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Original Research Article

Comparison of Various Techniques for the Diagnosis of Clinically Suspected Cases of Malaria with Special Reference to PCR

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Abstract:

Introduction: Malaria is one of the most successful and pervasive parasitic infections ever known to mankind attributable to its treatability. Its early diagnosis using rapid diagnostic tests contributes towards effective management. Increasing mortality and morbidity resulting from malaria makes it crucial that a rapid and accurate diagnosis be made, in order to initiate prompt treatment in clinically suspected cases.

Aims: To compare between various techniques for the diagnosis of clinically suspected cases of Malaria and also to evaluate the diagnostic utility of PCR.

Material and Methods: A total of 200 clinically suspected cases of malaria presenting with fever and chills were included in the descriptive study. The samples were processed for detection of malaria parasite or its antigen. Peripheral blood smear examination (PSMP) was done using Leishman's staining, Rapid Malaria Antigen Test (RMAT) by Accucare (Lab care diagnostics Pvt Ltd) and conventional PCR for detection of Plasmodium species (P. falciparum, P. vivax) using Malaria diagnosis kit (ACTIVETM, Imperial Life Sciences Pvt Ltd, Gurgaon, Haryana, India) were performed on each sample. The diagnostic efficacy of each test against the appropriate gold standard has been studied using indices such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy along with 95% CI as the precision of their estimates. The entire data was statistically analyzed using Statistical Package for Social Sciences (SPSS ver 16.0, Inc. Chicago).

Results: Out of 200 cases, PSMP detected and identified malarial parasites in 28(14%), RMAT in 35(17.5%) and PCR in 27(13.5%) of the cases. In 28 out of 200 samples, malaria positivity was seen by both PSMP and RMAT and speciation were in agreement (100%). Whereas PCR showed agreement in 22 cases in terms of speciation. Sensitivity of RMAT and PCR was 100% and 85.7% and specificity was 95.9% and 98.3% respectively. Additionally, the association between PSMP and RMAT, PSMP and PCR was statistically significant (P value=0.001).

Conclusions: Rapid diagnostic tests and PCR technique can be useful adjuncts to microscopy to diagnose malaria cases accurately.

Keywords: Malaria, Microscopy, Conventional-Multiplex PCR, P.falciparum, P.vivax.

Introduction

Malaria is one of the most successful and pervasive parasitic infections ever known to mankind. According to the latest WHO estimates, globally there were 241 million cases and 6,27,000 deaths in 2020 [1] while Indian statistics shows 46,809 cases and 6 deaths from malaria till June 2022.[2] Moreover a total of 5032 cases of malaria were reported from Mumbai in the year 2021.[3]

Out of the five species of Plasmodium causing malaria, P. *falciparum* can cause a lethal infection and often requires emergency interventions. Detaining the treatment of falciparum malaria may lead to serious repercussions including death.[4] *P.vivax* malaria can also present with severe manifestations like hepatic and renal dysfunction, severe anemia, ARDS and rarely cerebral malaria.[5]

Acute febrile illness, a characteristic feature of malaria is also a common manifestation of dengue, chikungunya, leptospirosis, typhoid, Japanese encephalitis, rickettsial infections and Influenza.[6] Differentiation of malaria from these conditions and to reduce the mortality and morbidity, a rapid and accurate diagnosis is imperative. Moreover, speciation of malarial parasite by diagnostic methods would enable the clinician to understand the pathogenesis and outcome of the disease.The quest for early and accurate diagnosis of malaria has led to the development of rapid diagnostic tests. In recent years, the development of molecular tests for malaria has introduced new diagnostic methods for the detection and speciation of malaria parasites. Therefore, the present study aims to compare peripheral smear, rapid immunochromatographic test and multiplex-nested PCR in diagnosis of malaria infection in clinically suspected cases and also to compare clinical and hematological correlates with microbiological findings and to evaluate the diagnostic utility of Multiplex PCR technique in malaria.

Material and Methods

Place of the Study

Department of Microbiology (Molecular lab and serology section), TNMC & BYL Nair Ch. Hospital.

Study design

Descriptive study.

Study period

12 months from June 2015 to October 2016.

Sample size

Sample size was determined by using the effect sizes from the previously published studies and with the help of following formula:

$N=Z^2 P (1-P)/ E^2$

Z=score at 95% confidence interval (1.96)
P=0.80 (approximate accuracy of PCR with PSMP)
1-P= 0.20
E=Absolute error (0.055)
N=required sample

Thus, the minimum sample size required according to this formula is 199.5=200 in our study.

Study Population

This study was carried out after obtaining Institutional ethics committee approval.

Inclusion Criteria

A total of 200 clinically suspected cases of malaria, comprising of 100 inpatients and100 outpatients more than/equal to 18 years of age, presenting with fever and chills were included in the study.

Exclusion Criteria

Patients who had already taken antimalarial treatment during the febrile period and who were positive for dengue, leptospirosis and typhoid using standard diagnostic tests were excluded from the study.

Sample Collection and Processing

Informed consent was taken from patients and by using standard aseptic precautions, 5 ml of blood (2 ml for peripheral smear examination and Rapid Malaria Antigen test and 3 ml for PCR) was collected by venipuncture in two sterile EDTA vacutainer tubes (Becton Dickinson). Dried blood spot was prepared for PCR by adding 20µl of blood on Whatman paper grade 3, air dried and stored at -20° Celsius in sterile aluminum foil.

Peripheral blood smear examination (PSMP) (Leishman's staining), Rapid Malaria Antigen Test (RMAT) (Accucare, Lab care diagnostics Pvt Ltd, Sarigam,India) and conventional PCR for detection of Plasmodium species ((P.falciparum, P.vivax) using Malaria diagnosis kit (ACTIVE^{TM} , Imperial Life Sciences Pvt Ltd, Gurgaon, Haryana, India) were performed on each sample.

Conventional microscopy for examination of peripheral blood smears (PSMP)

Thick and thin smears were prepared on the same slide and peripheral blood smear examination using Leishman's stain was carried out following standard guidelines. A minimum of 100 fields were examined taking 7-10 minutes per slide. Percent parasitemia (Parasitic index) was calculated and expressed as a percentage of erythrocytes infected.

Rapid Malaria Antigen Test (RMAT) for the detection of Malaria Pan/ Pv/ Pf

Rapid Malaria Antigen Test (RMAT) was performed in parallel as per manufacturer's instructions. Rapid ICT Accucare was used in this study which is a rapid, self-performing, qualitative two site sandwich immunoassay using principle of immunochromatography which detects *P.falciparum* specific histidine rich protein-2 (Pf HRP-2) and pan malaria specific pLDH of plasmodium species *(P.falciparum, P. vivax, P.malariae, P. ovale).*

Polymerase Chain Reaction (PCR) amplification

DNA was extracted from dried blood spot by saponin extraction.[7] Amplification was carried out on the

same day using amplification kit as per manufacturer's protocol. PCR mix was prepared in an eppendorf tube by adding Buffer (2.5 µl x n), dNTP (2 µl x n), Plasmodium spp forward primer and Plasmodium spp. reverse primer (0.8 µl x n) each, taq DNA polymerase (0.25 µl x n), Nuclease free water (18.65 µl x n), where n is the total number of samples run in each lot. 25 µl of PCR mix preparation was added to each eppendorf tube with the dried blood spots and then put in a thermal cycler. DNA was then amplified under the following cycling conditions: 35 cycles of initial denaturing period of 94° Celsius for 4 min, 55° Celsius for 2 min, 72° Celsius for 2 min and a final extension for 5 min. Primary PCR product was obtained. Nested PCR was performed with 2µl of primary PCR product and 23 µl of PCR mix. PCR mix was prepared by adding buffer (2.5 µl x n), dNTP (2 µl x n), P.vivax specific forward primer and P.vivax specific reverse primer (0.3 µl x n each), P. falciparum specific forward primer and reverse primer (0.3 µl x n each), . taq DNA polymerase (0.25 µl), Nuclease free water (17.05 µl). Second step PCR was done under the following conditions- 30 cycles of 94° Celsius for 40 sec, 58° Celsius for 1 min and 72° Celsius for 2 min. The amplified nucleic acid product was demonstrated by Gel electrophoresis. Positive control and negative control were used for each run. Positive control used was known *P.falciparum* culture and negative control was all the mix of reagents without sample. PCR products of both amplification procedures were analysed by 2% gel electrophoresis stained by ethidium bromide. Fluorescent bands were visualized by using UV illumination.

Statistical Analysis

The data on categorical variables was presented as number (n) and percentage (%) of cases. The diagnostic efficacy of each test against the appropriate gold standard has been studied using indices such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy along with 95% CI as the precision of their estimates. The entire data was statistically analyzed using Statistical Package for Social Sciences (SPSS version 16.0, Inc. Chicago) for MS Window.

Results

In our study, age of the study population ranged from 18 to 66 years with mean age being 42 years with male preponderance.

Table 1: Demographic features of study population (n=200)

			Gender			
Age(years)	**Total**		**Male**		**Female**	
	N	%	N	%	N	%
18-20	9	4.5	3	2.5	6	7.7
21- 30	74	37.0	44	36.1	30	38.5
31- 40	79	39.5	50	40.9	29	37.2
41- 50	27	13.5	20	16.4	7	8.9
51- 60	8	4.0	5	4.1	3	3.8
>60	3	1.5	0	0.0	3	3.8
Total	200	100	122	100	78	100

Malaria parasite was detected in 28(14%) of samples by peripheral blood smear while RMAT and PCR yielded positive results in 35(17.5%) and 27(13.5 %) cases respectively out of 200 clinically suspected cases of malaria.

Table 2: Distribution of study population (n=200) and PSMP positive cases (n=28) in outpatient department (OPD) and In patient department (IPD)

	Outpatient (OPD) No %	**Admitted patients (IPD)**	
		Ward No %	**Intensive care unit (ICU) No %**
Study population (n=200)	100 (50%)	89(44.5%)	11(5.5%)
PSMP positive cases(n=28)	4 (4%)	22 (24.71%)	2 (18.18%)

The study population presented with fever and chills (100%) besides profuse sweating (24%), abdominal pain (7.5%), headache(5.5%), anorexia(3.5%) and anemia(3%) in decreasing order. Malaria parasite was detected in 28(14%) of the samples by peripheral blood smear. Among the 28 PSMP positive cases, along with fever and chills (100%), profuse sweating (64.3%), headache (17.9%), abdominal pain(14.3%) and anemia (14.3%) were noted. None of the cases presented with complications like black water fever. Among the PSMP positive cases, thrombocytopenia was the predominant hematological finding in

32.14% cases, followed by neutrophilia in 25% cases, while anemia was seen in 21.42% cases.

While malaria parasite was detected in 28 (14%) samples by peripheral blood smear, RMAT and PCR yielded positive results in 35(17.5%) and 27(13.5 %) cases respectively (Table3). Out of the 28 cases positive by both PSMP and RMAT, only 24 were PCR positive. With regard to identification of species of malaria parasite, among the 28 PSMP positive cases, 23 were P.vivax, three were *P.falciparum* and two were mixed infections. In the 24 cases positive by all three tests, in 21 cases (75%), the results of PSMP, PCR and RMAT were in total agreement. They included 19 cases of *P.vivax,* one case each of *P.falciparum* and mixed infection. Results of 3 samples were discordant with respect to species identification which showed agreement by both PSMP and RMAT but gave different results by PCR. One sample showed mixed infection by PCR while PSMP and RMAT showed only *P.falciparum*. Another sample showed *P.vivax* infection in PCR but mixed infection in PSMP and RMAT. In the third sample, PCR detected *P.falciparum* while PSMP and RMAT showed *P.vivax* . Out of the seven RMAT positive and PSMP negative cases, five were positive for *P.vivax* and two were *P.falciparum* positive. However, PCR was positive in two out of 165 PSMP and RMAT negative cases (Table 3). Therefore, 163 cases were negative for malaria by all three tests. Considering PSMP as a gold standard, the sensitivity, specificity and accuracy of RMAT was 100.0%, 95.9% and 96.5% respectively while that of PCR was 85.7%, 98.3% and 96.5% respectively.

Table 3: Comparison of RMAT and PCR results with PSMP (n=200)

Group	Total	PCR positive %	PCR negative %
1.PSMP positive RMAT positive	28	24 (85.7%)	4 (14.3%)
2.PS negative RMAT positive	7	1 (14.3%)	6 (85.7%)
3.PS negative RMAT negative	165	2 (1.2%)	163 (98.8%)
Test	200	27 (13.5%)	173 (86.5%)

Table 4: Comparison of results of RMAT and PSMP Frequency Distribution

RMAT		PSMP (Gold Standard)		
		Positive	**Negative**	**Total**
Positive	n	28	7	35
	%	100.0	4.1	17.5
Negative	n	0	165	165
	%	0.0	95.9	82.5
Total	n	28	172	200
	%	100.0	100.0	100.0

Measures of Diagnostic Efficacy of RMAT

Measure	Value (%)	95% CI	
		Lower	**Upper**
Sensitivity	100.0	100.0	100.0
Specificity	95.9	92.9	98.9
Positive predictive value (PPV)	80.0	66.8	93.3
Negative predictive value (NPV)	100.0	100.0	100.0
Accuracy	96.5	93.9	99.0

The sensitivity, specificity and accuracy of RMAT compared to PSMP as a gold standard is 100.0%, 95.9% and 96.5% respectively.

Table 5: Comparison of results between RMAT and PCR (n=200).

RMAT		PCR (Gold Standard)		
		Positive	Negative	Total
Positive	N	25	10	35
	%	92.6	5.8	17.5
Negative	N	2	163	165
	%	7.4	94.2	82.5
Total	N	27	173	200
	%	100.0	100.0	100.0

Frequency Distribution

Measures of Diagnostic Efficacy

Measure	Value (%)	95% CI	
		Lower	Upper
Sensitivity	92.6	82.7	99.9
Specificity	94.2	90.7	97.7
Positive predictive value (PPV)	71.4	56.5	86.4
Negative predictive value (NPV)	98.8	97.1	99.9
Accuracy	94.0	90.7	97.3

The sensitivity, specificity and accuracy of RMAT against PCR as a gold standard is 92.6%, 94.2% and 94.0% respectively.

Frequency Distribution

Table 6: Comparison of results of PCR and PSMP (Gold Standard) (n=200)

PCR		PSMP (Gold Standard)		
		Positive	Negative	Total
Positive	n	24	3	27
	%	85.7	1.7	13.5
Negative	n	4	169	173
	%	14.3	98.3	86.5
Total	n	28	172	200
	%	100.0	100.0	100.0

Measures of Diagnostic Efficacy

Measure	Value (%)	95% CI	
		Lower	Upper
Sensitivity	85.7	72.8	98.7
Specificity	98.3	96.3	99.9
Positive predictive value (PPV)	88.9	77.0	99.9
Negative predictive value (NPV)	97.7	95.5	99.9
Accuracy	96.5	93.9	99.0

The sensitivity, specificity and accuracy of PCR against PSMP as a gold standard is 85.7%, 98.3% and 96.5% respectively.

Discussion

Malaria is one of the world's most prevailing parasitic infections and it inflicts a great socio-economic burden on humanity. Malaria afflicts 90 countries and territories in the tropical and sub-tropical regions and almost one half of them are in Africa, South of Sahara.[8] Clinical diagnosis and microscopy are the mainstay of treatment for most cases of malaria worldwide. Although microscopic examination of blood smears continues to be the gold standard, it has several drawbacks. It is time consuming, requires a trained microscopist and the results are poor in cases of low parasitemia. Under optimum conditions, microscopy can detect 20-50 parasites/µl blood (0.0004 to 0.001% parasitemia),[9] but under routine laboratory conditions, such sensitivity is seldom achieved. Moreover, clinically the signs and symptoms of malaria may mimic various other conditions like ty-

phoid, dengue, chikungunya, Leptospirosis, Japanese encephalitis, Rickettsial infections and Influenza. Hence a rapid, reliable, and sensitive method of diagnosis should be available to clinical laboratories for the detection of malaria. This would avoid under diagnosis, over treatment and also prevent the development of multidrug resistant malaria.[10]

Comparison of clinical and hematological correlates

Fever and chills are the classical presentation in malaria which is associated with synchronous rupture of blood schizonts and release of merozoites with high level of tumor necrosis factor-α (TNF-α) in the blood. Headache is also seen in malaria due to release of cytokine. Abdominal pain in malaria can result from enlargement of liver and spleen. Hepatomegaly is a feature of chronic malaria where periportal fibrosis, sinusoidal dilatation takes place. Reticuloendothelial hyperplasia is another factor causing hepatosplenomegaly.

In our study, among the PSMP positive cases, along with fever and chills (100%), other presenting features included headache (17.85%), abdominal pain (14.28%), anorexia (10.71%), hepatomegaly (7.14%) and splenomegaly (3.57%). Other studies like Taviad et al [11] and Muddaiah et al [12] have variously reported all these features.

In this study, anemia and thrombocytopenia were observed in 21.42% and 32.14% of the PSMP positive cases respectively. These features were in agreement with other studies like Bashwari et al,[13] Shrivastava et al [14] and Khuraiya etal.[15]

Lab diagnosis of malaria

In our study, malaria parasite was detected in 28(14%) out of 200 samples by peripheral blood smear while RMAT and PCR yielded positive results in 35(17.5%) and 27(13.5 %) cases respectively. A study by Gayar et al [16] in Saudi Arabia (2013) showed that 25.3% of the samples were positive for malaria by peripheral smear examination, 27.5% using HRP2-ICT and 31.9% by PCR out of 91 cases studied. Sharma et al[17] in Assam (2013) reported 21.82% positivity by PSMP, 28.19% positivity by rapid test and 25.53% positivity by PCR out of 188 cases studied.

Comparison of RMAT with PSMP

Considering PSMP as a gold standard, overall sensitivity, and specificity of RMAT was found to be 100% and 95.9% respectively. RMAT sensitivity in our study is closer to the sensitivity reported by Sharma et al [17] in 2013 from India (95.83%) and Khorashad et al[18] in 2014 from Iran (95%). However, it was higher than reports by Ashley et al [19] in 2009 in France (89.6%-95.2%), Gayar et al [16] in 2013 in Saudi Arabia (83%), Ayogu et al[20] in 2016 in Nigeria (82.2%) and Fransisca et al [21] in Indonesia, 2015 (72.9%-92%).

The reason for higher (100%) sensitivity of RMAT obtained in our study could be due to the quality of the RMAT kit used, the quality of kit varying between manufacturers [22]. Overtime newer kits have been developed with improved sensitivity.

Specificity of RMAT in our study was 95.9% which is similar to findings of Ashley et al [19] from France in 2009 (100%), Laban et al [22] from Zambia in 2015 (99%) and Ayogu et al [20] from Nigeria in 2016 (100%). Specificity reported in our study as well as others is higher than earlier studies which reported false positivity due to cross reaction with rheumatoid factor, but the issue has reportedly been addressed.[23] It is important to note that rapid diagnostic tests have the limitation that they cannot distinguish between the non-*P. falciparum* species (*P.ovale* and *P. malariae*), nor can they reliably distinguish pure *P. falciparum* infections from mixed infections as there is 90-92% analogy between pLDH of *P. falciparum* with *P.vivax*. Moreover, HRP2 antigen in *P. falciparum* remains detectable even up to 15 days following treatment.

Although we noted 100% sensitivity of RMAT, but false negative tests have been observed which has been attributed to possible genetic heterogeneity of PfHRP2 expression, deletion of HRP-2 gene, presence of blocking antibodies for PfHRP2 antigen or immune-complex formation, prozone phenomenon at high antigenemia or unknown causes. Occasional false positive tests can occur with RDTs for many reasons like PfHRP2 positivity, other than gametocytaemia, and include persistent viable asexual-stage parasitemia below the detection limit of microscopy (possibly due to drug resistance), persistence of antigens due to sequestration and incomplete treatment, delayed clearance of circulating antigen (free or in antigen-antibody complexes) and cross reaction with non-falciparum malaria or rheumatoid factor.[24, 25]In our study, four samples showed *P. vivax* and two showed *P. falciparum*by RMAT while both PS and PCR were negative. The false positivity in these cases needs to be ruled out.

Comparison of PCR with PS, considering PS as a gold standard, PCR showed overall sensitivity and specificity of 85.7% & 98.3% respectively. Olusola et al[26] from Nigeria in 2013 reported 97.3% sensitivity and 62.5% specificity of PCR compared to PSMP. Another study by Alam et al[27] in Bangla-

desh, 2011 reported sensitivity of 95.2% and specificity of 98.1% which is correlating with our study. A study by Datta et al[28]in Pune, India in 2010 showed a lower sensitivity (71.4%) compared to our study but specificity of 100% which is closer to that of our study (98.3%). Wang et al [29] from Myanmar, 2014 and Kanyi et al [30] from Nigeria, 2016 reported a PCR sensitivity and specificity of 100% each. In our study, four of the PCR negative samples were positive by both PSMP and RMAT. Parasitic index of these samples ranged from 6/ µl of blood to 400 / µl of blood. PCR could not detect those cases. False negative PCR results have been variously attributed to a considerable gap between the blood collection and DNA isolation (6-8 hours),[31] improper storage conditions, inadequate transportation of dried blood spots and loss of target DNA during extraction and interference during amplification due to presence of inhibitors in samples.[32]

Properly designed primers and probes specific for well conserved target are required for a successful PCR reaction. The target for multiplex PCR in our study was the highly conserved target sequence present in18srRNA gene resulting in high specificity. A study by Haanshuus et al [33]reported that PCR with another target i.e mitochondrial DNA showed 100% sensitivity and specificity. Its sensitivity and specificity were statistically non inferior to that of reference 18srRNA nested PCR.

Clinical outcome

In our study, out of 28 PSMP positive cases, 24 were admitted in the hospital of which two succumbed during stay while the remaining patients recovered. In this study the two deaths were due to multisystem involvement and they were diagnosed as one case each of *P.vivax* and mixed infection by all three methods. Mortality rate in our study was 7.14% which is correlating with other studies like Khuraiya et al,[15]in Madhya Pradesh, 2016 (8.65%), Kochar et al[5] in Bikaner, in 2009 (10.93%) whereas Galande et al[34]in Maharashtra, 2014 reported 3% which is lower than our study.

Conclusion

In conclusion, our results emphasize the role of laboratory in confirmation of diagnosis among clinically suspected cases of malaria. Our study also indicates that immunochromatographic techniques can usefully supplement microscopy, especially for screening of patients.

Though PCR is labour-intensive, and requires high level of expertise and standardization, it holds good promise in a clinical setting where there is high degree of clinical suspicion with smear and rapid test negative results. However, expert microscopy will continue to remain the mainstay in laboratory diagnosis of malaria.

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