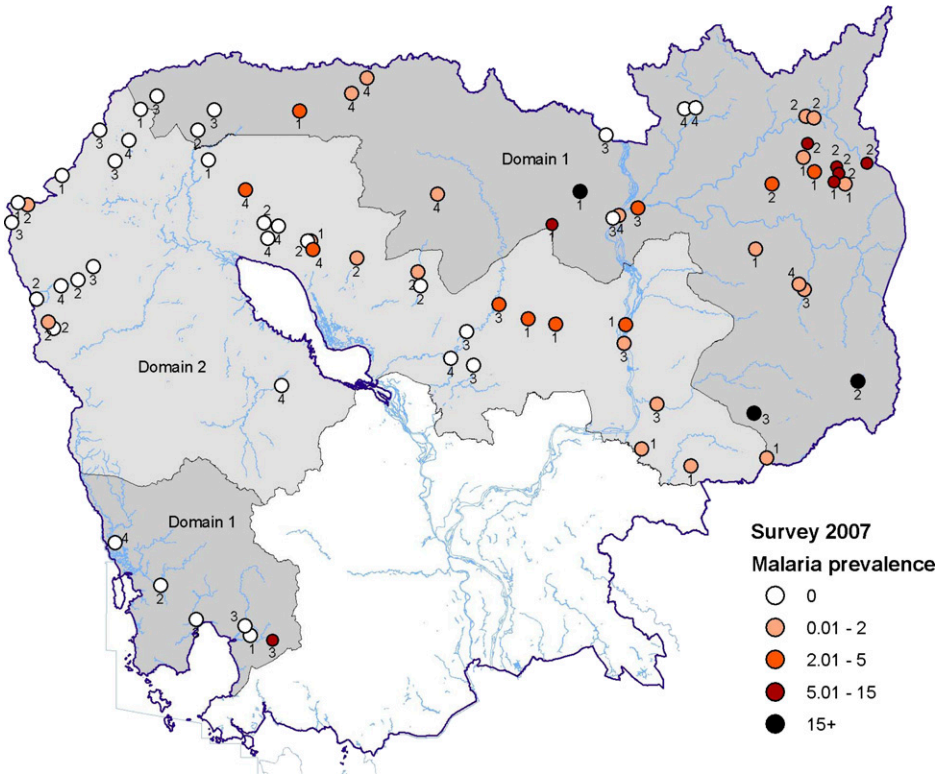

FIGURE 1. Malaria prevalence estimated by microscopy and distribution of the individual clusters, Cambodia, 2007. Domains 1 and 2 are shown in dark and light gray, respectively. The color assigned to each cluster corresponds to the malaria prevalence (%) estimated by microscopy (all species). The number beside each colored cluster indicates the risk zone: risk zone 1 (0–250 m from forest), risk zone 2 (250–1,000 m), risk zone 3 (1–2 km), and risk zone 4 (2–5 km).

three domains (Figure 1) based on the malaria prevalence results of the 2004 CNMS.[18] Domain 1 consisted of the northeastern provinces of Mondulkiri, Oddor Meanchey, Preah Vihear, Rattanakiri, and Stung Treng and the southern province of Koh Kong (high-transmission areas); Domain 2 consisted of the central and western provinces of Banteay Meanchey, Battambang, Kampong Thom, Kratie, Pailin, Pursat, and Siem Reap (low-transmission areas). The Domain 3 includes the remaining provinces around Phnom Penh (not sampled here due to the very low malaria prevalence found in the 2004 survey).

Each domain was further stratified into risk zones based on the distance from the nearest forest margin. Risk zones 1, 2, 3, and 4 correspond to distances from the forest of 0–250 m, 250–1,000 m, 1–2 km, and 2–5 km, respectively. Within Domains 1 and 2, 38 clusters were randomly sampled: 14 from risk zone 1 and eight from each of the other three risk zones. Within each cluster, 40 households were randomly chosen from the village census list maintained by the village leaders. Within each household, a fingerprick blood sample was taken from four individuals, one from each of the following groups: one aged 0–4 years, one aged 5–14 years, one adult female, and one adult male. If there was more than one person in any of these groups, one was sampled randomly from all individuals falling in that group. In addition, any pregnant woman in the household who was not already sampled was included in the blood sampling.

Random selection of clusters was not based on probability proportional to size, and so was not self-weighting. Weights were determined by census data collected at the time of sampling, and took into account the probability of selection of individual subjects based on both cluster size and the distribution of age categories in individual households.

All study subjects gave informed consent; the study was approved by the National Ethics Committee for Health Research of the Cambodian Ministry of Health (097 NECHR dated October 26, 2007), and was conducted in compliance with the international standards for the protection of human research subjects, the participation of Naval Medical Research Unit 2 was approved by the NAMRU-2 Institutional Review Board.

Blood sampling. Fingerprick blood samples from 8,067 individuals enrolled in a large-scale malaria prevalence survey in Cambodia conducted in October–December 2007 at the end of the rainy season, including 3,363 households in 13 provinces in Domains 1 and 2, were collected and used for microscopic examination of blood films and the detection of DNA using a PCR assay based on the *Plasmodium mitochondrial cytochrome b* gene from blood spots. The national malaria prevalence estimated both by microscopy and PCR was investigated and compared along with the main determinants associated with the malaria transmission in Cambodia.

Microscopic examination. Using blood from a fingerstick, thick and thin films for malaria microscopy were prepared by field workers in the households during the survey, and were stained with Giemsa stain on the day of collection, according to the WHO protocol.[19] Blood films were examined by a certified microscopist using × 1,000 oil immersion

LM. At least, 200 ocular fields were reviewed before a slide was considered negative. Parasites were counted per 200 leukocytes and reported as parasites/μL, assuming a white blood cell count of 8,000/μL. A second, senior certified microscopist reviewed all positive smears and 10% of negative smears. In the event of discrepancies, the senior microscopist's reading was recorded.

DNA extraction and PCR assay. From the same fingerprick, 2–3 drops of blood were blotted onto Whatman No. 3 filter paper (Sigma-Aldrich, Singapore), dried at ambient temperature, and placed in ziplock bags with a desiccant. DNA extraction, detection of *Plasmodium* DNA by nested PCR, and identification of the *Plasmodium* species (by sequencing the PCR products) were carried out as previously described.[20] In brief, two sets of primers were used to amplify an 815–base pair sequence of *Plasmodium mitochondrial cytochrome b*. The species were determined by analysis of single nucleotide polymorphisms at 11 sites within the PCR product.

Statistical analysis. All data were recorded on standardized case report forms, double entered into MS Access (Microsoft Inc., Redmond, WA), and exported for analysis to STATA (version 10.0; StataCorp LP, College Station, TX). The data were analyzed using survey analysis commands with eight strata (two domains and four risk zones in each domain); the primary sampling unit was the cluster, no finite population correction was used, and sample weights were calculated as the inverse of the probability of selecting each cluster. Linearized standard errors were calculated. Continuous data were compared by the unpaired *t* test (log transforming data, as needed), and proportional data were compared using the χ^2 test. All statistical tests were two-tailed, and significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

In total, 8,067 individuals were sampled from 3,363 households in 137 clusters in 13 provinces (Figure 1). Households numbered 2,110, 2,343, 1,820, and 1,794 from risk zones 1, 2, 3, and 4, respectively.

The male-to-female ratio was 0.89 and the median age was 23 years (95% confidence interval [CI]: 23–24 years). Of the sampled population, 63% was ≥ 15 years of age, 22% aged 5–14 years, and 15% were children aged from 0 to 4 years. Details of demographic data per domain and risk zone are provided in Table 1.

A total of 7,707 blood samples were tested both by microscopy and PCR among the 8,067 individuals enrolled in the national survey (95.4%). The distribution of the clusters sampled and the malaria prevalence in each cluster, determined by microscopy, are shown in Figure 1. The study did not sample enough sites in each province to produce reliable prevalence estimates at the provincial level. However, high prevalence clusters were mainly located in the northeastern provinces, known to be the highest malaria transmission settings in the country. As a consequence, the overall malaria prevalence rates estimated both by microscopy and PCR were significantly higher in high transmission areas: 4.5% and 9.8%, respectively, in Domain 1 compared with 0.7% and 2.2%, respectively, in Domain 2 ($P < 0.0001$).

Comparing the malaria prevalence rates between the risk zones, the risk of being malaria positive decreased with increasing distance from the forest margin, regardless of the

TABLE 1
Demographic data of the surveyed population, Cambodia, 2007

Demographic parameters	Sampling areas										*P* value
		Domain 1				Domain 2					
	Total	Risk zone 1	Risk zone 2	Risk zone 3	Risk zone 4	Total	Risk zone 1	Risk zone 2	Risk zone 3	Risk zone 4	Total	
Population sampled	8,067	992	1,299	1,039	934	4,264	1,118	1,044	781	860	3,803	–
No. of clusters	137	16	25	15	13	69	18	23	14	13	68	–
Mean of individuals sampled per cluster	58.9	62.0	52.0	69.3	718	61.8	62.1	45.4	55.8	66.2	55.9	–
No. of households	2,904	344	464	356	323	1,487	423	393	280	321	1,417	–
Mean of individuals sampled per household	2.8	2.9	2.8	2.9	29	2.9	2.6	2.7	2.8	2.7	2.7	–
Median age in years (range)	23 (0–95)	22 (0–90)	24 (0–84)	24 (0–85)	22 (0–90)	23 (0–90)	23 (0–95)	24 (0–86)	25 (0–87)	25 (0–81)	24 (0–95)	0.06
Population sampled by age group (%)												
0–4 years	14.9	15.5	14.8	15.6	16.2	15.5	15.8	10.0	10.0	13.3	14.4	0.75
5–14 years	21.8	21.4	20.3	22.6	22.8	21.7	21.3	20.0	23.3	21.9	20.0	
≥ 15 years	63.2	63.1	64.9	61.8	60.0	62.9	62.9	60.0	62.7	64.9	63.6	
Gender (% male)	47.1	47.7	49.2	47.5	45.9	47.7	45.6	46.8	46.5	46.6	46.4	0.75
Pregnant women among females (%)	11.6	14.7	13.1	9.2	12.1	12.3	10.8	11.4	11.6	9.2	10.8	0.42
Resident in the sampling area (%)	95.2	91.9	91.5	95.1	94.4	93.1	96.9	97.9	96.5	98.5	97.4	$P < 0.001$
Febrile individuals (Temperature ≥ 37.5°C, %)	13.1	14.2	14.1	12.5	12.3	13.3	12.8	12.6	12.7	1,300, 0%	12.8	0.84
Forest worker (%)	15.9	21.9	21.2	19.2	13.9	19.3	13.2	14.3	14.3	6.3	12.2	$P < 0.001$

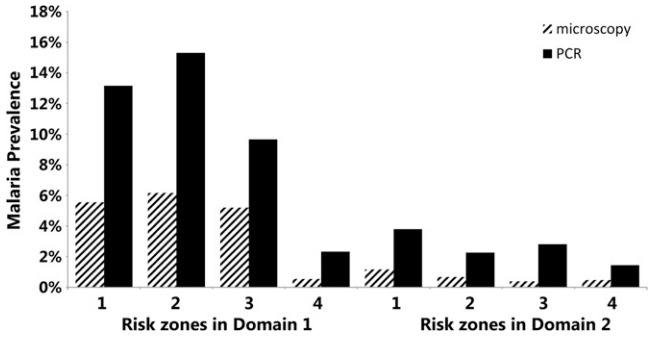

FIGURE 2. Malaria prevalence by domain and risk zone, Cambodia, 2007. The malaria prevalences (any species) by domain and risk zone as measured by microscopy and polymerase chain reaction (PCR) are shown. Risk zones 1, 2, 3, and 4 correspond to distances from the forest of 0–250 m, 250–1,000 m, 1–2 km, and 2–5 km.

domain and the method of detection. In Domain 1, malaria prevalence by microscopic detection ranged from 6.2% in risk zone 1 to 0.5% in risk zone 4 (χ^2 test for trend, $P < 0.001$), and from 15.3% in risk zone 1 to 2.3% in risk zone 4 by PCR (χ^2 test for trend, $P < 0.001$). In Domain 2, malaria prevalence by microscopic detection ranged from 1.2% in risk zone 1 to 0.5% in risk zone 4 (χ^2 test for trend, $P = 0.04$), and from 3.8% in risk zone 1 to 1.4% in risk zone 4 by PCR (χ^2 test for trend, $P < 0.01$; Figure 2).

On the basis of microscopy or PCR, malaria prevalence rates declined with increasing age in the high malaria transmission areas of Domain 1 (Figure 3): 6.3% in 0–14 years age group versus 3.5% in ≥ 15 years age group (microscopy, $P < 0.001$) and 11.4% versus 8.8% (PCR, $P < 0.01$). These data are consistent with other findings in Africa, where age has been strongly associated with malaria infection (detected by RDT or PCR), especially for children aged 0–10 years.[21] However, in low-transmission settings of Domain 2, the opposite trend was found for microscopy (0.2% in 0–14 years age group versus 1.0% in ≥ 15 years age group, $P < 0.01$). These data demonstrate how the malaria epidemiology can vary according to the ecological environment, the distribution of the vectors species, and human behavior, especially in unstable transmission areas.[22–25] This trend may be explained partly by the intense efforts at malaria control in Domain 2, where prompt malaria treatment is achieved through the widespread use of malaria RDTs and artemisinin-combined therapies in health facilities and at community level coupled with the increased coverage of LLITNs and indoor residual spraying that have led to a substantial fall in malaria transmission. This may have led to a slowing of the development of malaria immunity in adults who are still at a high risk of contracting malaria because of their work in the forests.

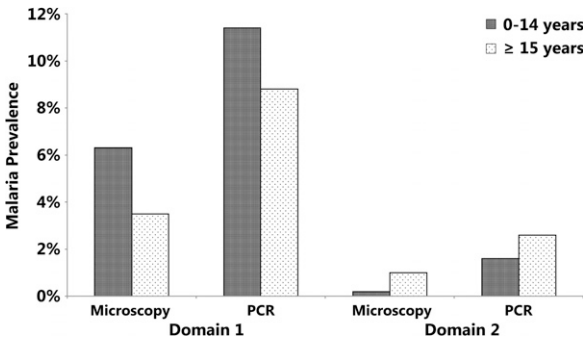

FIGURE 3. Malaria prevalence by diagnostic methods (microscopy and polymerase chain reaction [PCR]), Cambodia, 2007. The malaria prevalence rates (any species) found by microscopic examination and PCR detection are shown.

The distribution of parasite densities across the age groups is displayed in Figure 4. For individuals infected by *P. falciparum* (Panel A), higher parasitemia was overrepresented in the 0–14 years age group compared with ≥ 15 years age group, with a nonsignificant difference between the geometric mean values (1, 050 parasites/μL in the 0–14 years age group versus 410 parasites/μL in the ≥ 15 years age, $P = 0.1$). Although not significant, the trend of lower parasitemia with increasing age in Domain 1 suggests the development of some clinical immunity to malaria.[26,27]

By contrast, for individuals with vivax infections (Panel B), the geometric mean parasitemia was 50-fold lower and almost similar between age groups (84 parasites/μL in the 0–14 years age group versus 45 parasites/μL in the ≥ 15 years age, $P = 0.9$). Our parasitemia data are consistent with those of Baird and others, who demonstrated that the development of immunity to malaria was slower for *P. vivax* compared with *P. falciparum*.[28,29]

Overall malaria prevalence rates in both domains combined using microscopy or PCR are presented by species in Figure 5. PCR-determined malaria prevalence rates were

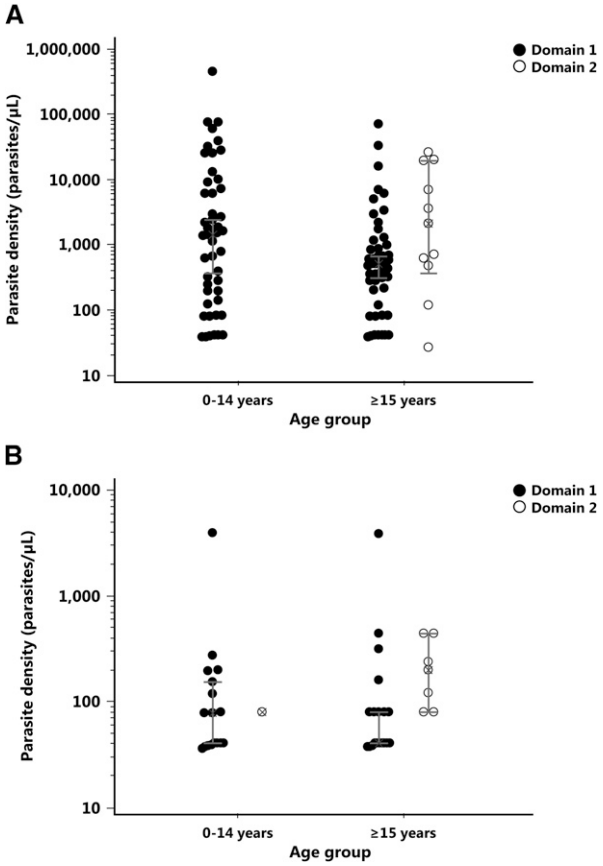

FIGURE 4. Distribution of microscopic parasitemias by age group, Cambodia, 2007. The microscopic parasitemias by age group for each domain are presented in Panel A for *Plasmodium falciparum* infection and in Panel B for *Plasmodium vivax* infection.

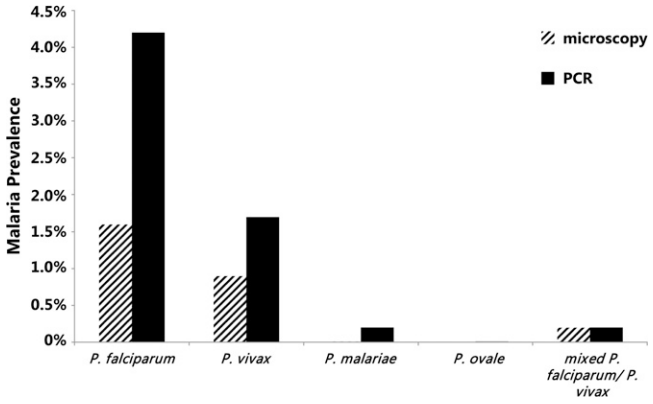

FIGURE 5. Malaria prevalence by species. The estimated prevalences for the entire surveyed area for any *Plasmodium* species (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and mixed *P. falciparum/P. vivax*), as measured by microscopy and polymerase chain reaction (PCR) are shown.

significantly higher for all malaria species compared with microscopy: 1) for *P. falciparum* (4.40%, 95% CI: 3.95–4.90% versus 1.6%, 95% CI: 1.4–2.0%, $P < 0.00001$), 2) *P. vivax* (1.89%, 95% CI: 1.60–2.22% versus 1.1%, 95% CI: 0.9–1.4%, $P < 0.001$), and 3) *P. malariae* infections (0.22%, 95% CI: 0.13–0.35% versus 0.01%, 95% CI: 0.0003–0.07%, $P < 0.0001$). Only one ovale infection was detected by PCR (0.01%) and missed by microscopy. The proportions of *P. falciparum/P. vivax* mixed infections detected by microscopy and PCR were not significantly different (0.20%, 95% CI: 0.12–0.32% versus 0.20%, 95% CI: 0.12–0.35%, $P = 1$). Among the samples tested as negative by microscopy, 3.85% (289/7,491) were found positive by PCR. This included 186 *P. falciparum*, 75 *P. vivax*, 16 *P. malariae*, 11 mixed *P. falciparum/P. vivax* and one *Plasmodium ovale* infections.

Our findings demonstrate that large reservoirs of submicroscopic infections are common in Cambodia, and are approximately two to three times more common compared with microscopy and represent a significant source of transmission.[30] It is worth noting that an elimination strategy based on microscopic mass screening and treatment would miss the majority of infected individuals, confirming our previous observations in Pailin Province.[31] Similarly, at the village level, more than a third of villages which appear free of malaria based on microscopy alone actually harbor infection. Factors associated with submicroscopic *P. falciparum* and *P. vivax* infections were individuals living in high-transmission settings (Domain 1: 3.9% versus Domain 2: 0.9%, $P < 0.0001$ for *P. falciparum* and Domain 1: 1.6% versus Domain 2: 0.6%, $P < 0.0001$ for *P. vivax*), and living close to forested areas (risk zones 1 and 2: 3.0% and 3.8% versus risk zones 3 and 4: 2.2% and 0.8% for *P. falciparum*, $P < 0.0001$ and risk zones 1, 2, and 3: 1.1%, 1.7%, and 1.2% versus risk zone 4: 0.4% for *P. vivax*, $P < 0.01$). Strategies to protect high-risk individuals from contracting malaria need to be developed, taking into account that the two main forest-related vectors, *Anopheles dirus* and *Anopheles minumus* are outdoor biting mosquitoes.[22,32]

Surprisingly, the PCR failed to detect 18 infections (0.23%) found positive by microscopy (five *P. falciparum*, 12 *P. vivax*, and one mixed *P. falciparum/P. vivax*). All samples missed by PCR had a parasite density < 100 parasites/μL, except for two *P. falciparum* (20,000 and 3,560 parasites/μL) and one *P. vivax* infections (3,880 parasites/μL), representing clear PCR false-negative results probably due to technical errors (presence of PCR inhibitors for instance). Finally, there were 29/7,707 (0.38%) cases in which microscopy and PCR gave different species identifications: half cases (15 cases) involved cases that were reported as mixed *P. falciparum/P. vivax* infections by microscopy but were classified as mono-infections by PCR (14 *P. falciparum* and one *P. vivax*; Table 2).

The data presented in this study are reliable baseline estimates from which to assess the trends in malaria prevalence rates with successive national malaria surveys. Our findings reinforce previous observations[11,33] in which the prevalence of infection measured by PCR was, on average, twice that measured by microscopy. The recent shift from control to elimination with elimination as the ultimate goal will require a rigorous assessment of reservoirs of infection, including submicroscopic infections. Cambodia has committed itself to the elimination of malaria by 2025. To reach this goal, asymptomatic reservoirs should be detected to assess the true prevalence

TABLE 2
Comparison of microscopy and PCR for the detection of asymptomatic malaria in 7,707 samples collected in endemic area in Cambodia in 2007

	Plasmodium species identification by	Microscopy						
		Negative	*P. falciparum*	*P. vivax*	*P. malariae*	*P. ovale*	*P. falciparum/P. vivax*	Total
PCR	Negative	7,202	5	12	0	0	1	7,220
	P. falciparum	186	116	7	0	0	14	323
	P. vivax	75	3	50	0	0	1	129
	P. malariae	16	0	0	1	0	0	17
	P. ovale	1	0	0	0	0	0	1
	P. falciparum/P. vivax	11	1	3	0	0	2	17
	Total	7,491	125	72	1	0	18	7,707

The following figures in the colored cells are: for green cells, concordant results between microscopy and PCR (95.6%); for yellow cells, microscopy positive/PCR negative results (0.2%); for orange cells, microscopy negative/PCR positive results and for black cells (3.7%), discordant results for Plasmodium identification (0.4%).

PCR = polymerase chain reaction; *P. falciparum* = *Plasmodium falciparum*; *P. malariae* = *Plasmodium malariae*; *P. ovale* = *Plasmodium ovale*; *P. vivax* = *Plasmodium vivax*.
In the table, green cells indicate concordant results between microscopy and PCR (95.6%); yellow cells indicate microscopy-positive/PCR-negative results (0.2%); orange cells indicate microscopy-negative/PCR-positive results; and black cells (3.7%) indicate discordant results for *Plasmodium* identification (0.4%).

of parasitemia to design an evidence-based elimination strategy and put in place a comprehensive surveillance system to assess the impact of the implemented strategies. Control methods based either on mass screening and treatment or targeted mass drug administration will only be effective if the screening method identifies submicroscopic reservoirs of infection. Cambodia may, therefore, need to shift its current emphasis on parasitological diagnosis based on RDTs and microscopy to high-throughput PCR with rapid communication to the field to find and eliminate hidden parasite reservoirs in transmission foci or to new molecular technologies implementable in the field, such as loop mediated isothermal amplification[34] or capture and ligation probe-polymerase chain reaction.[35] In addition, seroprevalence surveys of *P. falciparum* may complement current diagnostic tools and provide useful insight into malaria exposure levels and an estimation of the transmission intensity.[36] Lastly, to improve the malaria surveillance and monitoring, more data are needed to assess the role of submicroscopic infections in maintaining the transmission cycle through the low-transmission dry season.

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Authors' addresses: Dysoley Lek, National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia and School of Public Health, National Institute of Public Health, Phnom Penh, Cambodia, E-mail: soleyl@cnm.gov.kh. Jean Popovici and Didier Menard, Institut Pasteur in Cambodia, Phnom Penh, Cambodia, E-mails: jpopovici@pasteur-kh.org and dmenard@pasteur-kh.org. Frederic Ariey, Institut Pasteur in Cambodia, Phnom Penh, Cambodia, Department of Parasites and Insect Vectors, Institut Pasteur, Paris, France, Institut Cochin, Institut National de la Santé et de la Recherche Médicale U1016 (INSERM U1016), Faculté de Médecine, Université Paris Descartes Sorbonne Paris Cité, Paris, France, and Laboratoire de Parasitologie-Mycologie, Hôpital Cochin, Assistance Publique–Hôpitaux de Paris (AP-HP), Paris, France, E-mail: frederic.ariey@yahoo.fr. Seshu Babu Vinjamuri, World Health Organization Lao People's Democratic Republic Office, Vientiane, Lao PDR, E-mail: vinjamuriv@wpro.who.int. Sylvia Meek and Jan Bruce, London School of Hygiene and Tropical Medicine, London, United Kingdom, E-mails: s.meek@malariaconsortium.org and jane.bruce@lshtm.ac.uk. Walter R. J. Taylor, Service de Médecine Tropicale et Humanitaire, Hôpitaux Universitaires de Genève, Geneva, Switzerland and Mahidol Oxford Tropical Medicine Research Unit, Bangkok, Thailand, E-mail: bob@tropmedres.ac. Duong Socheat, National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia, E-mail: socheatd@cnm.gov.kh. William O. Rogers, Naval Medical Research Unit 2 Detachment, Phnom Penh, Cambodia, E-mail: rogerswo@namrutwo.org.

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