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Original Article

Microbiology Section

A Comparative Analysis of Four Different Diagnostic Techniques for Malaria

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ABSTRACT

Introduction: Malaria is one of the major parasitic diseases and laboratory diagnosis of malaria infection requires the availability of a rapid, sensitive, and specific test that is affordable. There is the availability of several laboratory procedures for malaria diagnosis.

Aim: To compare the performance of conventional microscopy using leishman staining against an immunochromatographic antigen detection test, fluorescent microscopy using Acridine Orange (AO), and molecular technique by Polymerase Chain Reaction (PCR) in the laboratory diagnosis of malaria.

Materials and Methods: In the present cross-sectional study, 432 specimens were collected from patients suspected of malaria attending Dr. Susheela Tiwari Government Hospital, Haldwani, Uttarakhand, India, during the study period from January 2018 to September 2019. Microscopic examinations of Leishman stained smears, immunochromatographic Rapid Diagnostic Test (RDT), fluorescent microscopy using AO, and PCR were done using appropriate statistical analysis.

Results: Out of the 432 specimens tested, a total of 208 (48.2%) were found positive for malaria using all four tests. Microscopy using leishman staining, RDT, and fluorescent microscopy were performed on all samples out of which 180 (41.6%) samples showed a positive result on Leishman staining, 186 (43%) were detected using AO fluorescent microscopy while the RDT had a yield of 208 (48.2%) positive results for malaria. While PCR was performed on 124 samples that yielded 79 (63.7%) positive results.

Conclusion: Though, peripheral blood smears are still considered the gold standard for malaria diagnosis. But, in this study, it was observed that newer RDT for malaria surpassed the diagnostic efficacy of clinical microscopy and hence, has a superior role in clinical practice and diagnosis.

Keywords: Acridine orange, Microscopy, Polymerase chain reaction, Rapid diagnostic tests

INTRODUCTION

Malaria is one of the major parasitic diseases which is annually affecting 300-500 million people globally and leads to over one million deaths [1]. Malaria has shown an increasing trend worldwide due to the emergence and spread of drug-resistant strains. This poses major health and economic problems for the population living in endemic areas and increases the risk of disease in travellers [2]. For laboratory diagnosis of malaria, a test is required that is rapid, sensitive, and specific, at a reasonable cost. There is the availability of several laboratory procedures for diagnosis of malaria such as microscopy of different types, immunological and molecular techniques [3]. The diagnostic techniques available for malaria ranges from conventional thick and thin smear to rapid modalities such as fluorescent staining and antigen detection tests detecting parasitic antigens like Histidine Rich Protein-2 (HRP-2), *Plasmodium* Lactate Dehydrogenase (pLDH), and pan-specific aldolase and molecular techniques like PCR [4-6]. Each of these techniques has advantages and disadvantages in terms of cost, ease of performance, sensitivity, and technical complexity [7].

Microscopic examination of blood film is considered the "gold standard" technique [8]. This method is relatively simple and inexpensive but is time-consuming, laborious, and has questionable sensitivity for the low level of parasitaemia and interpretation of mixed infection [9]. Most novel technology is based on antigen detection by Immunochromatographic (ICT) technique [10]. Many molecular techniques like species-specific Deoxyribonucleic Acid (DNA) probes, PCR, and ribosomal Ribonucleic Acid (rRNA) probes have been developed [11]. Among the several PCR methods, most often based on genus or species-specific sequences of the parasites 18s subunit rRNA gene [12]. Another method, fluorescent microscopy is based on the ability of fluorescent dyes to detect RNA and DNA of parasite. Various staining methods like Kawamoto AO staining [13] and Quantitative Buffy Coat (QBC) assay have been designed. This method has high sensitivity and specificity but needs considerable practice and expensive types of equipment [14].

Hence, the current study aims to compare the performance of conventional microscopy using Leishman staining against an immunochromatographic antigen detection test, fluorescent microscopy using AO and molecular technique (PCR) in the diagnosis of malaria.

MATERIALS AND METHODS

A cross-sectional study was conducted between January 2018 to September 2019 on the clinically suspected cases of malaria (a patient complaining of fever, along with any other symptoms suggestive of malaria including abdominal pain, nausea, vomiting, headache, bodyache, and signs such as anaemia and splenomegaly) who reported to Dr. Susheela Tiwari Government Hospital, Haldwani, Uttarakhand, India and every fifth patient was assessed for inclusion in the study. The approval of the study protocol was given by the Institute Ethical Committee of Government Medical College Haldwani, Nainital, India (IEC no- 393/GMC/IEC/2017/Reg.No.362/IEC/R-16-09-2017 dated: 10.10.2017).

Sample size calculation: As the prevalence of malaria among clinically suspected patients in the study area was not known the prevalence was assumed to be 50% to obtain the maximum sample size and taking absolute allowable error (d) as 5%, the sample size was calculated using the formula $(1.96)2p(1-p)/d^2$ at 5% level of significance. After adding a 10% non response rate, a sample size of 427 was obtained.

Inclusion criteria: The final study sample was 432, as all the suspect patients reporting on the last day of data collection were included according to recruitment strategy. Blood samples were collected from them with proper informed consent.

Exclusion criteria: Patients on prior antimalarial therapy were excluded from the study.

Diagnostic Procedure

Sample collection: Leishman staining was performed on thick and thin blood films prepared on precleaned glass slides with blood obtained by finger prick [4,5]. About 5 mL of venous blood was collected from every patient during the peak of fever and shipped to the laboratory for the remaining tests.

Rapid Diagnostic Test (RDT): It was performed using the Advantage Malcard (J. Mitra and Co. Pvt., Ltd.,) antigen detection test containing a strip, precoated with two polyclonal antibodies as two distinct lines across it. One polyclonal antibody (test line Pf) is specific to lactate dehydrogenase of *P.falciparum* while the other polyclonal antibodies (test line P.v/pan) are pan-specific to the lactate dehydrogenase of *Plasmodium* species. The test procedure was performed as per kit literature.

Acridine Orange (AO) staining: A 75 µL of blood was taken on a clean glass slide and 10 µL of AO stain was added using a pipette. A coverslip was placed over the mixture and pressed gently. This was followed by an examination of the wet mount under a fluorescent microscope for two minutes using exciter filter: LP 450 and barrier filter: LP 520 [15].

Molecular Method (PCR): For performing PCR, DNA extraction from blood was done using the QIAamp DNA minikit (Qiagen). A 400 µL of whole blood was added in an eppendorf tube followed by 40 µL of proteinase K and 400 µL of phosphate-buffered saline. It was mixed well in vortex and then 400 microlitres of lysis buffer was added. This was again vortexed for 15 seconds followed by incubation at 56°C for 10 minutes. Four hundred µL of 100% ethanol was added and this mixture was vortexed again for 15 seconds. The contents were transferred to a column and centrifuged at 8000 rpm for one minute. Thereafter, 500 µL of wash buffer AW1 was added and centrifuged at 8000 rpm for a minute, followed by the addition of 500 µL of AW2 and centrifugation at 14000 rpm for three minutes. The contents were taken in an eppendorf tube and 50 µL of elution buffer was added and centrifuged at 8000 rpm for 3 minutes. A total of 50 µL of DNA was collected and stored at -80°C. Genus-specific PCR followed by a nested species-specific PCR was carried out to amplify the 18S rRNA [16]. The amplification was performed as follows in a reaction mixture of 24 µL containing-Master mix (Fermentas, USA)-12 µg, Water-8 µL, Template DNA-50 ng, Primers Rplu1, and Rplu5-250 nm, PCR reaction performed without template served as the negative control.

The PCR product was checked using 2% agarose gel electrophoresis stained with ethidium bromide.

Amplification conditions were as follows:

- Initial denaturation at 94°C for four minutes

This was followed by 35 cycles of-

- Denaturation at 94°C for 30 seconds.
- Annealing at 55°C for one minute.
- Extension at 72°C for one minute.
- Final extension at 72°C for five minutes

For nested PCR, the template used was 2 µL of the amplification product of the first PCR. Similarly, the primers and master mix concentration in nested PCR, as well as the amplification conditions, were the same as in the first PCR [16].

Finally, the amplification products of nested PCR were subjected to agarose gel electrophoresis and ethidium bromide staining to distinguish among all five species. In both the PCR, the primer sequences were as shown in [Table/Fig-1].

STATISTICAL ANALYSIS

The data was entered in Microsoft Excel and the software Epi Info 7.0 was used for the analysis. The Chi-square test was applied for the assessment of the significance of differences among the proportions. A p-value <0.05 was taken as statistically significant.

Target	Primers	Band size
Genus specific *Plasmodium*	rPLU1: 5′-TCAAAGATTAAGCCATGCAAGTGA-3′ rPLU5: 5′-CCTGTTGTTGCCTTAAACTCC-3′	1200 bp
Species specific *P. falciparum*	rFAL1: 5′- TTAAACTGGTTTGGGAAAACCAAATATATT-3′ rFAL2: 5′- ACACAATGAACTCAATCATGACTACCCGTC-3′	205 bp
P.vivax	rVIV1: 5′- CGCTTCTAGCTTAATCCACATAACTGATAC-3′ rVIV2: 5′- ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3′	120 bp
P.malaria	rMAL1: 5′- ATAACATAGTTGTACGTTAAGAATAACCGC-3′ rMAL2: 5′- AAAATTCCCATGCATAAAAAATTATACAAA-3′	144 bp
P.ovale	rOVA1: 5′- ATCTCTTTTGCTATTTTTTAGTATTGGAGA-3′ rOVA2: 5′- GGAAAAGGACACATTAATTGTATCCTAGTG-3′	800 bp

[Table/Fig-1]: Primer sequences in PCR.

RESULTS

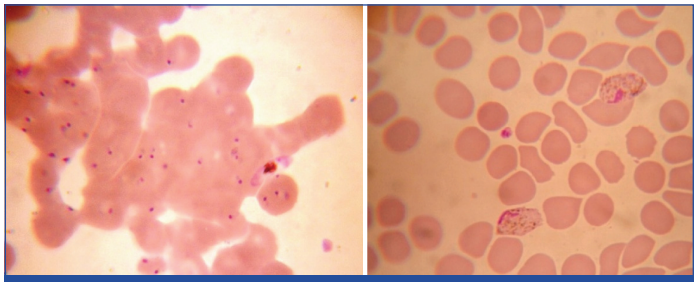
The present study was conducted on a total of 432 subjects, clinically suspected of malaria, of all age groups and both sexes. Among the subjects, 241 were males and 191 were females with a male to female ratio of 1.26:1 in favour of males. The maximum number of test subjects (175) belonged to the age group of 16-30 years [Table/Fig-2].

Age (in years)	Number	Percentage
<1	7	1.62
1-15	112	25.93
16-30	175	40.51
31-45	76	17.59
46-60	43	9.95
>60	19	4.4
Total	432	100.00

[Table/Fig-2]: Age distribution of patients N=432.

A total of 208 (48.2%) were positive for malaria and 224 (51.8%) were negative. Among the 208 cases confirmed for malaria, 126 were males and 82 were females with a male to female ratio of 1.54:1.

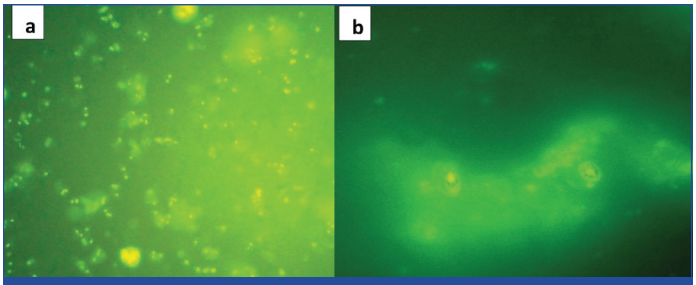
Among the 432 samples collected, RDT using ADVANTAGE MAL-CARD by J.Mitra and Co. Pvt., Ltd., conventional microscopy using Leishman staining and fluorescent staining using AO were performed on all samples. A total of 208 samples were positive for malaria using the RDT out of which 187 were positive for pan malaria while 21 cases had Pf/Pan nfection, whereas Leishman staining detected 180 positive cases out of 432 [Table/Fig-3,4].



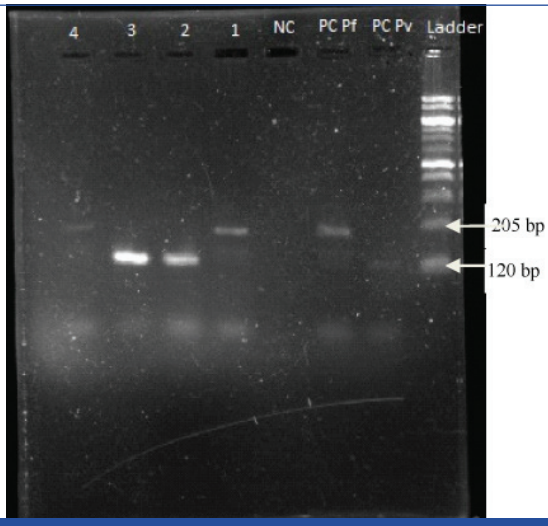
[Table/Fig-3]: Showing gametocyte and multiple ring forms of *Plasmodium falciparum* at 100X (oil immersion) on Leishman staining; **[Table/Fig-4]:** Showing gametocytes of *Plasmodium vivax* at 100X (oil immersion) on Leishman staining. (Images from left to right)

Fluorescent microscopy using AO detected 186 positive samples out of the 432 tested [Table/Fig-5]. PCR was done for confirmatory identification of *Plasmodium* spp. on 124 random samples out of which 79 were positive for malaria [Table/Fig-6].

Six samples that were negative on conventional microscopy were found to be positive on PCR. All samples positive on PCR were positive on RDT as well. The positivity rate for malaria parasite found on peripheral blood smear was compared with other diagnostic techniques and it was found to be significantly different on RDT and PCR tests [Table/Fig-7].



[Table/Fig-5]: Showing (a) Multiple ring forms and (b) Gametocytes of *Plasmodium falciparum* on fluorescent microscopy using Acridine Orange (AO).



[Table/Fig-6]: Agarose gel electrophoresis of nested PCR showing 100bp DNA ladder: PCPF *Plasmodium falciparum* positive control, PCPV *Plasmodium vivax* positive control NC Negative control. Samples 1,4 positive for *Plasmodium falciparum* (205bp) and samples 2,3 positive for *Plasmodium vivax* (120bp).

Diagnostic method	Positive for malaria parasite		Negative for malaria parasite		p-value*
	No (%)	95% CI	No (%)	95% CI	
Peripheral blood smear (N=432)	180 (41.67)	37.11-46.37	252 (58.33)	53.63-62.89	Reference test
RDT (N=432)	208 (48.2)	43.47-52.86	224 (51.8)	47.14-56.33	0.05
Fluorescent staining (N=432)	186 (43.06)	38.47-47.77	246 (56.94)	52.23-61.53	0.67
PCR (N=124)	79 (63.71)	54.95-71.64	45 (36.29)	28.36-45.05	0.00002

[Table/Fig-7]: Malarial parasite detection rate by different diagnostic methods. *Chi-square test; CI: Confidence interval

Among the 208 laboratory-confirmed cases, 187 (89.9%) cases were *P.vivax* followed by 12 (5.8%) patients with *P.falciparum* and 9 (4.3%) patients showing mixed infection with *P.vivax* and *P.falciparum*. However, *P.malariae* and *P.ovale* were not observed in any of the samples.

DISCUSSION

In this study, a total of 208 (48.2%) out of the 432 subjects were found to be positive for malaria. Among these 208 laboratory-confirmed cases, 187 (89.9%) were *P.vivax* followed by 12 (5.8%) patients with *P.falciparum* and 9 (4.3%) patients showing mixed infection with *P.vivax* and *P.falciparum*. A similar finding was observed by Muddaiah M and Prakash PS where *Plasmodium vivax* was the most common parasite type found in 52.54% followed by *P.falciparum* and mixed malarial infection detected in 33.75% and 13.69% patients respectively [17]. Patel JM and Godara N, in their study found that out of the cases positive for malaria, 84% were *Plasmodium vivax* while 16% were *P.falciparum* [18]. While Pachpute S et al., found that of the 339 cases, 40.7% were positive for malarial parasites [19]. Among which 49.3% had infection with *P.falciparum*, 36.9% had *P.vivax* infection and 13.8% mixed infection. Another study from Karnataka, conducted by Karumbaiah P et al., revealed that out of the 105 children suspected of malaria, 63.5% were positive for *P.vivax*, 31.7% were infected with *P.falciparum* while 4.8% had mixed infection with *P.vivax* and *P.falciparum* [20].

In the present study, four different diagnostic modalities for the diagnosis of malaria were employed. Leishman staining, fluorescent staining using AO, and RDT using an ICT (ADVANTAGE MAL-CARD by J. Mitra and Co. Pvt., Ltd.,) done for all samples while molecular technique (nested PCR) could only be done on 124 samples due to resource constraints. In the present study, it was found that 180 (41.6%) samples showed a positive result on Leishman staining, 186 (43%) were detected using AO fluorescent microscopy while the RDT had a yield of 208 (48.2%) positive results for malaria, while PCR yielded 79 positives out of 124 samples. The findings of PCR coincide with the results of the RDT showing that none of the samples that were negative on RDT were found positive on PCR and vice versa. All the samples negative on fluorescent staining (AO staining) and by RDT were found negative on Leishman staining as well. Hemvani N et al., in their study from Indore found 10.21% positive by using the AO staining method while only 4.48% were positive on Leishman staining [15]. Rubio JM et al., from Equatorial Guinea in their study compared peripheral blood smear examination with semi-nested PCR and detected malaria parasite using peripheral blood smear and PCR in 69.4% and 79.3%, respectively [21].

Though the light microscopic examination with the Giemsa or Leishman stained blood smear has been recognised as the gold standard test for the malaria diagnosis, it needs a well-trained and skilled microscopist along with considerable time. However, PCR analysis is also more expensive requiring costly laboratory equipment installment and maintenance. The technique is also labour-intensive and requires high-level expertise and standardisation.

Immunochromatographic tests (RDTs) provide a quick, non microscopic method for malaria detection, therefore saving both time and training. These tests are easy to perform and interpretation of results is possible with little training [10]. Kim JY et al., in their study from South Korea also found comparable results of RDTs with PCR [22]. The performance of the RDTs was found to be similar to microscopic examination and nested-PCR. However, these RDTs cannot differentiate *Plasmodium* species other than *P.vivax* and *P.falciparum*. Overall, RDTs were found to be less time-consuming, cost-effective, feasible, and can be performed without any technical expertise. The new generation of RDT offers a convincing practical solution to shift the diagnosis of malaria closer to the patient in the community setting away from the traditional laboratory setting.

Limitation(s)

In this study, molecular technique (nested PCR) could not be performed on all samples due to certain resource constraints which make it difficult to rule out all the false negatives. Also, in many patients, the samples were received only once when the requisition was made for RDT and fresh repeat samples could not be collected for microscopy as some of the patients were discharged before that. This could be the reason for the lower number of samples showing positive results on conventional microscopy.

CONCLUSION(S)

The RDTs were better in diagnosing malaria in comparison to the AO technique and leishman staining. The RDTs as compared to other techniques are simple, quick, and can be utilised as a bedside diagnostic test. It also does not need special types of equipment, extraordinary setup, and technical expertise. The PCR detected the maximum percentage of malaria cases as it is a more specific tool and can detect the parasite at very low concentrations but still, the facility is not widely available at every institute and is a much expensive option than the other methods making it an

unsuitable choice for a malaria-endemic developing country like India. Thus, it is recommended that RDTs may be used for the routine diagnosis of malaria to ensure prompt treatment and decrease morbidity and mortality.

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