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Usefulness of Polymerase Chain Reaction to Supplement Field Microscopy in a Pre-Selected Population with a High Probability of Malaria Infections

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Abstract. This study determines the use of nested PCR as a diagnostic tool to supplement field microscopy in symptomatic individuals suspected of being positive for malaria, and it explores its role in active case detection to identify asymptomatic parasite carriers. In symptomatic individuals, compared with PCR, microscopy had a sensitivity of 86.6% (95% confidence interval [CI] = 77.8–92.4) and specificity of 100% (95% CI = 96.9–100). During active case detection, two asymptomatic persons were diagnosed as having vivax malaria by polymerase chain reaction (PCR) but not microscopy. Currently, PCR is being carried out in Sri Lanka only for population surveys to estimate the hidden reservoir of malaria. Based on the results of this study and because of cost considerations, pooled PCR will be used in the future to screen samples from clinically suspected foci to increase the proportion of malaria cases detected. This strategy will assist the success of the malaria elimination program in Sri Lanka.

INTRODUCTION

With the decline in the number of malaria cases being reported from Sri Lanka since the year 2000 and only 670 microscopically confirmed cases being reported in 2008, Sri Lanka has been working on eliminating malaria along with 10 other countries.[1,2] After the successful ending of the long-running civil conflict in the country in May 2009, the goal of the National Program was phased elimination (that is, elimination of *Plasmodium falciparum* malaria by 2012 and *P. vivax* malaria by 2014).[3,4]

In view of achieving this target, disease surveillance has been strengthened in previously malaria-endemic regions, which are traditionally confined to the dry zone of the country, and the Anti-Malaria Campaign (AMC) is targeting 100% case detection and confirmation by microscopy or rapid diagnostic tests (RDT). Because fever is the most frequent clinical symptom seen in malaria infections, screening of fever patients with signs and/or symptoms of malaria attending medical institutions by passive case detection (PCD) and screening of asymptomatic individuals through active case detection (ACD) (through mobile malaria clinics at village level in regions where there is a high risk of malaria transmission or suspected malaria foci) are being carried out. However, symptomatic patients are not routinely subjected to blood smear examination or RDT for confirmation or exclusion of malaria diagnosis as the low prevalence of the disease has resulted in malaria being moved far down the list of differential diagnosis used by healthcare providers. Currently, polymerase chain reaction (PCR) is being carried out in Sri Lanka only for population surveys to estimate the hidden reservoir of malaria in previously high-risk communities or to confirm diagnosis of malaria on a request made by a physician. Several constraints are present in carrying out this procedure, including the high cost (US $10 per test) and the limited availability of molecular diagnostic facilities only in central laboratories.

Accurate diagnosis is essential for a patient to receive proper treatment. After a change in the drug policy for treatment of malaria in 2008,[5] treatment of *P. vivax* malaria is now with chloroquine and a 14-day course of primaquine, whereas *P. falciparum* is treated with artemisinin combination therapy (ACT) and a single dose of primaquine. Considering the fact that the two main parasite species present in the country are being treated with two different drugs and treatment regimens, the importance of correct diagnosis at the time of patient presentation is unquestionable, because the treatment strategy adopted by the doctor will depend on laboratory confirmation of the malaria species. The current study examines the need to establish PCR to supplement field microscopy for routine diagnosis of malaria.

This study also assesses the use of PCR for diagnosis of malaria in asymptomatics. An individual may be harboring a submicroscopic infection that may not be detected by microscopy. This may result in a negative result because of low parasite density of one species in a monoinfection, or a wrong diagnosis of a monoinfection because of low parasite density of one species in a mixed infection. Resurgence of malaria originating from hidden reservoir hosts harboring parasites having submicroscopic parasitemia is a matter of serious concern for malaria eliminating programs.

MATERIALS AND METHODS

Study area. This study was carried out between the months of June and November in 2008 in the Anuradhapura district. Anuradhapura is the largest district in Sri Lanka, with a population of 773,227 at risk of contracting malaria (S. R. Jayanetti, Regional Malaria Officer, personal communication) and malaria transmission occurring throughout the year. The district is divided into 19 Medical Officer of Health (MOH) areas and is composed of 692 Grama Niladari areas (a GN area is the smallest administrative unit in the country).

Study population and sample collection. Samples were collected from two pre-selected groups of individuals: (1) patients with signs and/or symptoms suggestive of malaria attending selected medical institutions (PCD) and (2) asymptomatic individuals attending mobile malaria clinics (ACD). Written consent was obtained from both groups. In the case of minors less than 16 years of age, consent was obtained from the parents or guardians.

Doctors in charge of the three selected hospitals were requested to refer all patients suspected of having malaria for confirmation of diagnosis. Sample collection was carried in a

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consecutive series of patients that were referred to the AMC's Public Health Laboratory Technicians (PHLTs) working in the hospitals selected for the study, namely the General Hospital, Anuradhapura, the largest tertiary care unit in the entire North Central Province and the Rural Hospital, Rambewa, which was selected based on the high number of cases reported from this MOH area over the past 5 years. Medical Laboratory Technicians (MLTs) were responsible for sample collection at the Military Hospital, Anuradhapura, which is the main military hospital in the region.

Blood samples were collected from asymptomatic individuals attending mobile malaria clinics in three high malaria risk MOH areas with suspected malaria foci (namely, Kekirawa, Padaviya, and Madhyama Nuwaragama).

Considering that the population at risk of contracting malaria in the Anuradhapura district is 773,227, the calculated sample size was 384 with 5% precision level and 95% confidence level. Based on the proportions screened by the two case detection methods, within the past 5 years, a stratified sample of 390 was selected (250 by passive case detection and 140 by active case detection). Ethical clearance was obtained from the Ethics Review Committee, Faculty of Medicine, University of Colombo.

Laboratory diagnosis of malaria. *Microscopy.* Thick and thin blood smears were prepared using finger-prick blood and were stained with 10% Giemsa stain for 10 minutes. The slides were reported as negative for malaria parasites if no parasites were detected in 200 thick-film fields under light microscopy at 500× magnification. If a smear was positive for malaria parasites, an additional 100 microscopic fields were screened to confirm the parasite species. The parasite density was calculated from the thick smear as indicated in the World Health Organization (WHO) Bench aids for malaria microscopy by counting the number of parasites against 200 leukocytes (assuming that there are 8,000 leukocytes/µL), and it was expressed as parasites per microliter.[6]

Nested PCR assay based on the 18 ssrRNA gene of Plasmodium spp. Approximately 500 µL finger-prick blood was collected from the patient into microtubes coated with acid citrate dextrose (ACD), stored at −20°C, and transported to the Genetech Research Institute in Colombo, where extraction of DNA and PCR assays was carried out blinded to the microscopy results. PCR assays were repeated for all PCR-positive and microscopically negative samples.

DNA template preparation and nested PCR. Phenol-chloroform extraction of DNA from the blood samples was done according to the method given by Beck,[7] with 0.05% saponin lysis and Proteinase K (20 µL/mL) treatment. A nested PCR was performed according to the method described by Snounou and Singh[8] for the genus- and species-specific PCR amplifications of 18 ssrRNA malaria genes. Genus-specific primers: rPLU1 and rPLU5 amplified a 1.6- to 1.7-kb fragment depending on the species. The product of the first amplification reaction (nest 1) was used as the template for the genus-specific second amplification reaction (nest 2), with primers rPLU 3 and rPLU 4, which produced a 235-bp fragment that clarified the malaria PCR positivity. These positive samples were then subjected to species-specific primers, namely rFAL1 and rFAL2, rVIV1 and rVIV2, and rMAL1 and rMAL2, and rOVA1 and rPLU2 which were used to amplify *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* 18ssrRNA genes, respectively. These amplifications produced PCR fragments of 206, 121, 145, and 226 bp for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, respectively. All precautions given by Beck[7] and Snounou and Singh[8] were taken to minimize contamination in performing PCR, including having a negative control.

The PCR products were electrophoresed in 2% Tris-acetic acid and EDTA agarose gel for 30 minutes followed by ethidium bromide staining for 15 minutes and visualized under UV light.

Determination of analytical sensitivity of PCR. The sensitivity of the nested PCR to determine the lowest parasite density in the clinical samples was evaluated by measuring a blood sample positive by PCR and microscopy, with a parasite density of 2,000 parasites/µL. DNA was extracted as described by Beck,[7] and six 10-fold serially diluted DNA samples were prepared to obtain DNA concentrations equivalent to parasite densities of 200 parasites/µL, 20 parasites/µL, 2 parasites/µL, 0.2 parasites/µL, 0.02 parasites/µL, and 0.002 parasites/µL. Genus-specific nested PCR assays were done using 2 µL serially diluted DNA samples as templates.

Cross-checking of blood smears and treatment of malaria positives. All blood smears were cross-checked, and the parasite density was reestimated at the AMC headquarters by a PHLT who was blinded to the initial results. The average of the parasite density was taken as the final result. The result of the PCR was obtained within 1 month of taking the corresponding blood smear. The Regional Malaria Officer was given information on the positive cases, and the patients were treated according to the AMC guidelines.[5]

Statistical analysis. Minitab 14 statistical software was used. Differences in the relative prevalence obtained by PCR and microscopy were analyzed using the χ^2 test. Sensitivity, specificity, and negative and positive predictive values of microscopy compared with PCR were estimated.

RESULTS

Characteristics of the study group. The study group comprised a total of 390 individuals; 250 individuals were from the Medical Institutes, and 140 individuals were from mobile malaria clinics. In all individuals, positivity for malaria was determined by blood-smear examination and PCR. Characteristics of the population and the four main clinical manifestations that they presented with to be referred for microscopy are given in Table 1.

Analytical sensitivity of the nested PCR assay. It was noted that the lowest limit of detection of *Plasmodium* species was 2 parasites/µL. In this study, PCR is considered as the gold standard for malaria diagnosis because of its advantage in detecting submicroscopic infections, and the microscopy results were validated against this.

Malaria diagnosis by passive case detection. Characteristics of the 97 individuals (6 females and 91 males) who presented with fever and signs and/or symptoms of malaria and tested positive for malaria parasites are shown in Table 2. A majority (84%) of malaria-positive patients was from the armed forces, and 34 of the infections were diagnosed at the military hospital. PCR diagnosed all 97 infections, giving a prevalence rate of 39%, whereas microcopy confirmed 87% (N = 84) of these infections. The difference in proportion of cases detected by microscopy and PCR was not statistically significant.

Blood samples that were diagnosed as malaria-positive by PCR but were microscopy-negative were cross-checked, and

Table 1
Study population characteristics

Category of population screened	Medical institutions		Mobile malaria clinics	
	N	Percent	*N*	Percent
Sex				
Males	188	75.2	113	80.7
Females	62	24.8	27	19.3
Occupation				
Armed forces	110	44	75	53.57
Other	140	56	65	46.43
Mean age (years)				
Males	29	26		
Females	27	40		
Clinical picture				
Symptomatic*	250	100	0	
Fever	250	100		
Head ache	238	95.2		
Myalgia	210	84.0		
Back ache	208	83.2		
Asymptomatic†	0		140	100

* Symptomatic = with fever and/or signs and/or symptoms suggestive of malaria.
† Asymptomatic = without fever and signs and symptoms suggestive of malaria.

there was no discrepancy in the microscopic result between the initial reading and cross-checking result. Thereby, 13% (13/97 × 100) of blood smears examined gave false-negative results. The parasitemia of microscopically positive infections ranged from 80 to 12,320 parasites/μL.

Given that the nested PCR presented higher positivity (PCR = 39% versus microscopy = 33%), it was considered as the gold standard to calculate the power of microscopy. In the diagnosis of symptomatic malaria, microscopy presented a sensitivity of 86.6% (95% CI = 77.8–92.4), specificity of 100% (95% CI = 96.9–100), positive predictive value (PPV) of 100% (95% CI = 94.5–100), and negative predictive value (NPV) of 92.2% (95% CI = 86.7–95.6).

Comparison of species diagnosis between species-specific nested PCR and microscopy was assessed (Table 3). The nested PCR detected 82 *P. vivax* infections, and microscopy diagnosed 72 of these cases. Similarly, of the eight *P. falciparum* infections diagnosed by PCR, only five positives were detected by microscopy. Of the six *P. vivax* and *P. falciparum* mixed infections, four were reported as being mixed infections by microscopy, and the other two mixed infections were reported as *P. falciparum* monoinfections. However, the only *P. malariae* infection found in this study was correctly identified by microscopy.

Table 2
Characteristics of symptomatic individuals who tested positive for malaria in the different hospitals

	Number		
	Military hospital	General hospital	Rural hospital
Diagnostic method			
Microscopy	28	55	1
PCR	34	59	4
Sex			
Males	34	55	2
Females	0	4	2
Occupation			
Armed forces	34	46	2
Other	0	13	2
Clinical manifestations			
Fever	34	59	4
Headache	32	54	4
Myalgia	24	45	4
Back ache	25	34	0

Thus, of the 84 cases detected by microscopy, species identification was accurate in 98% of the instances (82/84).

Compared with nested PCR, sensitivity of microscopy in species identification among symptomatic individuals was 87.8% (95% CI = 78.2–93.6) for *P. vivax* and 62.5% (95% CI = 25.8–89.7) for *P. falciparum*, whereas the specificity was 100% (95% CI = 97.2–100) for *P. vivax* and 99.1% (95% CI = 96.7–99.8) for *P. falciparum*. The PPV was 100% (95% CI = 93.6–100) for *P. vivax*, because all 72 cases of vivax were identified correctly by microscopy. However, because two mixed infections had been misdiagnosed as falciparum, the PPV of *P. falciparum* was 71.4% (95% CI = 30.25–94.88). The NPV was 94.3% (95% CI = 89.6–97.1) for *P. vivax* and 98.7% (95% CI = 96.1–99.6) for *P. falciparum*.

Malaria diagnosis by active case detection. During active case detection at mobile malaria clinics, two asymptomatic persons (males) were diagnosed as having vivax malaria by PCR depicting a prevalence rate of 1.4%. Microscopy failed to detect any malaria infections in this group. The sensitivity, specificity, PPV, and NPV were not determined because of the low number of positives.

Both of these cases were detected in persons from the security forces who were on home leave.

DISCUSSION

Accurate malaria diagnosis and treatment with appropriate drugs is mandatory for an elimination strategy to be successful. Microscopy and RDTs provide cost-effective, rapid diagnostic tools that can easily be applied in the field.[9] Nonetheless, these methods are prone to misdiagnosis, especially in the cases of mixed infections and low-level parasitemia.[10–12] Nested PCR and real-time PCR are known to have a higher sensitivity and specificity for malaria diagnosis compared with light microscopy.[13,14] The detection of *P. vivax* and *P. falciparum* parasitemia by PCR, at levels undetectable by microscopy, has been reported by several authors.[15–21]

PCR has been used for malaria diagnosis in community-based studies carried out in Sri Lanka (one study in the Anuradhapura district and the other study in the Kurunegala and Trincomalee districts).[22,23] Neither of these studies reported any malaria positives by microscopy or PCR. However, a high malaria positivity rate was expected and diagnosed in the current study population, which was preselected. First, symptomatic patients from the military hospital were recruited for the study, and this is the main hospital catering to the persons from the armed forces working in the Northern Province where malaria transmission continues to occur at a higher rate than the rest of the country.[1] Second, the period of study coincided with the annual increase in transmission associated with the North East monsoon.

The analytical sensitivity of the nested PCR assay was high, being 2 parasites/μL, although it did not reach the sensitivity limit of 0.1 parasites/μL blood (0.000002% parasitemia) stated by Snounou and Singh.[8] This discrepancy may be because of the quantity and nature of the initial DNA template. Significant inhibition of PCR by excess of human DNA has been reported[7,8,24] which may be the case in this study as well. This is because the DNA used to determine the analytical sensitivity was from patient-derived blood and not from a parasite culture that is free of human DNA. However, this analytical sensitivity confirmed that PCR was able to detect even

TABLE 3
Comparison of parasite detection by species-specific nested PCR and microscopy among symptomatic individuals

Microscopy	Nested PCR				
	P. falciparum	*P. vivax*	*P. falciparum + P. vivax*	*P. malariae*	Negative
Negative	3	10	0	0	153
P. falciparum	5	0	2	0	0
P. vivax	0	72	0	0	0
P. falciparum + P. vivax	0	0	4	0	0
P. malariae	0	0	0	1	0
Total	8 (3.2%)	82 (32.8%)	6 (2.4%)	1 (0.4%)	153 (61.2%)

submicroscopic malaria infections, and thereby, the use of the PCR assay as the gold standard test is considered justifiable. The analytical sensitivity also indicates that the amount of blood collected in the field, the method of collection and transport to the central laboratory, the reagents used, and the PCR procedure are suitable to carry out a sensitive nested PCR assay to screen suspected malaria patients presenting to peripheral hospitals or asymptomatics in the community. However, mechanisms are in place to increase the analytical sensitivity by optimizing the sample collection procedure when implementing PCR assay for routine diagnosis of malaria under field settings. Compared with PCR, which was able to detect 2 parasites/μL, the parasite detection threshold for microscopy was 80 parasites/μL. Thereby, microscopy alone would have missed the submicroscopic infections, leading to higher morbidity and the formation of a reservoir of infection that could potentially jeopardize elimination efforts.

Furthermore, the majority of the malaria infections was reported from persons of the armed forces, where a high probability of malaria infections is known to occur because of the transmission of malaria occurring in the jungles of the Northern Province (unpublished data). Due to repeated malaria infections occurring in this population of individuals (185 military persons, which amounts to 47% of the study population), a low-density parasitemia could exist within this group, giving rise to the high percentage of submicroscopic infections detected in this study. However, the sample comprised mainly of residents from Anuradhapura, where malaria incidence has decreased significantly over the years (Annual Parasite Incidence [API] = 0.01 in 2008). This probably explains why there is no significant difference in the number of positives detected through microscopy and PCR. The findings of this study have been used by the National Program to increase malaria surveillance among military personnel in its drive to malaria elimination.

The current study shows that the results obtained by PCR were superior to those obtained by microscopy, because PCR was able to detect all microscopy-positive samples and additional *Plasmodium*-positive samples that were negative (10 vivax and 3 falciparum) or misdiagnosed (2 mixed infections wrongly diagnosed as monoinfections) by microscopy. In clinically suspected malaria patients, sensitivity of microscopy in identifying *P. falciparum* infections was as low as 62.5 (95% CI = 25.8–89.7), probably because of sequestration of mature parasites. This indicates that the PCR test may provide a more precise diagnosis of patients infected with *P. falciparum* malaria by detecting parasites that would be missed by traditional blood-film screening. Thus, achieving 100% sensitivity for a diagnostic test would be necessary in accurate diagnosis of *P. falciparum* and *P. vivax* considering the dangerous nature of the former and the relapse and morbidity pattern associated with the latter.

All individuals diagnosed as being malaria-positive were treated. Clinically symptomatic patients diagnosed with falciparum infection were admitted to the General Hospital Anuradhapura and treated with ACT and primaquine. However, it is unlikely that the three *P. falciparum* patients who were not diagnosed by blood-smear examination would have received appropriate treatment had their diagnosis not been confirmed by PCR. Two coinfections of *P. falciparum* and *P. vivax* were diagnosed as *P. falciparum* by microscopy. The treatment strategy extended to *P. falciparum* would have eliminated the blood stages of *P. vivax*. Because the single dose of primaquine given to patients with falciparum malaria would not have been sufficient to eliminate the liver stages of vivax, a relapse may have occurred at a later date.

Similar situations could occur because of misdiagnosis of the patients with vivax malaria, which was shown in this study where PCR confirmed vivax infections in 10 clinically symptomatic patients who were not diagnosed by microscopy. The clinical implications of misdiagnosis, missing falciparum infections, or not treating relapses are severe not only for the patients concerned but also for the elimination efforts that are underway in the country.

Microscopy detected the only case of *P. malariae* in this study, which was confirmed by PCR. This individual had acquired the infection outside the country, and this detection can be considered significant, because *P. malariae* is not normally reported in this country.

Unlike the two previously reported community-based studies carried out in Sri Lanka,[22,23] this study detected two *P. vivax* infections during the screening of 140 asymptomatic individuals, indicating that asymptomatic carriers still exist, although not on a large scale. The current focus of the malaria elimination strategy is carrying out approximately two mobile malaria clinics per district, focusing on the smallest administrative units of the country where cases have been reported to detect individuals with patent parasitemia who may be within the incubation period. A larger, non-stratified sample would have allowed for calculations of sensitivity, specificity, and predictive values of microscopy for this group.

Because PCR is not affected by the subjectivity of the observer, it is an excellent tool for obtaining accurate epidemiological data and also ensuring correct treatment and follow-up of all positives. However, this does not indicate that the quality of microscopy should not be improved. Since the launch of the elimination program, a considerable strengthening of the malaria surveillance system, including the retraining of laboratory staff, has commenced. Establishment of PCR

facilities for diagnostic purposes is limited by financial constraints and lack of transport facilities. PCR may not be cost-effective in large-scale applications such as community surveys in a country such as Sri Lanka where the API is 0.01, unless pooling of samples is being done to reduce the cost of PCR.[25] Over the past few years, a variety of PCR-based techniques have been developed for genus- or species-specific diagnosis of malaria.[24,26] The recent advance of a real-time quantitative PCR technique has proven useful in various applications, including parasite detection, species differentiation, gene expression and regulation, and allelic discrimination,[27,28] and it is more sensitive than nested PCR. However, the large majority of developed real-time PCR assays use many sets of primers and/or probes to analyze each sample, thus increasing cost along with reliability, which may not be feasible for a resource-poor country such as Sri Lanka.

Based on the results of this study, PCR assay, which is considered as the optimal diagnostic strategy to increase the proportion of malaria cases detected, can be used in the future to screen samples from suspected foci. Because of the high cost involved, routine diagnostic activities will commence with pooled samples, which will reduce the cost significantly. This strategy will assist in the success of the malaria elimination program in Sri Lanka by the year 2014.

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