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Comparative evaluation of microscopy, OptiMAL[®] and 18S rRNA gene based multiplex PCR for detection of *Plasmodium falciparum* & *Plasmodium vivax* from field isolates of Bikaner, India

Deepak Pakalapati¹, Shilpi Garg¹, Sheetal Middha², Abhishek Kochar², Amit Kumar Subudhi¹, Boopathi Pon Arunachalam¹, Sanjay Kumar Kochar², Vishal Saxena¹, Pareek RP⁴, Jyoti Acharya², Dhanpat Kumar Kochar³, Ashis Das^{1*}

¹Center for Biotechnology, Dept of Biological Sciences, Birla Institute of Technology and Science, Pilani–333031, Rajasthan, India

²Department of Medicine, Sardar Patel Medical College, Bikaner–334003, Rajasthan, India

³Kothari Medical and Research Institute, Bikaner, Rajasthan–334003, India

⁴Medical Centre, Birla Institute of Technology and Science, Pilani–333031, Rajasthan, India

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ABSTRACT

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Objective: To evaluate microscopy, OptiMAL[®] and multiplex PCR for the identification of *Plasmodium falciparum* (*P. falciparum*) and *Plasmodium vivax* (*P. vivax*) from the field isolates of Bikaner, Rajasthan (Northwest India). **Methods:** In this study, a multiplex PCR (*P. falciparum* and *P. vivax*) was further developed with the incorporation of *Plasmodium malariae* (*P. malariae*) specific primer and also a positive control. The performance of microscopy, plasmodium lactate dehydrogenase (pLDH) based malaria rapid diagnostic test OptiMAL[®] and 18S rRNA gene based multiplex PCR for the diagnosis of *P. falciparum* and *P. vivax* was compared. **Results:** The three species multiplex PCR (*P. falciparum*, *P. vivax* and *P. malariae*) with an inbuilt positive control was developed and evaluated. In comparison with multiplex PCR, which showed the sensitivity and specificity of 99.36% (95% CI, 98.11%–100.00%) and 100.00% (95% CI, 100.00%–100.00%), the sensitivity and specificity of microscopy was 90.44% (95% CI, 88.84%–95.04%) and 99.22% (95% CI, 97.71%–100.00%), and OptiMAL[®] was 93.58% (95% CI, 89.75%–97.42%) and 97.69% (95% CI, 95.10%–100.00%). The efficiencies were 99.65%, 95.10% and 95.45% for multiplex PCR, microscopy and OptiMAL[®], respectively. **Conclusions:** Our results raise concerns over the overall sensitivities of microscopy and OptiMAL[®], when compared to the multiplex PCR and thus stress the need for new molecular interventions in the accurate detection of the malarial parasites. This further highlights the fact that further developments are needed to improve the performance of rapid diagnostic tests at field level.

1. Introduction

Half of the world's population is at risk from malaria and about 25% of the population has been considered as at high risk. An estimated 94 million were effected, leading

to nearly 345 960 deaths in 2010. India alone contributes to 65% of the 2.4 million cases seen in Southeast Asia^[1], which is still considered as underestimated^[2].

The changing clinical manifestations with multi-organ involvement in *Plasmodium falciparum* (*P. falciparum*)^[3], emerging trends of complications in *Plasmodium vivax* (*P. vivax*)^[4–6] and *Plasmodium knowlesi* malaria^[7], and burden of malaria in pregnancy are other important issues that merit attention and formulation of suitable intervention strategies^[8]. Parasite resistance to antimalarial medicines and mosquito resistance to insecticides are major threats to

*Corresponding author: Ashis Das, PhD, Professor, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Rajasthan, 333031, India.

Tel (R): +91-1596-244118

Fax: 91-1596-244183

E-mail: ashisd28@gmail.com; ashdas28@gmail.com

achieving global malaria control^[1].

The detection of human malarial parasites by Giemsa-stained thick and thin films still remains the official gold standard for malaria diagnosis. Although simple and economical, its reliability is questionable particularly at low parasitemia^[9] and requires well trained personnel in parasite morphological differentiation even at low densities^[10,11] and in elucidating mixed infections^[12]. Rapid identification of the human malaria species is thus imperative for accurate diagnosis and appropriate therapy. Because of an increasing occurrence of drug-resistant parasites even to the newly developed drugs like artemisinin combination therapies^[13,14], appropriate diagnosis is essential. Presumptive treatment of malaria also results in significant overuse of antimalarials. Thus, a reliable test which is able to differentiate the various malaria species and to detect mixed infections would aid in effective management of the disease.

Various alternative techniques have also been developed to address these problems such as immunochromatographic tests (RDTs) based on detection of antigens such as HRP II and plasmodium lactate dehydrogenase (pLDH). Histidine-rich protein 2 (HRP2) and pLDH are the most widely used antigens for parasite detection and quantification^[15]. However, these tests have their significant advantages and also drawbacks. Persistence of the HRP2 antigens in the circulation post treatment and gene deletions limits its use^[16]. Because pLDH is a product of viable parasites only^[17] and not a residual metabolite like HRP2, the OptiMAL[®] assay might be a simple way to monitor parasitemia in the malaria infected patients at a field level and also to monitor antimalarial therapy^[18].

Various sophisticated molecular techniques have also been used in the process of finding a best alternative to the gold standard, out of which PCR has been adopted routinely in various laboratories worldwide. The 18S rRNA gene (SSU rRNA gene) has been widely used as a molecular target for the species specific identification of human *Plasmodium* species by nested PCR^[19], multiplex PCR^[6,20] and realtime PCR^[21].

In India, no study has been done comparing microscopy, antigen and antibody based dipstick assays and other techniques. This paper reports the comparative field evaluation of microscopy, antibody based dipstick (OptiMAL[®]) and a multiplex PCR^[20] for the simultaneous detection of *P. falciparum* and *P. vivax*.

2. Materials and methods

2.1. Study site

The study was performed at the SP medical Hospital at Bikaner, India, a district in Rajasthan where severe

manifestations were reported to be caused by *P. vivax* along with *P. falciparum*^[4,6,22]. The study was approved by the Institutional Ethical Committee of the (SPM) and BITS–Pilani. Informed written consent was obtained from either the patient or a relative/guardian in all the cases. The testers were blinded to the patient status/diagnosis during the study.

2.2. Patients

Samples collected from 292 adults and children attending the medical hospital from Bikaner district, Rajasthan (India) were used for the study. Only those who had a presumptive diagnosis of clinical malaria were eligible for the study. A history of fever, symptoms, and drugs taken, if any, was recorded for each subject. Each selected patient blood sample was examined by the three methods: microscopy, OptiMAL[®], multiplex PCR and various other routine serological tests. The samples of patients who refused to consent were omitted from the study.

2.3. Microscopy

Thick and thin films were prepared directly from finger prick blood samples, and immunochromatographic testing was performed directly with the finger prick blood samples. Thick and thin films were stained with the standard 10% Giemsa solution and examined at 100× by two expert microscopists. The microscopists were unaware of the patient's diagnosis or immunochromatographic test result. The initial slide was considered negative if no parasites were seen in at least 100 high-power fields.

2.4. OptiMAL[®]

OptiMAL[®] test (DiaMed AG, Switzerland) is a rapid malaria detection test (RDT), which utilizes a dipstick coated with monoclonal antibodies against the intracellular metabolic enzyme pLDH. Differentiation of malaria parasites is based on antigenic differences between the pLDH isoforms. Since pLDH is produced only by live *Plasmodium* parasites, this test has the ability to differentiate live organisms from dead ones^[23]. All whole blood samples were tested with the OptiMAL test according to manufacturer's instructions. Interpretation of the assay test strip results was done as: 1) When one control band and two test bands appeared, the test was considered to be positive for *P. falciparum*; 2) When one control band and one test band appeared, the test was considered positive for *P. vivax* (No other species are found in this region); 3) When only one control band appeared at the top of the test strip without test band, the test was considered to be negative (Figure 1).

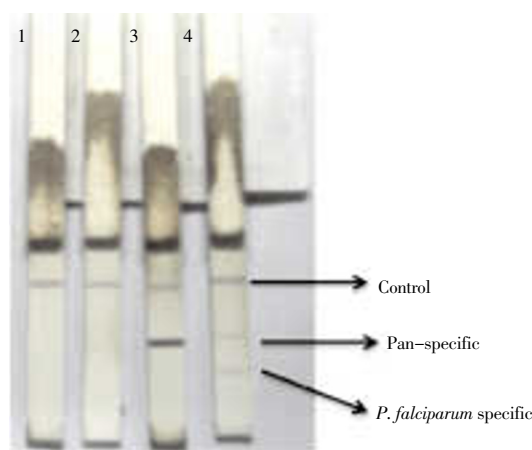


Figure 1. OptiMAL® strips depicting the plasmodial infection status in blood.

Strips 1 and 2 showing the control band alone were negative. Strip 3 shows the pan-specific band alone, confirming the presence of *P. vivax* and strip 4 shows the *P. falciparum*-specific band along with the pan-specific band, confirming the presence of *P. falciparum*.

2.5. DNA isolation

Blood samples were collected on informed consent by trained clinicians, from patients. They were collected in ethylenediamine tetra acetic acid (EDTA) or acid citrate dextrose (ACD) anticoagulants and stored at -20°C . Complete DNA was isolated from these samples^[24] by treating them with lysis buffers containing NaCl, Tris-HCl (pH 8.0), EDTA and SDS (1%) at 37°C for 45 min, and with proteinase K (Sigma) at 5°C for 2 h, followed by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and overnight ethanol precipitation at -20°C . The precipitated DNA was washed with 70% ethanol and the pellet was air dried and resuspended in TE buffer (pH 8.0). The DNA was stored at -20°C until processed. This was used directly for PCR reactions.

2.6. 18S rRNA gene based multiplex PCR

The PCR studies were targeted against the 18S ribosomal RNA gene of the parasite and were based on conditions reported earlier^[6,20], utilizing one genus-specific forward primer and two species-specific reverse primers in the same reaction. Additional *Plasmodium malariae* (*P. malariae*) specific reverse primer and also the 18S rRNA gene based PCR positive control were devised and tested (Table 1). The positive control could be run in the same reaction or separately, but the latter was preferred as this targets a specific region of human and also the plasmodial parasite 18S rRNA gene. This served as an internal DNA positive control.

PCR amplifications were performed with $4\ \mu\text{L}$ of purified DNA, $200\ \mu\text{M}$ each of dNTPs (dATP, dTTP, dGTP, and dCTP) (Finzymes), $200\ \text{ng}$ of each primer except the genus-specific one which was $300\ \text{ng}$, $1\ \text{U}$ of *Taq* DNA Polymerase (Bangalore Genei) and $1\times$ of *Taq* Buffer (Bangalore Genei) for a $50\ \mu\text{L}$ reaction. The resulting amplicons were analyzed on a 1% agarose gel (Figure 2).

Table 1

Details of the PCR primers used for the multiplex PCR for the detection of *P. falciparum*, *P. vivax* and *P. malariae*.

S.No	Primer sequence (5' -3')	Detection	Orientation
1	ATC AGC TTT TGA TGT TAG GGT ATT	Genus	Forward
2	TAA CAA GGA CTT CCA AGC	<i>P. vivax</i>	Reverse
3	GCT CAA AGA TAC AAA TAT AAG C	<i>P. falciparum</i>	Reverse
4	CAC TCA TAT ATA AGA ATG TCT C	<i>P. malariae</i>	Reverse
5	CCG GAG AGG GAG CCT GAG AAA	Positive control	Forward
6	CCT GCT TTG AAC ACT CTA ATT TTT T	Positive control	Reverse

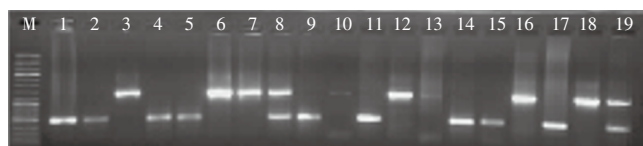


Figure 2. Field evaluation of the diagnostic PCR assay.

Agarose gel image of the multiplex diagnostic PCR amplicons identified as *P. vivax* (~500 bp) from lanes 1, 2, 4, 5, 9, 11, 14, 15 and 17; as *P. falciparum* (~1 400 bp) from the lanes 3, 6, 7, 10, 12, 13, 16 and 18; as mixed infection (both the species, *P. falciparum* and *P. vivax*) from the lanes 8 and 19. M, 1 kbp DNA ladder mix.

2.7. Calculation

Diagnostic performance characteristics of the RDT and microscopy were compared with multiplex PCR, which was used as the gold standard. The test indices measured were the number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). Sensitivity was calculated as $\text{TP}/(\text{TP} + \text{FN})$, specificity as $\text{TN}/(\text{TN} + \text{FP})$, positive predictive value (PPV) as $\text{TP}/(\text{TP} + \text{FP})$, and negative predictive value (NPV) as $\text{TN}/(\text{TN} + \text{FN})$. The test efficiency was determined as $1 - (\text{FN} + \text{FP}/\text{Total}) \times 100$ ^[25], J index, (which is the overall measure of reliability of a diagnostic test) was calculated as $[(\text{TP} \times \text{TN}) - (\text{FP} \times \text{FN})]/[(\text{TP} + \text{FN})(\text{TN} + \text{FP})]$. The test with the J index value approaching 1 is termed to be more reliable. Also, the 95% confidence interval (CI) for sensitivity and specificity was calculated according to the following equation:

$$p \pm 1.96 \times \sqrt{(p(1-p)/n)}$$

where, p = sensitivity (or specificity) and n = number of infected samples (or, for specificity, from uninfected sample)^[26]. The data were analyzed in Microsoft Excel 2007.

3. Results

Out of the total of 292 samples, six samples were excluded from the study as they were negative with the DNA positive

control. The rest of the 286 samples were included into the study.

3.1. Comparison of the tests under evaluation

There were significant discrepancies between microscopy and multiplex PCR. These discrepant samples ($n=17$) were also analyzed by our in-house nested PCR assay (under review). Upon review, this discrepancy was resolved in favor of multiplex PCR assay result except one sample ($n=1$) which was even positive by the other two tests under consideration. Thus, multiplex PCR was taken as a comparative standard and sensitivities, specificities, PPVs, NPVs and relative test efficiencies were calculated (Tables 2 and 3).

Table 2

Measures of test performance of microscopy, OptiMAL and multiplex PCR.

Items	Microscopy	OptiMAL [®]	Multiplex PCR
True positive	142	146	156
True negative	128	127	129
False positive	1	3	0
False negative	15	10	1

3.2. Microscopy

Microscopy detected 149/286 (52.09%) of the samples as positive (infected) and 137/286 (47.90%) of the samples as negative (uninfected). Out of the 149 infected samples, 61 (40.93%) were *P. falciparum* positive, 72 (48.32%) were *P. vivax* positive and 16 (11.03%) were identified as mixed infections (*P. falciparum* + *P. vivax*).

3.3. Multiplex PCR

Multiplex PCR detected 155/286 (54.19%) of the samples as positive (infected) and 131/286 (45.80%) of the samples as negative (uninfected). Out of the 155 infected samples, 60 (38.70%) were *P. falciparum* positive, 72 (46.45%) were *P. vivax* positive and 23 (14.83%) were identified as mixed infections (*P. falciparum* + *P. vivax*).

3.4. OptiMAL[®]

OptiMAL[®] detected 151/286 (52.79%) of the samples as positive (infected) and 135/286 (47.20%) of the samples as negative (uninfected). Out of the 151 infected samples, 57 (37.74%) were *P. falciparum* positive and 72 (47.68%) were *P. vivax* positive. Mixed infections (*P. falciparum* + *P.*

vivax) detected as *P. falciparum* (3 bands) were taken into consideration as mixed and only *P. vivax* (2 bands) were considered as falsely negative for *P. falciparum* ($n=2$).

With OptiMAL[®], 10 samples were undetected (falsely negative), which includes 5 *P. falciparum* and 2 *P. vivax* and 1 *P. falciparum* + *P. vivax* infection. and 2 *P. falciparum* + *P. vivax* infections were detected as only *P. vivax*. As a mixed infection showed *P. falciparum* band along with the *P. vivax*, these two samples were considered as false negatives for *P. falciparum*. Three samples (2 *P. vivax* and 1 *P. falciparum*) were falsely positive by this assay which was negative by microscopy and multiplex PCR. By microscopy, 7/23 mixed infections (as identified by PCR) were identified as 4 *P. falciparum* and 2 *P. vivax* mono-infections respectively and 1 as uninfected. One *P. vivax* sample as falsely positive and 13 falsely negative (Table 2). One *P. falciparum* infection remained undetected by multiplex PCR, which was tested positive with the other two methods.

4. Discussion

As a major health problem in the developing countries, malaria proved to have a negative impact on the socio economic development. In India, nearly 2 million people are effected[8]. Prompt and effective diagnosis is the key to effective disease management. The multiplex PCR[20], which targets the 18S rRNA gene of *P. falciparum* and *P. vivax*, was further developed by including a *P. malariae* specific primer (Figure 3) along with a DNA positive control. The advantage of multiplex PCR compared to other nested PCRs is the amplification of target in a single tube (*P. falciparum*, *P. vivax* and *P. malariae*), thus preventing contamination, saving time and reagents used.

Because it was difficult to determine whether microscopy or PCR was the more accurate assay, all non-concordant samples were retested by an in-house developed nested PCR. Out of the samples analyzed, six were negative with positive control for the multiplex PCR assay which targets the 18S rRNA gene of human and plasmodial genus were omitted from the study. This could have occurred either due to the degradation of DNA during shipment or inhibitors present with the product. Positive controls were used to check the negative amplification results, thus ruling out the failure of the PCR reactions. The DNA positive control used here amplifies the 18S rRNA gene segment of human and also the plasmodial species alike, at the same reaction

Table 3

Comparison between microscopy and OptiMAL tests for detection of *P. falciparum* and *P. vivax* in the samples with the multiplex PCR test as the gold standard.

Test	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Efficiency (%)	J Index
Microscopy	90.44 (88.84–95.04)	99.22 (97.71–100.59)	99.30 (97.99–100.60)	89.51 (87.22–94.79)	95.10	0.89
OptiMAL [®]	93.58 (89.75–97.42)	97.69 (95.10–100.28)	97.98 (95.78–100.18)	92.70 (88.21–97.18)	95.45	0.91
Multiplex PCR	99.36 (98.11–100.60)	100.00 (100.00–100.00)	100.00 (100.00–100.00)	99.23 (97.72–100.70)	99.65	0.99

95% CI levels are included in the parentheses.

conditions to the diagnostic PCR, thus saving time. This could be used as an external or as an internal positive control. In Bikaner, *P. malariae* infections are not reported till date. No other spurious amplicons were observed with the negative controls (uninfected DNA), thus substantiating the specificity of the primers used.

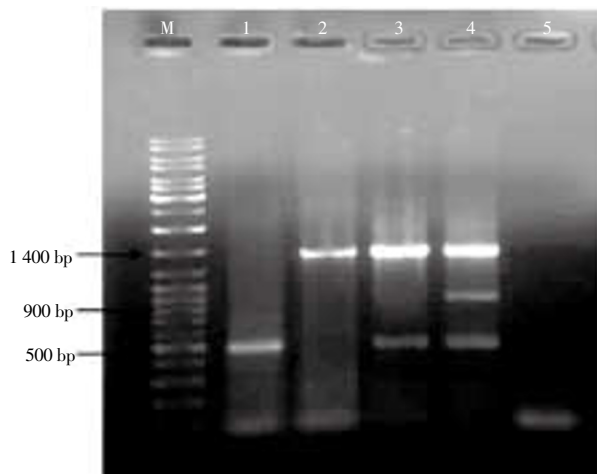


Figure 3. Amplicons were detected as (1) *P. vivax* (~500 bp), (2) *P. falciparum* (~1 400 bp), (3) mixed infection (*P. falciparum* + *P. vivax*) and (4) experimental mixed infection with *P. malariae* (*P. falciparum* + *P. vivax* + *P. malariae*).

P. malariae amplicon was visible at ~900 bp. M, 1 kbp DNA ladder mix (#SM0331; Fermentas Inc., Glen Burnie, MD).

When the data were analyzed, mixed infections of *P. vivax* and *P. falciparum* were classified as *P. falciparum* with OptiMAL®. This is because the *P. falciparum* band and pan-plasmodial (*P. vivax* band) must be visible if infected by both the species. Those mixed samples (as proved by PCR) with only *P. vivax* band ($n=2$) were considered as false negative for *P. falciparum*. OptiMAL® was not useful in detecting mixed infections based upon the high intensity of the *P. vivax* band when compared to the *P. falciparum*. As *P. falciparum* infected sample shows two bands apart from the control, if the sample has a mixed infection, high intensity of the *P. vivax* band has been observed earlier in earlier studies which has not been significantly observed in our study. Various studies all over the world reported sensitivities of OptiMAL® from as low as 25% to as high as 100% [18,23,27–30]. Although, high temperature (up to 60 °C) and humidity (up to 70%) were found not to affect the test results [15], false negatives were also reported even up to parasite densities of 2 500 parasites/μL [28,30]. OptiMAL® has also been used in parasite detection by non invasive method like saliva, but with limited sensitivities [31].

Reliance on microscopy alone in disease endemic areas may result in misleading interpretation of the values of alternative forms of diagnosis, and underestimate mixed-species infections [32–34]. Instances of prevalence of mixed infections detected by PCR and missed by microscopy were also reported earlier in Amazonia [35] and Cambodia [36]. The PCR analysis of these Bikaner field samples emphasizes

the importance of the characteristics of the comparative standard when interpreting diagnostic and epidemiologic studies. These results also suggested that there are a considerable number of cases with mixed infections in the study area that mainly remain undiagnosed by microscopy. This may occur because of abundance of one species over the other in the same sample (mixed infection) or when the peripheral parasitemia is below the threshold of microscopy or when parasites might sequester in deep microvasculature and evade the circulation [37]. In such areas, the use of RDTs with species specific antibodies would be preferable to pan-specific counterparts, to diagnose mixed infections.

Although microscopy remains the mainstay for routine diagnosis of malaria in India, RDTs are a valuable adjunct in cases of emergency. But, as per the results from our analysis, OptiMAL® could not be reliably used to detect the mixed infections and it would be recommended to use parasite specific antibodies to the respective antigens, rather than the pan-specific antibodies by the RDT. This presses the need for novel molecular interventions, which when used along with microscopy, resulting in detection limits up to 1 parasite/μL. Although the use of PCR at the field level is questionable, the capacity to establish a species specific diagnosis and recognize mixed infections makes PCR a very effective screening tool for epidemiological purposes.

We have developed the multiplex PCR (*P. falciparum* and *P. vivax*) with additional *P. malariae* specific primer and positive control and further evaluated microscopy, OptiMAL® and the multiplex PCR for diagnosis of malarial infections from Bikaner. Microscopy and OptiMAL® missed many mixed infections. Although the PCR required more time for completion, it surpassed the other two methods with the propensity to diagnose mixed infections in multiple samples. Thus, this PCR could be used for mass screening and epidemiological purposes as an efficient diagnostic tool that is highly sensitive and specific.

Conflict of interest statement

We declare that we have no conflict of interest.

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