To determine sensitivity & specificity between microscopy and polymerase chain reaction methods in patients presenting with acute undifferentiated fever and clinically suspected of malaria

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Abstract:

To overcome some of the limitations associated with microscopy, molecular assays that detect Plasmodium specific nucleic acid sequences are increasingly being used. Aim: The aim of the present study was to perform peripheral blood smear examination for malaria parasite by Leishman stain in patients presenting with acute undifferentiated fever and clinically suspected of malaria and to compare them with Real-Time Polymerase Chain Reaction (PCR) for diagnosis of malaria in clinically suspected patients considering microscopy as gold standard. Materials & Methods: After obtaining Institutional Ethics Committee permission, the present study was conducted and informed consent from all the participants of the study was taken. The sample size of the present study is 154. Results: Despite the fact that 64 samples were identified as P.Falciparum by microscopy, only 46 were confirmed by PCR; one was negative and the other was P.Vivax. However, three samples identified as P.Falciparum by PCR were not identified as P.Falciparum by microscopy. he results of PCR of negative samples detected by microscopy (n=90) as a diagnostic quality. Between the samples detected by microscopy18 (14.7%) were false negatives based on PCR. Conclusion: The present study also demonstrated a highly sensitive and specific molecular method for detection of low level parasitemia in a targeted high-risk population which may prove it cost effective too.

Keywords: Polymerase Chain Reaction (PCR), Malaria

Introduction:

Malaria is a disease of parasites caused by an infective bite by female Anopheles mosquitoes that transmits Plasmodium species. In tropical countries including India, it is a foremost reason of febrile illness and death, particularly in children below 5 years of age [1 – 4]. Between the years 2005 and 2015, the global malaria problem reduced substantially, because of active regulatory policies by governments [5]. In the past recent years, nevertheless, this trend has stagnated in few countries, and an increased problem in certain countries are reported [1, 4]. The diagnosis is the basis for any treatment, from drugs to surgery. A precise identification of a disease is critical to prevent squandering precious time on the wrong choice of treatment [6, 7]. The diagnostics tools play a crucial role in assisting the accurate diagnosis.At present, malaria diagnosis in most of the health care centers including primary health care level is conducted by either microscopy or rapid diagnostic tests [8, 9]. The gold standard method for malaria diagnosis is microscopy, however numerous challenges associated with performing quality microscopy may lead to variation in assay sensitivity and specificity affecting patient diagnostic outcome and clinical trial results [10, 11]. Microscopy is highly operator dependent and proficiency testing is required to achieve reproducible, highly quality data [12, 13]. PCR is useful in detection asymptomatic malaria, and evaluation of anti- malarial therapy and drug resistance [14]. PCR is useful especially in patients with low parasitemia, who might serve as a reservoir for transmission of malaria. They may not suffer from clinical malaria [15, 16]. PCR can detect parasitemia as low as one gene copy and allows differentiation of all five Plasmodium species [17]. Real time PCR detection of malaria is considered as a confirmatory test for malaria diagnosis due to its high sensitivity and specificity as well as rapid processing time [15, 16].

To overcome some of the limitations associated with microscopy, molecular assays that detect Plasmodium specific nucleic acid s equences are increasingly being used. These assays are several orders of magnitude more sensitive than microscopy or antigen det ection tests [14, 18]. Therefore, the aim of the present study is to evaluate the efficacy of RT-PCR for diagnosis of malaria among clinically suspected patients visiting a tertiary care hospital in Central India. To attain the aim of this study the following objectives have been taken into consideration, to perform PBS examination for malaria parasite by Leishman stain in patients presenting with acute undifferentiated fever and clinically suspected of malaria and to compare them with Real-Time PCR for diagnosis of malaria in clinically suspected patients considering PBS as gold standard.

Materials & Methods:

The study was conducted in the department of microbiology, Index Medical College Hospital & Research Centre, Indore, MP. The present study is a descriptive cross-sectional study. The duration of the study two years (July 2019- July 2021) including six months of data analysis. After obtaining Institutional Ethics Committee permission, the present study was conducted and informed consent from all the participants of the study was taken. The sample size of the present study is 154. All patients attending various out-patient departments and or admitted to Index hospital and clinically suspected of suffering from malaria were included in the study. The exclusion criteria were patients of all age groups and both gender including infants, children under 5 years of age, pregnant women and patients with HIV/AIDS undergoing evaluation for malaria were enrolled in the study.

Patients with acute febrile illness suspected with causes other than malaria and those with a localized cause of fever were excluded. The study proforma was filled up for each study subject. Following tests were performed for each patient i) Peripheral blood smear (PBS) & ii) Real time PCR. Blood was taken by pricking a fingertip before starting treatment with Antimalarial drugs. Both, thick and thin smears of the blood were prepared on the different glass slides. A thick drop of blood will be taken by finger prick on a slide and spread with a needle with the corner of another slide to form an area of about 12 mm square. De-hemoglobinization of thick smear is done by keeping the slide in distilled water in Koplin's jar in vertical position for 5-10 minutes. The smear was covered with 5-10 drops of Leishman stain. After 2 minutes, the stain was then diluted by adding twice as many drops of buffered distilled water. The diluted stain was allowed to remain on the slide for 15- 20 minutes for staining. The slide was washed with buffered distilled water, dried in atmospheric air and examined under oil- immersion lens. At least 200-300 oil-immersion fields was examined before the smears are considered negative.

Statistical Analysis:

For categorical variables frequencies with 95% confidence intervals (CI) were used. To know the associations between two or more investigative procedures we used Chi-square test or Fischer's exact test. $P < 0.05$ was taken as the level of significance. Frequencies with 95% confidence intervals (CIs) were used for categorical variables. All the tests were performed on SPSS software. Specificity and sensitivity were also calculated. Percentages were also calculated.

Results:

Microscopy and PCR were used to analyze 154 samples. Table 1 summarizes the findings. Two positive samples were unable to be identified by microscopy, and six samples were unable to determine whether they were positive or negative due to improper staining or the slide fading. When the authors of the current study compared three different investigative methods, they discovered significant differences between positive and negative samples. Despite the fact that 64 samples were identified as P.Falciparum by microscopy, only 46 were confirmed by PCR; one was negative and the other was P.Vivax. However, three samples identified as P.Falciparum by PCR were not identified as P.Falciparum by microscopy.

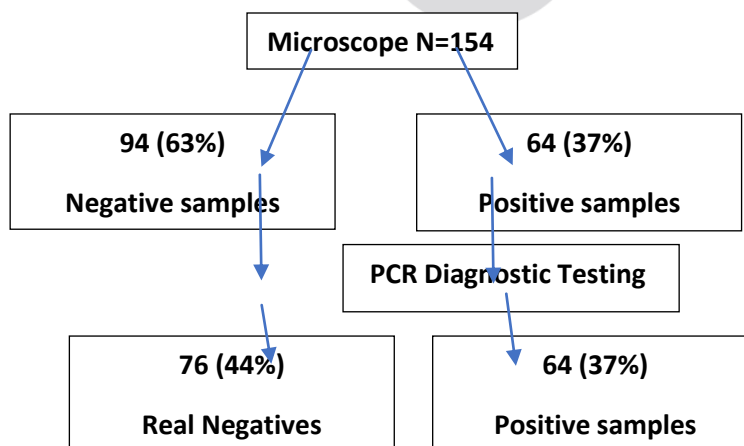**Table 1: Investigation results with each method**

	Microscope			PCR			
	N	%	95% CI	N	%	95% CI	P-value
Negative samples	90	63%	60.3-65.3	14	09%	07.5-12.2	<0.001
Positive samples	64	37%	34.3-39.6	136	91%	88.3-94.1	<0.001
P.Falciparum	61	97	94.1-99.4	132	98	95.1-99.8	<0.001
P.Vivax	03	---	---	04	0.8	0.7-1.4	0.002
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Table 2 shows the results of PCR of negative samples detected by microscopy (n=90) as a diagnostic quality. Between the samples detected by microscopy18 (14.7%) were false negatives based on PCR (Figures 1). Figure 1 shows the processing of negative samples found in RDT and microscopy with PCR. The results have been notified as according to the results derived.

Table 2: Analysis of negative samples by PCR

	Microscopy (n=90)			p-value
	N	%	95% CI	
P.Falciparum	17	96.4	93.4-98.6	<0.178
P.Vivax	1	3.6	2.1-5.6	<0.367
Total positives	18	14.7	12.1-17.4	<0.0001

The comparison of microscopy with PCR in terms of specificity and sensitivity. Microscopy has shown (54.3; 95% CI 52.1- 58.1) less sensitivity in diagnosing malaria problem. Microscopy has shown less specificity (81.8; 95% CI 79.2-84.5) in diagnosing malaria problem. Microscopy has shown less specificity of (56.4; 95% CI 53.2-59.4) in in detecting P. Falciparum. Microscopy has shown less specificity of (56.4; 95% CI 53.2- 59.4) in detecting P. Falciparum than PCR.
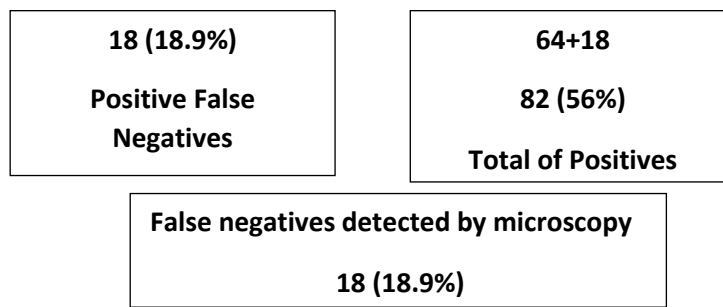
18 (18.9%) Positive False Negatives	64+18 82 (56%) Total of Positives

False negatives detected by microscopy 18 (18.9%)

Figure: Flow chart of diagnosis testing, Shows the processing that has been done to the 158 samples, both microscopy and PCR

Discussion:

The absolute necessity of lucid therapy in the face of widespread medication resistance sets a premium on malaria diagnosis accuracy. Microscopy and rapid diagnostic tests (RDTs) are the two investigative technologies most likely to have the greatest influence on malaria control today. These two tools, each with its own set of strengths and limitations, represent the best hope for accurate diagnosis as a fundamental component of malaria control and eradication success. The current study looked at the problems with current malaria detection techniques and represented data from various procedures. Every malaria-affected and prone-areas-prone country must invest in the development of malaria drugs or vaccines, as well as a commitment to improve malaria diagnostic techniques.

Our present study tried to know the investigative deficiencies in terms with microscopy in and around Indore region of India. The present study observed a false negative rate of 18.9% was found for microscopy investigative method. The present study samples have shown positivity in 62 samples by microscopy. The other samples yielded negative result this is due to the parasite load in the blood. The positivity in the samples is due to the parasitic load detected by microscopy technique [19, 20]. Parasite load in the blood is influenced by age, gender, socio-economic status and immunity of the individual [19, 20]. In malaria prone tropical countries, submicroscopic infections are undetected by microscopy is due to the strength of acquired immunity [19 – 21].

The low parasite density along with the high number of false negatives detected in microscopy (18.9%), indicates that in many occasions it is difficult to give a good microscopic diagnosis. The false negatives could infer two reasons, one is due to less expertise of the microscopists in the laboratory and hospital and the second may be due to improper staining of the slide. As the microscopy need staff who are better expertise at these techniques. Thus, better training is needed at these technical staff. It was detected misdiagnosed due to erroneous readings performed by the laboratory technicians, bad staining of the slide, and stain artefacts, or wrong species diagnoses. Odhiambo et al., 2014, conducted a study on factors associated with malaria microscopy diagnostic performance following a pilot quality-assurance programme in health facilities in malaria low-transmission areas of Kenya and observed that the diagnostic accuracy of malaria microscopy was positively associated with refresher-course in microscopy. Therefore, the refresher training and QA programme should be systematically implemented together to improve parasitological diagnosis of malaria by microscopy [22]. There are studies [23 – 25] which suggests that individuals with subclinical or lower parasitemia level were difficult and not possible to give a positive diagnosis by microscopy or RDT and such negative diagnosis have been shown in studies conducted in tropical regions. These studies have shown a inverse relationship with parasitemia level and clinical malaria in the subjects of studies concerned [23 – 25]. Studies [26 – 28] have been shown to treat lower levels of parasitemia in the blood and eliminate submicroscopic infections. Subclinical cases of malaria with submicroscopic infection with lower parasitemia withholds a strong basement that argues the strategies shall be designed to remove all densities of malaria parasitemia [29 – 32]. Considering the samples with coinciding diagnosis in PCR, more diagnostic matches were found with RDT than microscopy. PCR allows for the detection of subclinical or low-density infections and, even more importantly, specific parasite infection which are routinely missed in microscopy, as this PCR has a somewhat lower limit of detection [32], thus making it an ideal confirmatory test for malaria diagnosis.

One disadvantage of the current assay, along with a number of other malaria quick diagnostic techniques, is its inability to distinguish between the four Plasmodium species. To determine the Plasmodium sp. involved, a positive result must currently be supported with a malaria smear or nested PCR. Future versions of this assay would need to incorporate a species identification component because medication is dependent on the infecting species, though this may not be as significant if the assay is used simply as a screening test or blood products. Another possible drawback of this strategy is the existing scarcity of real-time PCR platforms.

Conclusion:

The present study explored the enormous potential of detecting asymptomatic individuals harboring malaria by a sensitive molecular method such as RT- PCR. The present study also demonstrated a highly sensitive and specific molecular method for detection of low level parasitemia in a targeted high-risk population which may prove it cost effective too. PCR as a reference method provides a more accurate information about prevalence and species distribution.

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