Prevalence of Malaria at a Tertiary Care Hospital in Mumbai, India

Rashmita Das[1], Shilpa Patil[1*], Kavita Balakrishnan[2], Chaitali Bhagat[2], Arunagiri Subramanian[2], Rajas Warke[2] and Abhay Chowdhary[1]

[1]*Department of Microbiology, Grant Government Medical College and Sir J.J. Hospitals, Mumbai, India.*
[2]*Department of Molecular Biology, HiMedia Laboratories Pvt. Ltd., Mumbai, India.*

Authors' contributions

This work was carried out in collaboration among all authors. Authors RD, SP designed the study and wrote the protocol. Authors KB, CB, AS performed the tests. Authors RD, SP and AS performed the statistical analysis and author RD wrote the first draft of the manuscript. Author RD and SP managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: Malaria is caused by parasites of genus *Plasmodium*. It remains a major public health concern around the world. Though various diagnostic tools are available, there is an urgent need to use a more sensitive diagnostic method for early diagnosis to prevent unwanted outcomes.
Objectives: (i) To assess the prevalence of malaria in a tertiary care hospital in Mumbai (ii) To detect and speciate *Plasmodium* by Peripheral Blood Smear (PBS), Rapid Diagnostic Test (RDT) and real-time Polymerase Chain Reaction (PCR) and to compare their performance characteristics.
Methodology: A hospital-based diagnostic study was carried over a period of 18 months. A total of 550 non-duplicated blood samples from clinically suspected cases of malaria were collected and subjected to three tests- PBS by Field's staining, RDT and PCR to detect *Plasmodium* species. The agreement and the differences between the three tests were analyzed and the statistical significance was assessed using Chi-square test.

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Corresponding author: E-mail: shilpapatil0207@gmail.com;

Results: Out of the 550 samples, 166 (30.2%) were positive for malaria by either of the three tests, of which 92 (55.42%), 119 (71.69%) and 161 (96.99%) samples were positive by PBS, RDT and PCR, respectively. *P. vivax* was the predominant *Plasmodium* species. The most significant finding was PCR detected mixed infections in 8.43% of cases whereas PBS and RDT could detect 0% and 1.20% of mixed infection, respectively. Considering PBS as gold standard, sensitivity of RDT and PCR was 100% and 97.83% and specificity was 94.10% and 84.50% respectively.

Conclusions: In this study, 30% of the population was infected with malaria with *P. vivax* being the predominant *Plasmodium* species. PCR helped identify more mono and mixed infections than conventional methods and would be a helpful adjunct for malaria diagnosis in tertiary care setup.

1. INTRODUCTION

Malaria is a vector borne disease caused by the parasites of genus *Plasmodium*. They are transmitted mostly through the bites of infected female Anopheles mosquitoes. There are 5 parasite species known to cause malaria in humans and two of these species pose a greater threat- *P. vivax* and *P. falciparum* [1]. It is the leading cause of morbidity and mortality around the globe, especially in tropical, sub-tropical and mildly temperate countries. It is associated with mankind since time immemorial. As per the World Malaria report in November 2020, there were 229 million malaria cases and an estimated 409 000 malarial deaths worldwide in 2019. The report states that WHO Africa region accounted for approximately 94% and WHO South East Asia Region accounted for 3% of global malaria burden. India accounted for 88% of malaria cases and 86% of malaria deaths in the WHO South East Asia Region in 2019 and accounts for 47% of all *P. vivax* cases globally. However, India has reported the largest reduction in cases in the South East Asia Region, with a decrease in malaria cases from 20 million cases in 2000 to about 5.6 million cases in 2019 [2]. This has been possible due to stringent enforcement of strategic plans for malaria elimination developed by National Vector Borne Disease Control Programme (NVBDCP) in support of WHO.

In 2018, *P. falciparum* accounted for 99.7% of malaria cases in the WHO African Region and 50% of cases in the WHO South-East Asia Region [1]. Globally, 3.3% of estimated cases are caused by *P. vivax* [2]. It is responsible for 53% of cases in the WHO South-East Asia Region with majority being in India (47%) [3]. The conducive climatic condition, topography, and malaria vector diversity in India, promotes the growth and spread of malarial parasites.

However, the distribution of these parasite species is not uniform across the Indian localities. Therefore, there may be presence of mixed species infection due to these two species as reported from different parts of the country [4].

There are various diagnostic tools currently available for identification of malaria parasites in human samples. It includes light and fluorescence microscopy, immuno-chromatographic lateral flow assays which are commonly known as Rapid Diagnostic Tests (RDTs), serology, Nucleic Acid Amplification Techniques (NATs) like Polymerase Chain Reaction (PCR) and isothermal amplification techniques which includes Loop Mediated Amplification Technique (LAMP) and Nucleic Acid Sequence-Based Amplification (NASBA), microarrays, flow cytometry etc. [5]. However, at the primary health care levels, the most commonly used tests are microscopy and RDTs. The gold standard microscopy test is labor intensive and time consuming. It also requires skilled personnel and good quality technical equipment. The commercially available RDTs differ widely in their sensitivity and specificity. Both these methods fail to diagnose malaria at low parasite counts. Also, under field conditions, these tests fail to detect mixed infection due to low sensitivity in diagnosing multiple species. In recent years, PCR has emerged as a superior method in detecting malarial parasites even at low parasite levels. However, its use is limited to laboratories only and thus serves as a tool for epidemiological study of malaria [6].

Therefore, the present study was aimed to determine the prevalence and the demographic profile of malaria at a tertiary care hospital in Mumbai and to detect and speciate the parasite in clinically suspected cases of malaria. The agreement and the differences between three

tests namely, microscopy, RDT and real-time PCR, used in identification of *Plasmodium* species was also studied.

2. MATERIALS AND METHODS

2.1 Study Design

A cross- sectional study was carried out in the Department of Microbiology in a tertiary care hospital in Mumbai, India, over a period of 18 months from January 2017 to June 2018. A total of 550 non-duplicated blood samples collected from clinically suspected cases of malaria admitted in the hospital were included in the study. All the samples were subjected to microscopy by Field's stain, rapid diagnostic test (PARAMAX-3, Tulip Diagnostics (P) Ltd, Goa, India) and real time probe-based single plex PCR (MBPCR 111 Plasmodium genus Detection Kit, HiMedia Labs Pvt Ltd, Mumbai, India) for genus detection and multiplex PCR (MBPCR135 Plasmodium species Detection Kit, HiMedia Labs Pvt Ltd, Mumbai, India) for species identification.

2.2 Study Tool

It included information regarding their socio-demographic factors, symptoms experienced during the sickness, presence of any underlying illness and the treatment received during the admission.

2.3 Malaria Diagnosis

After their consent, 3 mL of whole blood sample was collected in EDTA vacutainer from each patient. Of this 3 mL, 2 mL was aliquoted and stored in cryovials at -20°C before they were shipped to molecular laboratory for screening of malaria using real-time PCR.

A single drop of blood was used for preparation of thick and thin smears on glass slide and were stained by Field's stain. The microscopical examination of the blood smears were done using light microscope following the standard protocol for identification of various stages of different species of malaria parasite.

Lateral flow immunochromatography was done using a bivalent RDT kit, PARAMAX-3, Tulip Diagnostics (P) Ltd, Goa, India for identification of *P. vivax* and *P. falciparum* and was interpreted according to manufacturer instructions.

Genomic DNA was extracted using a spin-column based kit- HiPurA™ Blood Genomic DNA Miniprep Purification Kit (MB504, HiMedia

Laboratories, Mumbai, India). The extracted DNA was subjected to real time single plex PCR (MBPCR 111, HiMedia Laboratories, Mumbai, India) for amplification of 18S rRNA to detect genus *Plasmodium*. Samples positive for *Plasmodium* genus were differentiated into *P. vivax* and *P. falciparum* using multiplex probe-based real time PCR (MBPCR 135, HiMedia Laboratories, Mumbai, India). The PCR assays were performed and the amplification data was interpreted based on Ct values as recommended by the manufacturer (Ct value greater than 35 was considered negative, Ct value less than 35 was considered positive).

2.4 Statistical Analysis

The statistical significance was assessed using the Chi-Square test, with a *p* value of < 0.05 indicating significance. The results of RDT and PCR were compared with the microscopy results (gold standard test) for malaria diagnosis. The data was analyzed by a 2 x 2 contingency table. Sensitivity, specificity, negative predictive value, and positive predictive value were calculated using MedCalc for windows, version 14.6 (MedCalc Software, Ostend, Belgium).

3. RESULTS

3.1 Gender Wise Distribution of Malaria Positive Patients

Out of 550 malaria suspected patients, 166 (30.2%) were positive for malaria by either of the three tests, i.e., PBS, RDT and real-time PCR. Males were affected more (62.6%) than the females (Table 1). However, there was no statistically significant difference in gender-wise distribution pattern among malaria patients with *P* > .05 by chi-square test.

3.2 Age Wise Distribution of Malaria Positive Patients

Patients in the age group 10 to 50 years were mostly affected with 20-29 years being the predominant age group to be affected (Table 2). However, there was no statistically significant difference in age-wise distribution pattern among malaria patients with *P* > .05 by chi-square test.

3.3 Clinical Presentation of Malaria Suspected Patients

The most common presenting symptoms of the enrolled patients were fever (100%), body-ache and weakness (55.45%), chills and rigor

(53.45%), headache (52.18%), vomiting (23.27%), joint pain (11.63%), convulsions (1.27%) and rash (0.72%).

3.4 Seasonal Variation in Cases of Malaria

To study the seasonal variation of AFI cases, seasons were classified as Summer (March to May), Monsoon (June to September), Post-Monsson (October to December) and Winter (January to February) as per the Indian Meteorological Department [7]. In our study, the cases of Malaria started to rise from the month of June and peaked in the months of August and September which corresponds to rainy season (Fig. 1). Short peaks were observed in the months of October to December. This corresponds to the post-monsoon period when increase in relative humidity is favorable for mosquito and parasite development as well as for disease transmission. Towards the end of the year there was gradual decline in the number of positive cases. The seasonal variation seen in AFI cases was statistically significant with $P <$ .05 by chi-square test.

Table 1. Gender-wise distribution of malaria positive patients

Gender	Number of patients
Male	104 (62.7%)
Female	62 (37.3%)
TOTAL	166 (100%)

Table 2. Age-wise distribution of malaria positive patients

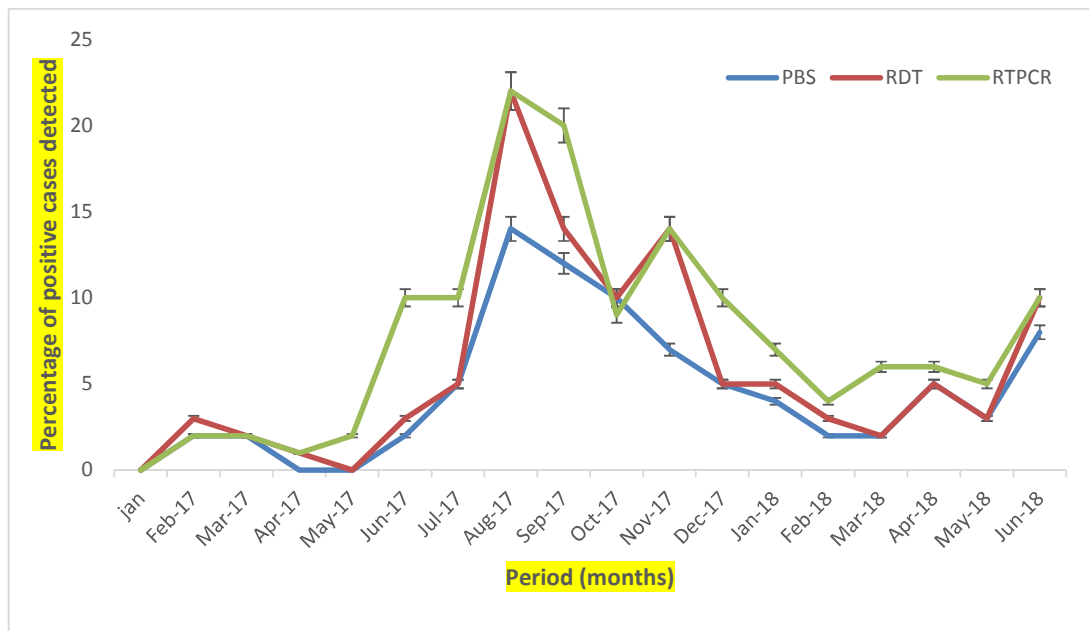| Age (years)	Total number of patients (%)
< 9	11 (6.6%)
10 -19	28 (16.9%)
20 - 29	47 (28.3%)
30 - 39	23 (13.9%)
40 - 49	25 (15.1%)
50 - 59	16 (9.6%)
>60	16 (9.6%)
TOTAL	166 (100%)

Fig. 1. Seasonal variation seen in cases of malaria

3.5 Species Distribution by PBS, RDT and PCR

Out of the 166 cases positive for malaria by either of the tests, PBS examination could detect 92 (55.42%) positive cases; 82 (89.13%) were *P. vivax* mono-infections, 10 (10.87%) were *P. falciparum* mono-infections. There were no mixed infections detected by PBS.

Similarly, RDT could detect 119 (71.69%) positive cases; 100 (84.03%) were *P. vivax* mono-infections, 14 (11.76%) were *P. falciparum* mono-infections, 2 (1.68%) were *P. vivax* and *P. falciparum* mixed infection and 3 (2.52%) were positive for other species (Pan positive).

However, real time PCR could detect 161 (96.99%) positive cases; of which 122 (75.78%) were *P. vivax* mono-infections, 16 (9.94%) were *P. falciparum* mono-infections and 14 (8.69%) were *P. vivax* and *P. falciparum* mixed infection. There were 9 (5.59%) cases which were positive for *Plasmodium* species but could not be speciated by PCR (Table 3).

In this study, it was found that *P. vivax* as the predominant *Plasmodium* species detected by all three tests, i.e., PBS (49.4%), RDT (60.2%) and PCR (73.5%).

3.6 Performance of RDT and PCR Compared to Microscopy

The total malaria prevalence detected by microscopy, RDT and real-time PCR is 16.7%, 21.6% and 29.3%, respectively. Considering microscopy as the "gold standard", results of RDT and PCR were compared with microscopy as shown in Table 4 and Table 5, respectively.

There were 458 samples that were negative for malaria by PBS of which 431 samples were in concordance with RDT. Similarly, of the 82 samples positive for *P. vivax* by PBS, 80 were in concordance with RDT. There were 2 mixed

infections (*P. vivax* + *P. falciparum*) detected by RDT which was otherwise diagnosed as *P. vivax* mono-infection by PBS (Table 4). RDT could detect 20 new *P. vivax*, 4 *P. falciparum* and 3 *Plasmodium* species (Pan positive) infections which were otherwise negative by PBS. In case of *P. falciparum* infection, both PBS and RDT gave concordant results.

Similarly, comparing the results with PCR, of 458 samples that were negative by PBS, 387 showed concordance with qPCR. Of 82 samples positive for *P. vivax* by PBS, 69 showed agreement with PCR and 1 was negative. There were 12 mixed infections (*P. vivax* + *P. falciparum*) detected by PCR which were misdiagnosed as *P. vivax* mono-infection by PBS. In case of *P. falciparum* infection, of 10 samples, 9 showed concordance with qPCR and 1 was negative. It could detect 53 new *P. vivax*, 7 *P. falciparum*, 2 mixed infections (*P. vivax* + *P. falciparum*) and 9 *Plasmodium* species which were otherwise negative by PBS. However, it failed to detect 2 samples which were otherwise positive by PBS (Table 5).

On comparing the performance characteristics of RDT and PCR with microscopy as the "gold standard" test, RDT had a sensitivity of 100% whereas PCR had a sensitivity of 97.83%. The specificity of each test was 94.10% and 84.50%, respectively. The Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of each test was 77.31%, 55.90% and 100%, 99.49%, respectively (Table 6).

4. DISCUSSION

In this hospital-based malaria prevalence study, 30% of the study population was positive for malaria. Many studies across India, conducted between 2016 to 2018, have reported malaria prevalence rates of 10.4% to 35.44% [8-11]. Two studies from Maharashtra have reported a prevalence rate of 6.37% and 12% in 2017 and 2008, respectively [12,13]. On the other hand, in

Table 3. Species distribution by microscopy, RDT and PCR

Species Distribution	PBS	RDT	PCR
P. vivax	82 (49.4%)	100 (60.2%)	122 (73.5%)
P. falciparum	10 (6.02%)	14 (8.4%)	16 (9.6%)
P. vivax + *P. falciparum*	0 (0%)	2 (1.2%)	14 (8.4%)
Plasmodium species	0 (0%)	3 (1.8%)	9 (5.4%)
Total positive	92 (55.4%)	119 (71.7%)	161 (96.9%)
Total negative	458 (83.27%)	431 (78.36%)	389 (70.73%) *

* *There were 5 samples which were negative by PCR but positive by RDT or PBS. The reasons for this discrepancy have been addressed in the discussion section. Therefore, total samples positive for malaria were 166 (161+5) and negative were 384*

Table 4. 2 x 2 Contingency table comparing microscopy and RDT

Test		RDT					
		Negative	***P. vivax***	***P. falciparum***	***P. vivax + P. falciparum***	***Plasmodium* species**	**Total**
PBS	Negative	431	20	04	-	03	458
	P. vivax	-	80	-	02	-	82
	P. falciparum	-	-	10	-	-	10
	Total	431	100	14	02	03	550

Table 5. 2 x 2 Contingency table comparing microscopy and RT-PCR

Test		PCR					
		Negative	***P. vivax***	***P. falciparum***	***P. vivax + P. falciparum***	***Plasmodium* species**	**Total**
PBS	Negative	387	53	07	02	09	458
	P. vivax	01	69	-	12	-	82
	P. falciparum	01	-	09	-	-	10
	Total	389	122	16	14	09	550

Table 6. Performance characteristics of RDT and RT-PCR vs microscopy

Performance characteristics	RDT (%)	PCR (%)
Sensitivity	100	97.83
Specificity	94.1	84.5
Positive predictive value (PPV)	77.31	55.90
Negative predictive value (NPV)	100	99.49

a multicentric study conducted by Haanshuus et al. in the year 2016, a higher rate of malaria (35%) was reported in Ratnagiri district of Maharashtra [10]. *P. vivax* (75.78%) was the predominant *Plasmodium* species to be detected in the study followed by *P. falciparum* (9.94%), which is consistent with the studies conducted by Singh et al. [14] and Dayanand et al. [15] in 2015 and 2017, where *P. vivax* infection was seen in 65.51% and 81.7% cases, respectively. On the contrary, in studies by Haanshuus et al. and Silwal et al., *P. falciparum* infection was the predominant *Plasmodium* species in India. The above studies conducted in 2016 and 2018 have reported a dramatic shift in malaria burden from *P. vivax* to *P. falciparum* infection which has been attributed to increasing chloroquine resistance in *P. falciparum* [10,11]. A regional bias was seen in dominance of two species across India in a study by Silwal et al. in the year 2018, where *P. vivax* was dominant in northern and southern parts of India whereas *P. falciparum* was predominantly seen in eastern, north-eastern and central parts of India [11]. Globally, *P. vivax* is responsible for 36% of malaria cases except in African countries where only 4% of cases are due to *P. vivax* [3].

Microscopy could detect only 55.42% of malaria positive cases. Various studies, conducted in 2001, 2003 and 2016, in and outside India have reported different positivity rates of 3.07% to 42.1% using microscopy [16-20]. The reason for this large variation could be due to multiple factors which include proper slide preparation and staining techniques, training and skills of the microscopists, maintenance of microscopes and quality of the laboratory reagents as stated by Santhy et al., Silwal et al. and the WHO [19,11,21]. It has a low sensitivity especially among individuals with sub-microscopic infection as it fails to detect parasites at counts below 100 parasites/ µL of blood [10,21]. In case of mixed infections, it failed to detect 2 mixed infections and mis-identified 12 mixed infections as *P. vivax* mono-infection. This phenomenon was also observed by Krishna et al. in 2015, where microscopy could not identify 17.4% of mixed infections. A possible explanation to this could be as one parasite predominates over the other, microscopy fails to detect parasite with low counts [22]. The low species-specificity of microscopy as compared to PCR has also been seen in study by Haanshuus et al. [10]. Misdiagnosis of mixed infections can be attributed to facts like i) time of blood collection, ii) similarity in the ring stages of both the species,

iii) presence of all stages of life cycle of *P. vivax* compared to only ring and gametocyte stages of *P. falciparum*, iv) deformities of infected RBCs caused by *P. vivax* is more prominent than *P. falciparum* [11].

Similarly, RDT could detect 71.69% of malaria positive cases. Silwal et al. [11], Santhy et al. [19], Mfuh et al. [23] and Ojurongbe et al. [24] reported detection rates of 31.41%, 52.4%, 45% and 38.7%, respectively. However, there were 5 (3%) cases which were negative by RT-PCR. Similar phenomenon was also seen in a study by Mahende et al. in 2016, where 15% of the samples were positive by RDT but were negative by PCR [25]. These could be false positive results given by RDT as they are known to give false positive results due to persistence of HRP-II antigen even after treatment as per WHO or due to presence of immunological factors like Rheumatoid factor in blood [21,26]. It failed to detect 47 positive cases which is consistent with finding by Haanshuus et al. [10]. Two potential causes for such finding could be i) the sensitivity of RDT markedly decreases below the level of 100 parasites per µL of blood ii) RDTs use Histidine Rich Protein (HRP-II) for *P. falciparum* diagnosis and Lactate Dehydrogenase (LDH) protein for *P. vivax* diagnosis. Deletions and mutations are commonly reported in *Pfhrp-2* gene as claimed by various studies. Thus, misdiagnosis by RDT is inevitable [21,11].

PCR could detect 96.99% of malaria positive cases. Studies by Haanshuus et al. [10], Silwal et al. [11] and Santhy et al. [19] have reported detection rates of 19%, 35.44% and 50.8%, respectively. Of these PCR positive samples, 8.69% were mixed infection due to *P. vivax* and *P. falciparum*. Mixed species infection is more prevalent in the middle and the south-west coast of India as stated by Silwal et al. [11]. Singh et al. has reported a higher prevalence of mixed infection (27.93%) in their study from Navi Mumbai [27]. Haanshuus et al. reported a high prevalence of mixed infection from Anantapur, Tezpur and Ambur region with rates being 19%, 14% and 16%, respectively. In the same study, in Ratnagiri, the prevalence rates were 8% for *P. vivax* and *P. falciparum* co-infection, 1% for *P. falciparum* and *P. malariae* co-infection and 1% for *P. vivax* and *P. malariae* co-infection. Cases of mixed infection have also been reported from countries like Thailand, Cambodia, Papua New Guinea [10].

There were 5 cases which were positive either by PBS or RDT but were negative by RT-PCR. This

phenomenon has also been observed by Santhy et al. [19] and Rantala et al. [28] (2010) where 9.52% and 9.09% of the samples were positive by microscopy but were negative by PCR. This false negative result may be due to failure to amplify the target DNA which can be attributed to low copy number of target sequence to primer [19]. However, there were 9 cases which were positive for Plasmodium species but could not be speciated by RT-PCR. These cases require further study.

Despite the advantages of PCR in detecting disease even at low parasite counts, its use in routine diagnostics is limited. It requires sophisticated equipment, training and is significantly more expensive than conventional methods. Another challenge in using PCR for clinical diagnosis is that it may detect low parasitemia in semi-immune individuals. Therefore, actual pathogen causing fever may be overlooked. Such sub-microscopic and asymptomatic infections are not only seen in high endemic areas but have also been reported from regions of low endemicity [10]. Therefore, in support of conventional methods, this assay is of great value in accurately identifying malaria species and mixed infections. It is an important epidemiological tool in assessing the malaria burden in the country.

Compared to the microscopy, RDT had a sensitivity of 100% and specificity of 94.10 %. This finding was consistent with studies conducted by Santhy et al. and Adesanmi et al. (2011) where RDT had a sensitivity of 95.2% and 82% and a specificity of 85% and 91.5% respectively [19,29]. On the contrary, in 2016 and 2015, studies by Garba et al. [30] and Elechi et al. [31] showed that RDT was less reliable than microscopy in diagnosis of malaria in children less than five years with sensitivity as low as 9.09% and 8.3%, respectively. In this study, PCR had a sensitivity of 97.83% and a specificity of 84.50%. Similar findings were also seen by Santhy et al. [19], Rantala et al. [28] and Wangai et al. (2011) [32] where PCR had a sensitivity of 90.5%, 90.9% and 100% and a specificity of 85%, 91.2% and 79%, respectively. Coleman et al., in their study in 2002, concluded that PCR was both sensitive and specific at parasite counts above 500/ µL but both the parameters dropped markedly at counts below 500/ µL [33].

There are few studies where, PCR was considered as the gold standard test and the performance of microscopy and RDT was assessed against it. Under such consideration, Dinko et al. (2016) and Andrade et al. (2010) found that the sensitivity and specificity of both the tests were lower than expected with RDT performing superiorly than microscopy [26,34]. On the other hand, in 2013, Ojurongbe et al. found PCR to be more sensitive and specific than microscopy followed by RDT using composite reference method as the gold standard [24].

The WHO states that there is a need to identify a better "gold standard" test against which other tests can be assessed as microscopy is an imperfect diagnostic tool for malaria diagnosis. With the currently available methods for diagnosis, further studies are required to address questions such as whether combination of diagnostic tests would be a better approach to a gold-standard test and whether the same gold-standard can be used in all epidemiological situations [21].

5. CONCLUSION

Malaria diagnosis can be done using conventional methods like microscopic examination of thick and thin smears, rapid diagnostic tests, and molecular methods like polymerase chain reaction. Though conventional microscopy remains the "gold-standard" test for malaria diagnosis, it requires skilled personnel and is less sensitive in diagnosing mixed infection than more recent molecular methods like PCR.

Rapid diagnostic methods, though are more expensive than microscopy, are quick and easy to use. Therefore, these conventional methods remain the mainstay in malaria diagnosis in field conditions.

Molecular methods are better suited to research laboratories as they are expensive, require sophisticated laboratory set-up and well-trained staff. However, they are helpful in identification of mixed infections and parasite at very low counts.

Therefore, there is a dire need to identify a better "gold standard" test for malaria diagnosis. However, the choice of most appropriate method is largely determined by the endemicity of the disease, the urgency of diagnosis and the availability of skilled staff and financial resources.

DISCLAIMER

The products used for this research are commonly and predominantly used products in

our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

An informed consent was obtained from the patients and a structured questionnaire was prepared to collect information about the study population.

ETHICAL APPROVAL

The study was discussed and approved by the Institutional Ethics Committee of the hospital.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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