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COMPARISON OF DIAGNOSTIC METHODS FOR THE DETECTION AND SPECIATION OF MALARIAL PARASITE IN URBAN KOLKATA, INDIA

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Abstract- Objectives: The present study was done to compare RDT, species and specific multiplex PCR-based diagnosis with conventional microscopic peripheral blood smear examination for detection of malarial parasite in urban Kolkata. **Methods:** We collected whole blood samples (N = 100) from febrile patients attending Malaria Clinic, CSTM, Kolkata, India. Uncomplicated malaria was diagnosed by microscopy, rapid diagnostic test (RDT) and polymerase chain reaction (PCR) to compare RDT, genus and specific multiplex PCR targeting plastid-like large subunit ribosomal-RNA and 18S small-subunit RNA genes respectively for conventional microscopic peripheral blood smear examination (PBS). **Results:** We identified 39 infections, 18 of which were caused by *Plasmodium vivax*, 18 by *Plasmodium falciparum*, and 3 were mixed infections harbouring both *Plasmodium vivax* and *Plasmodium falciparum*. The sensitivity and specificity of each method to detect plasmodial parasite varied as 87.25% and 92.4% for microscopy, 79.5% and 88.4% for RDT and 100% and 100% for genus specific PCR, 80.95% and 97.5% for P vivax specific multiplex PCR and 100% for falciparum specific multiplex PCR respectively. All samples showing disagreement among the methods were reevaluated by repeat testing. PCR detected parasites in the 5 false-negative samples as found both by microscopy and RDT. The mixed infections were detected as *Plasmodium falciparum* by PCR, while the other methods diagnosed them as mixed infections. Two cases of *vivax* infections were detected as genus *plasmodium* by PCR. **Conclusion:** The use of the microscopic method for malaria detection is suitable for its low cost but diagnostic accuracy can be enhanced by PCR-based method where available thus facilitating proper treatment. RDT can be applied in fieldwork.

Keywords- Microscopy, Antigen, PCR, Malaria, *Pf*, *Pv*.

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Introduction

Malaria is a highly significant global public health problem. Its greatest burden is imposed on the world's poorest countries [1]. It is the third leading cause of death from infectious diseases for children under age of five worldwide [2] and the fourth leading cause for all ages [3].

Malaria case management constitutes a vital component of the malaria control strategies [4, 5] in which proper diagnosis plays an important role. This is more relevant in areas where more than one species are prevalent in endemic countries like India with *Plasmodium vivax* and *Plasmodium falciparum* having equal distribution [6]. Proper species identification is essential for treatment where different drug combinations are in use for different parasitic infections [7]. Microscopy is still considered as gold standard for malaria diagnosis [8,9] but the method is prone to misdiagnoses especially in cases of mixed infections and wrong diagnosis in case of small rings of *Plasmodium vivax* [10]. Several new methods have been developed as RDT based on antigen detection [11] and more accurate molecular diagnosis by PCR-based detection [12-15]. New technology were compared with accepted gold standard that makes comparisons of sensitivity and specificity between different methods [16-18]. The present study was done to compare RDT, PCR-based diagnosis with conventional microscopic peripheral blood smear examination for detection of uncomplicated malaria in urban Kolkata.

Materials and Methods

Study site and sample collection

Blood samples were collected from 100 patients attending the Malaria Clinic under Protozoology Unit of the Calcutta School of Tropical Medicine in October 2012. In this part of the country two major human malarial parasites, *Plasmodium vivax* and *Plasmodium falciparum* are equally prevalent [19]. A detailed clinical history regarding the duration of fever, its nature and associated symptoms was taken from each patient. Febrile patients with no obvious focus of bacterial, viral or fungal infection irrespective of age and sex were included in this study. 2-3 ml blood samples were collected from the recruited patients for this study.

Informed written consent was obtained from the patients and/ or guardians of the children enrolled in the study. The report and treatment of patients with malarial infection detected by microscopy and/ or RDT were made available on same day. Those found to be negative were asked to report to our Clinic on next two consecutive days to collect their report of PCR-based diagnosis and for repeat testing if required.

The Institutional Ethics Committee of the Calcutta School of Tropical Medicine approved the study protocol.

Microscopic diagnosis and parasite count

Thick and thin peripheral blood smears were examined for detection of malaria parasite and determination of species following standard Giemsa staining by analyzing the slides under a 100X objective. Smears with no visible parasites in 100 oil-immersion fields were considered to be negative for this test. All slides were crosschecked by two other microscopists for this study.

Parasite counts were done on Giemsa-stained thick films and the number of parasites per 200 WBC was counted by light microscope, by experienced microscopists. Assuming a WBC count to be 8,000/µl of blood, parasitaemia was calculated and expressed as per µl of blood.

RDT

The blood samples were also assayed with dual antigen kit Bioline manufactured by SD Bio Standard Diagnostics Pvt. Ltd., Gurgaon, Haryana. The kit detected *Plasmodium vivax* specific pLDH (plasmodial lactate dehydrogenase) and *Plasmodium falciparum* specific HRP-II (histidine-rich protein) of malaria parasites in lysed blood based on immunochromatographic methods according to the manufacturer's instructions. It is a qualitative test; one can diagnose the presence of parasite only but quantity. The strips were independently examined and the results of each assay were recorded as positive or negative on the basis of the observation of the precipitated band.

PCR-based diagnosis

DNA isolation: Genomic DNA of plasmodial parasite was isolated from 200µl EDTA blood that were collected on day 0, using QiaAmp DNA Mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions.

PCR amplification

PCR was performed in a two-step method. Detection of malarial infection was done with genus-specific primers targeting a 595bp fragment within the plastid-like large subunit rRNA [LSU-rRNA] gene [20]. Two species of the genus Plasmodium, vivax and falciparum prevailing in the study area were detected with primers targeting the conserved 18S small-subunit RNA genes of the parasites by multiplex PCR [21] The oligonucleotide primers and PCR conditions are summarised in [Table-1]. PCR products were run on 2% agarose gel and visualised under UV illumination following ethidium bromide staining and documented by Gel-Doc system. A 595-bp product in genus specific PCR indicates presence of Plasmodium parasite. Detection of 276-bp and 300-bp product in species specific multiplex PCR indicates infection of *P. falciparum* and *P. vivax* respectively.

Data Analysis

True positive was identified by any one of all four methods and true negative was in agreement with all four methods. Mixed infections were considered as two separate infections to facilitate the calculations.

Sensitivity was calculated as the number of true positives divided by the number of true positives and false negatives combined. Specificity was similarly calculated as the number of true negatives divided by the number of true negatives and false positives combined.

The Z-test values were calculated for the comparison of the sensitivity and specificity of the four diagnostic tools by stastical software "R" (version 2.13.1).

Results

In our study among enrolled 100 patients attending the clinic with malaria symptoms, only 34% (34/100) had a *Plasmodium* spp. infection as detected by microscopy. Among them 21% (21/100) of patients were positive for *Plasmodium vivax*, 15% (15/100) *Plasmodium falciparum* and 2% (2/100) had a mixture of both species [Table-2].

By RDT malaria parasite were diagnosed in 31 cases of them 13 for *P. falciparum*, 16 for *P. vivax* and 2 for both *P. falciparum* and *P. vivax*. Genus specific PCR could detect *Plasmodiam sp.* in 39 (39%) cases. Eighteen cases of *P. falciparum*, seventeen *P. vivax* and two mixed infection with both *P. falciparum and P. vivax* were detected by species specific multiplex PCR method.

There was agreement among 4 methods in 27 cases which included 13 cases of *falciparum* malaria & 14 cases of *vivax* malaria. All conflicting speciation results were present in 12 samples. Those samples showed either no infection or mono-infection by one or two methods, which were detected positive in having mono-infection, & mixed infection respectively by other methods. There was a case of *vivax* malaria detected only by microscopy, which was detected as genus *plasmodium* was negative by RDT and multiplex PCR. Two cases of *vivax* determined by both PCR methods & microscopy were found to be negative by RDT. One case of *vivax* detected by both microscopy & RDT was detected as genus plasmodium by PCR. There was another case which showed *vivax* infection by both microscopy & RDT but multiplex PCR detected it to be a case of mixed infection. Both the PCR methods showed positive result of *falciparum* infection in 5 samples which could not be detected by other 2 methods. On the other hand 2 mixed infections determined by microscopy and RDT were detected as falciparum infection only by multiplex PCR. So a consensus was established among three diagnostic methods to detect maximum number of positive samples with species identification [Table-2].

The sensitivity for the detection of genus *Plasmodium* was recorded to be highest for PCR (100.0%), followed by microscopy (87.2%) and RDT (79.5%). Significant difference in sensitivity of PCR vs RDT (Z = - 2.9857, P = 0.00278) and also of PCR vs microscopy was observed (Z= - 2.3114, P = 0.02088). No Significant difference of sensitivity between microscopy and RDT (Z = 0.9115, P = 0.36282) was noted.

In case of species determination for vivax, microscopy proved to be superior to multiplex PCR (Z = 2.1026, P = 0.03572) without any significant difference between microscopy vs RDT (Z = 1.7974, P = 0.07186) and multiplex PCR vs RDT (Z = 0.414, P = 0.6818).

For identification of falciparum species, multiplex PCR showed similar significant difference over microscopy and RDT (Z = - 2.6458, P = 0.00804). However sensitivity of microscopy and RDT was equal with no significant difference (Z = 0, P = 1) [Table-3].

Table-1 *Primers & PCR conditions for genus and species specific diagnosis of malaria parasites*

Target gene	Primer name	Primer Sequence (5'-3')	Mg^{2+} Conc. (mM)	PCR program						
				Denaturation		Annealing		Elongation		No. of cycles
				Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	
LSU-rRNA (Plasmodium specific)	L1	*GACCTGCATGAAAGATG*	2.5	90	1	56	2	72	1	40
	L2	*GTATCGCTTTAATAGGCG*								
18S small-subunit RNA gene (Species specific multiplex PCR)	Reverse	*GTATCTGATCGTCTTCACTCC C*	2	94	0:45	60	1:30	72	5	43
	PF	*AACAGACGGGTAGTCATGATT GAG*								
	PV	*CGGCTTGGAAGTCCTTGT*								

Table-2 *Results of all samples according to 4 tests & consensus for diagnosis of malarial parasite in 100 blood samples collected from the Clinic, CSTM,Kolkata*

Total isolates	Microscopy	RDT	PCR		Consensus
			Genus specific	*Species specific*	
13	*P. falciparum*	*P. falciparum*	*Plasmodium*	*P. falciparum*	*P. falciparum*
14	*P. vivax*	*P. vivax*	*Plasmodium*	*P. vivax*	*P. vivax*
2	*P. vivax*	Negative	*Plasmodium*	*P. vivax*	*P. vivax*
1	*P. vivax*	*P. vivax*	*Plasmodium*	*Plasmodium*	*P. vivax*
1	*P. vivax*	*P. vivax*	*Plasmodium*	*P. vivax* + *P. falciparum*	*P. vivax* + *P. falciparum*
1	*P. vivax*	Negative	*Plasmodium*	Plasmodium	*P. vivax*
2	*P. vivax* + *P. falciparum*	*P. vivax* + *P. falciparum*	*Plasmodium*	*P. falciparum*	*P. vivax* + *P. falciparum*
5	Negative	Negative	*Plasmodium*	*P. falciparum*	*P. falciparum*
61	Negative	Negative	Negative	Negative	Negative

Discussion

In India *P. falciparum* and *P. vivax* are almost equally prevalent [19]. As per National Vector Born Disease Control Programme (NVBDCP) there are two different regimens of antimalarial drugs used for the treatment of two different species of malarial parasite, chloroquine (25mg/kg body weight in three days) plus Primaquine (0.75mg/kg body weight for 14 days) for vivax malaria and Artesunate (4mg/kg body weight) for 3 days, Plus Sulphadoxine (25mg/kg body weight) - Pyrimethamine (1.25mg/kg body weight) on day1and a single dose of Primaquine at 0.75mg/kg body weight on day2 for falciparum malaria. So, proper diagnosis is essential for providing proper treatment. Microscopy is used for this purpose in well-equipped laboratory but in peripheral setup detection of antigen based on immune-chromatographic method is applied. Recently both genus and species specific PCR developed and showed highly sensitive and specific [12-15].

In the present study, genus specific PCR showed the highest overall sensitivity of 100% followed by microscopy (87.2%) & RDT (79.5%). Species specific multiplex PCR method was unable to detect vivax infection in two cases with vivax alone and two with mixed infection as detected by microscopy. Boonma *et al*. 2007[22] also observed that multiplex PCR missed three times the vivax parasite in three cases of mixed infections. The probable reason could be that band sizes of vivax & falciparum differed by only 24-bp.

In the present study genus Plasmodium was detected in five cases by genus specific PCR and were diagnosed as falciparum by multiplex PCR but were negative for both microscopy and RDT. It showed that both the PCR methods are more sensitive and specific for detection of *P. falciparum* than microscopy perhaps due to low levels of parasitaemia [23]. Those five cases were not administered any anti-malarials on the day of their first visit to our Clinic. A repeat sample on next two consecutive days was collected from them for both microscopy and RDT. Interestingly, four out of five showed *Plasmodium falciparum* by microscopy but all were negative by RDT on day 2 also. So in endemic areas PBS examination on three consecutive days definitely yields better results.

RDT sensitivity is directly related to parasite density, health care providers cannot rely on a negative RDT result to rule out malaria. However, a positive result may be helpful in making management decisions before a microscopic diagnosis can be made.

Hence microscopic diagnosis is preferred in patient care service where facility is available. PBS examination for three consecutive days from symptomatic patients in endemic areas is recommended for better diagnostic accuracy. PCR-based diagnosis will be useful for detection of malaria infection at low level of parasitaemia and even in microscopically negative cases. As RDT showed more or less equal sensitivity as that of microscopy its use in health care settings where microscopy facility is unavailable is justified.

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Conflicts of interest: The authors have no conflicts of interest concerning the work reported in this paper.

Authors' contributions: AKM, SG conceptualized and designed the study protocol; PS, SG, PS, and MC performed the PCR and other diagnostics and interpretation of data; AKM, SG and PKK drafted the manuscript. All authors read and approved the final manuscript.

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Abbreviations

RDT: Rapid Diagnostic Test
PCR: Polymerase Chain Reaction
rRNA: ribosomal-RNA

Table-3 *Comparative statement of the 4 diagnostic methods for malarial genus and species identification in 100 blood samples collected from our Clinic*

Detection of genus/ species of malarial parasites	Method	Positive	Negative	Sensitivity*	Specificity#
Malaria/ Genus *Plasmodium*	Microscopy	34	66	87.2%	92.4%
	RDT	31	69	79.5%	88.4%
	PCR	39	61	100%	100%
P. vivax	Microscopy	21(19+2)	79	100%	100%
	RDT	18 (16+2)	82	85.7%	96.3%
	PCR	17 (16+1)	83	80.95%	97.5%
P. falciparum	Microscopy	15 (13+2)	85	71.4%	94.04%
	RDT	15 (13+2)	85	71.4%	94.04%
	PCR	21 (19+2)	79	100%	100%

*# Sensitivity and specificity were calculated as per criteria mentioned in the Material and Methods section.