DIAGNOSIS OF MALARIA BY REAL-TIME POLYMERASE CHAIN REACTION IN CASES OF NEGATIVE MALARIAL PARASITE ON MICROSCOPY

Hina Mushtaq, Mariam Raza*, Makki Muhammad, Tarique Arain***, Imran Khan****, Nighat Afridi******

Rehman Medical Institute, Peshawar Pakistan, *Pakistan Naval Ship Shifa Hospital, Karachi Pakistan, **Makki Diagnostic Centre Mardan Pakistan, ***Dow University of Health Sciences Karachi Pakistan, ****Combined Military Hospital Peshawar/National University of Medical Sciences (NUMS) Pakistan

ABSTRACT

Objective: To determine the frequency of malaria by polymerase chain reaction in malarial parasite negative samples on microscopy.

Study Design: Cross-sectional study.

Place and Duration of Study: Haematology department of Pakistan Naval Ship, Shifa Hospital, from Jan 2018 to Oct 2018.

Methodology: This study involved 150 adults of both genders with suspicion of malaria but negative malarial parasite on microscopy. Polymerase chain reaction was performed on blood samples from selected patients as per protocol. Outcome variable was the frequency of polymerase chain reaction positivity for malarial parasite. A pre-designed proforma was used to collect the data that was analyzed through SPSS version 20.0.

Results: The mean age of patients was 29.51 ± 8.73 years. The majority (75.3%) of the patients had undiagnosed fever. Only a small proportion of patients had hemolytic anemia (4.0%), splenomegaly (4.0%), acute renal failure (1.3%) and jaundice (1.3%). Polymerase chain reaction was positive in 3 (2.0%) cases. There was no significant difference in the frequency of positive polymerase chain reaction across underlying signs/symptoms except splenomegaly. Patients with splenomegaly had a significantly higher frequency of positive polymerase chain reaction (16.7% vs. 1.4%; p=0.009).

Conclusion: The frequency of positive polymerase chain reaction was found to be 2.0% in malarial parasite negative samples on microscopy. It was significantly higher among patients with splenomegaly.

Keywords: Malarial parasite, Microscopy negative samples, PCR diagnosis of malaria, Real-time PCR.

INTRODUCTION

Malaria is considered a perilous health problem with major prevalence in the developing part of the world. Globally around 3-4 million peoples are threatened to face this hazardous ailment.

World Health Organisation (WHO) reported 219 million cases of malaria globally (92% in Africa) and 435,000 deaths principally among children (61%) in the year of 2017[1]. Out of total cases, around 1.3 million cases reported in Mediterranean region[1]. Moreover, 5 countries of the developing world (Nigeria, India, Congo, Mozambique, and Uganda) are facing the main burden that comprises around 50% of worldwide malaria cases[1]. A total of 374,513 (P. Vivax: 84.0%, P. Falciparum: 14.9%) confirmed malaria cases have been reported in Pakistan in the year of 2018[2].

Complications related to malaria can only be avoided by the early diagnosis and commencement of effective therapy. Microscopy is regarded as gold standard for diagnosis[2,3]. It is an inexpensive, simple and economical method for parasite detection. However, this method is laborious, trained eye dependent and time-consuming. Its role is particularly questionable in its reliability of detecting low parasitemia lesser than 50 parasites /µl of blood or mixed infections[3-6]. Detection of low parasitemia is particularly significant in the screening of donors for blood transfusion in endemic areas where asymptomatic carriers are a risk for disease transmission for the recipients[7].

PCR has been introduced as a molecular diagnostic modality for malaria. Since the inception

Correspondence: Dr Hina Mushtaq, Department of Heamatology, Rehman Medical Institute Peshawar Pakistan
Received: 29 Feb 2019; revised received: 18 May 2020; accepted: 21 May 2020

of PCR based techniques for the diagnosis of malaria, several modalities have been developed. These methods have higher sensitivity in cases with low parasitemia and combined parasitic infection[8,9]. These methods were designed to create more accuracy in the diagnosis of malaria. Among these introduced methods, Real-time PCR has replaced the conventional PCR methods like nested and semi-nested[10]. The automated PCR method is simpler and rapid one with more specificity and higher sensitivity levels. One of the studies conducted in Bangladesh and Singapore showed sensitivity of Real-time PCR 95.2% with 98.1% specificity[11] and 94.1% sensitivity with 100% specificity for P. Falciparum respectively. Real-time PCR has the sensitivity to detect lower parasitemia levels that may be as low as 0.01 to 1 parasite/µl of blood[12,13] which makes it superior over microscopy in terms of diagnosis of malaria. Among the disadvantages, this method is comparatively expensive; it requires trained staff; and high uncontaminated technique. Although, its ability to detect low parasitemia overcomes the pitfalls[14].

In the available literature, very few studies exist that determine the frequency of PCR based diagnosis of malaria in microscopy negative cases. This study is designed to determine the significance of PCR based investigation to confirm the Malaria in symptomatically probable cases.

METHODOLOGY

This cross-sectional study was conducted at the Hematology department of PNS Shifa Hospital Karachi, from January 2018 to October 2018. Approval to conduct the study was taken from the ethics committee of the hospital. Written consent was obtained from each patient. This study included 150 adults with ages between 15 to 60 years, who were provisionally diagnosed as a case of malaria based on history and examination, but had negative malarial parasite on microscopy of blood smear. Non-probability consecutive sampling was employed for the selection of the patients. WHO calculator was used to determine the sample size, taking the sensitivity 95.2%[11], confidence level 95% with a margin of error 4%.

Samples were collected from indoor & outdoor patients. In the EDTA tube, a sample of venous blood (3ml) was obtained. DNA extraction was performed from the blood sample by using the DNA purification kit Gentra USA. The samples were collected as per the manufacturer's instructions. The amplification of the extracted DNA was done in batches of 36 samples along with positive and negative controls simultaneously. Real-time PCR was performed by employing the Taqman probe following the protocol as prescribed by Lee *et al*[5]. The Primers used were Genus specific, Gen Bank accession no M 19172 was used for Plasmodium Falciparum and X 13926 for P. Vivax. PCR was done by 25 pi reaction mixture which had DNA sample, DNA Taqman probe, PCR Mix and Primer mix. Realtime PCR amplification was done by thermal cycler Rotor-Gene Q-series software 1.7 (QIAGEN company). Denaturing was initially done at 95 degrees Celsius for 5 minutes. 40 cycles of denaturation at 95 degrees Celsius for 15 seconds and annealing at 60 degrees Celsius for 60 seconds were performed alternately. Preparation of positive control was done by pooling samples which were positive for Plasmodium vivax and Plasmodium Falciparum on microscopy. For negative control, samples from healthy, afebrile individuals who were negative on microscopy were used. To calculate the sensitivity, the cyclical threshold (c) of fluorescence was determined by serial dilutions. The result was interpreted by analysis of PCR quantization curves.

The data collected was entered into SPSS version 20 and analyzed. Age being a numerical variable presented as ± 2. While percentage and frequency were used to present categorical variables including gender, underlying signs and symptoms and positive or negative results on PCR. Data was arranged for age, gender, and underlying signs/symptoms. After stratification of data, a chi-square test was applied. A $p \leq 0.05$ was considered as significant.

RESULTS

The mean age of the patients was 29.51 ± 8.73 years, ranging from 15 years to 50 years. Majority 90 (60%) were >25 years. 117 (78.0%) males and 33 (22.0%) females were included in the study. Table-I outlines the age variable. The majority (75.3%) of the patients had undiagnosed fever followed by recurrent fever (7.3%) and vomiting (6.0%). Only a small proportion of patients had hemolytic anemia (4.0%), splenomegaly (4.0%), acute renal failure (1.3%) and jaundice (1.3%). 1 patient had hematuria (0.8%) as shown in table-II.

Table-I: Demographics of study participants.

Characteristics	Participants (n=150)
Age (years)	29.51 ± 8.73 (15-50)
Age Groups	
≤25 years	60 (40%)
>25 years	90 (60%)
Gender	
Male	117 (78%)
Female	33 (22%)

Table-II: Frequency of various underlying signs/ symptoms.

Underlying Sign/ Symptom	Frequency	Percentage
Undiagnosed Fever	113	75.3
Recurrent Fever	11	7.3
Vomiting	9	6.0
Hemolytic Anemia	6	4.0
Splenomegaly	6	4.0
Acute Renal Failure	2	1.3
Jaundice	2	1.3
Hematuria	1	0.8
Total	150	100

Table-III: Frequency of Malaria on PCR.

PCR Diagnosis of Malaria	Frequency	Percentage
Yes	3	2.0
No	147	98.0
Total	150	100

PCR was positive in 3 (2.0%) cases as shown in table-I. The positive PCR frequency across age (p=0.812) and gender (p=0.353) was not significantly different. Similarly, positive PCR frequency across underlying signs/symptoms was also not significantly different. Patients with splenomegaly had exception with a significantly higher frequency of positive PCR (16.7% vs. 1.4%; p=0.009). As shown in tables-IV & V.

DISCUSSION

This study was designed with objective to determine the frequency of malaria by PCR in patients with malarial parasite negative samples on microscopy. The targeted outcome variable was the frequency of PCR positivity for the malarial parasite.

In this study, 2% of the malarial parasite negative samples on microscopy were found positive on PCR, among them the significantly higher proportion of patients was with splenomegaly (16.7% with p-value 0.009). Our results

Table-IV: Frequency of Malaria on PCR across undiagnosed fever (n=150).

Underlying Sign/Symptom	PCR Diagnosis		p-value
	Malaria (n=3)	No Malaria (n=147)	
Fever Not diagnosed (n=113)	2 (1.8%)	111 (98.2%)	0.725
Others (n=37)	1 (2.7%)	36 (97.3%)	
Total	3 (2%)	147 (98%)	

Chi-square test, Insignificant statistical difference

Table-V: Frequency of Malaria on PCR across Spleenomegaly (n=150).

Underlying Sign/Symptom	PCR Diagnosis		p-value
	Malaria (n=3)	No Malaria (n=147)	
Splenomegaly (n=6)	1 (16.7%)	5 (83.3%)	0.009*
Others (n=144)	2 (1.4%)	142 (98.6%)	
Total	3 (2%)	147 (98%)	

Chi-square test, *Insignificant statistical difference

are comparable with another local study by Iqbal *et al*[15] (2014) who reported that 3% of patients with negative malarial parasite on microscopy were found positive on PCR. Nesha *et al*[16] (2018) conducted a study on a comparatively bigger sample size of 2333 patients also reported 3.3% more cases by PCR method[16]. Wang *et al*[17] (2014)

reported a frequency of 2.47% more patients among the Korean population-based study. Nicastri *et al*[18] in 2009 (2.63%) in Africa, Coleman *et al*[19] in 2006 (1.17%) in Thailand, Rodulfo *et al*[20] in 2007 (0.49%) in Venezuela also observed a similar frequency of PCR positivity in microscopy negative samples. But few others observe higher frequencies of positive PCR cases. Johnston *et al*[21] (2006) observed this frequency to be 9.09% in the USA. Ojurongbe *et al*[22] (2013) and Mawili-Mboumba *et al*[23] (2013) observed a much higher frequency and reported it to be 17% and 23% respectively among African patients. Billy *et al*[24] (2019) detected a difference of 10% between the two methods. This conflict among studies can be a result of the difference in personal skills as microscopy is purely an operator-dependent technique. Moreover, researchers use different modalities of the PCR method that may impact on the outcome of the studies.

There are very few studies conducted in the Pakistani population to determine the number of cases missed during routine workup but deemed as a malaria case. Such cases pose a diagnostic challenge to a physician and lead to treatment failure due to improper management. It leads to dissatisfaction on the part of the patient and poses an economic burden over the society. Although in this region vivax related malaria is prevalent and it considered having benign outcomes, but few cases of cerebral malaria are also reported with a background of Vivax specie[25]. So the possibilities of life-threatening morbidities can't be ruled out with benign considering malaria. These findings made it prudent to diagnose malaria in earlier stages to avoid the adverse consequences of this disease.

Although the overall positive rate was quite low to conclude using the PCR technique in all microscopy negative patients. But considering the morbidities and mortalities associated with malaria, it can be advocated that in patients where microscopy negative patients with strong clinical suspicion of malaria; PCR should be considered to confirm the diagnosis. The result of this study also reflects that the symptomatic patients with splenomegaly have more probability of malaria infection. So in such a patient PCR method should be performed for accurate diagnosis of Malaria.

This study has a limitation that, it is conducted on a small sample size of 150 cases in a single center. So the conclusion drawn from this study should be validated in a multicenter study with larger sample size.

ACKNOWLEDGEMENT

We thank all patients for donating blood samples. We also thank the laboratory staff of PNS Shifa for their unconditional technical assistance in the conduction of study.

CONCLUSION

The PCR method may be used in Microscopy negative symptomatic patient to avoid delay in the diagnosis and to avoid morbidities associated with malaria.

Disclaimer

The views mentioned in this article and the conclusion drawn from the article is those of the authors. This article does not reflect the official policy of hospital.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

REFERENCES

1. World Health Organization. ((2018 World malaria report . World Health Organization. [Internet]. Geneva: World Health .2018 Available from .[14 cited Jul] 2018 ;Organizationhttps://apps. who.int/iris/handle/10665/275867.9789241565653_ eng.pdf
2. Pakistan Malaria Annual Report 2019. (2019). [online] Available at: http://mc.gov.pk/documents/pdfs/Pakistan%20Malaria %20Annual%20Report%202019%20(002).pdf
3. Baird JK, Valecha N, Duparc S, White NJ, Price RN. Diagnosis and treatment of plasmodium vivax malaria. Am J Trop Med Hyg 2016; 95(Suppl-6): 35–51.
4. Das S, Jang IK, Barney B, Peck R, Rek JC, Arinaitwe E, et al. Performance of a high-sensitivity rapid diagnostic test for plasmodium falciparum malaria in asymptomatic individuals from uganda and myanmar and naive human challenge infections 2017; 97(5): 1540–50.
5. Osoga J, Waitumbi J, Guyah B, Sande J, Arima C, Ayaya M, et al. Comparative evaluation of fluorescent in situ hybridization and Giemsa microscopy with quantitative real-time PCR technique in detecting malaria parasites in a holoendemic region of Kenya. Malar J 2017; 16(1): 1–7.

6. Donald W, Pasay C, Guintran JO, Iata H, Anderson K, Nausien J, et al. The utility of malaria rapid diagnostic tests as a tool in enhanced surveillance for malaria elimination in Vanuatu. PLoS One 2016; 11(11): 1-14.
7. Zakeri S, Kakar Q, Ghasemi F, Raeisi A, Butt W, Safi N, et al. Detection of mixed Plasmodium falciparum and P. vivax infections by nested PCR in Pakistan, Iran, and Afghanistan. Indian J Med Res 2010: 132: 31-35.
8. Vincent JP, Komaki-Yasuda K, Iwagami M, Kawai S, Kano S. Combination of PURE-DNA extraction and LAMP-DNA amplification methods for accurate malaria diagnosis on dried blood spots 11 Medical and Health Sciences 1108 Medical Microbiology. Malar J 2018; 17(1): 1–7.
9. Echeverry DF, Deason NA, Davidson J, Makuru V, Xiao H, Niedbalski J, et al. Human malaria diagnosis using a single-step direct-PCR based on the Plasmodium cytochrome oxidase III gene. Malar J 2016; 15(1): 1–12.
10. Komaki-Yasuda K, Vincent JP, Nakatsu M, Kato Y, Ohmagari N, Kano S. A novel PCR-based system for the detection of four species of human malaria parasites and Plasmodium knowlesi. PLoS One 2018; 13(1): 1–15.
11. Klian SA, Ahmed S, Mushahid N, Anwer M, Saeed S, Khan FA, et al. Comparison of real time polymerase chain reaction with microscopy and antigrn detection assay for the diagnosis of Malaria. J Coll Physicians Surg Pak 2013; 23(11): 787-92.
12. Wanja EW, Kuya N, Moranga C, Hickman M, Johnson JD, Moseti C, et al. Field evaluation of diagnostic performance of malaria rapid diagnostic tests in western Kenya. Malar J 2016; 15(1): 1–10.
13. Mfuh KO, Achonduh-Atijegbe OA, Bekindaka ON, Esemu LF. A comparison of thick-film micro-scopy, rapid diagnostic test, and polymerase chain reaction for accurate diagnosis of Plasmodium falciparum malaria. Malar J 2019; 18(1): 1-8.
14. Mokuolu OA, Ntadom GN, Ajumobi OO, Alero RA, Adedoyin OT. Status of the use and compliance with malaria rapid diagnostic tests in formal private health facilities in Nigeria. Malar J 2016; 15(1): 1–11.
15. Iqbal A, Mushtaq R, Hussain F, Saleem M, Naz M, Hashmi BK. Comparison of conventional and PCR based detection of Plasmodium falciparum and Plasmodium vivax infection in human blood. JAB 2014; 3(3): 248-51.
16. Siwal N, Singh US, Dash M, Kar S, Rani S, Rawal C, et al. Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by plasmodium falciparum and p. vivax in India. PLoS One 2018; 13(3): 1–15.
17. Wang B, Han SS, Cho C, Han JH, Cheng Y, Lee SK, et al. Comparison of microscopy, nested-PCR, and Real-Time-PCR assays using high-through put screening of pooled samples for diagnosis of malaria in asymptomatic carriers from areas of endemicity in Myanmar. J Clin Microbiol 2014; 52: 1838-45.
18. Nicastri E, Bevilacqua N, Sañé Schepisi M, Paglia MG, Meschi S, Ame SM, et al. Accuracy of malaria diagnosis by microscopy, rapid diagnostic test, and PCR methods and evidence of antimalarial overprescription in non-severe febrile patients in two Tanzanian hospitals. Am J Trop Med Hyg 2009; 80: 712-17.
19. Coleman RE, Sattabongkot J, Promstaporm S, Maneechai N, Tippayachai B, Kengluecha A, et al. Comparison of PCR and microscopy for the detection of asymptomatic malaria in a Plasmodium falciparum/vivax endemic area in Thailand. Malar J 2006; 5: 121.
20. Rodulfo H, De Donato M, Mora R, González L, Contreras CE. Comparison of the diagnosis of malaria by microscopy, immunochromatography and PCR in endemic areas of Venezuela. Braz J Med Biol Res 2007; 40: 535-43.
21. Johnston SP, Pieniazek NJ, Xayavong MV, Slemenda SB, Wilkins PP, da Silva AJ. PCR as a confirmatory technique for laboratory diagnosis of malaria. J Clin Microbiol 2006; 44: 1087-89.
22. Ojurongbe O, Adegbosin OO, Taiwo SS. Assessment of clinical diagnosis, microscopy, rapid diagnostic tests, and polymerase chain reaction in the diagnosis of plasmodium falciparum in Nigeria. Malar Res Treat 2013; 2013: 308069.
23. Mawili-Mboumba DP. Karine Bouyou-Akotet M, Mbouoronde CO, Kombila M. Analysis of malaria diagnosis discrepancies between RDTs and microscopy by nested PCR. J Biomed Sci Eng 2013; 6: 967-72.
24. Ngasala B, Mutemi DD, Mwaiswelo RO. Diagnostic performance of malaria rapid diagnostic test and microscopy compared with PCR for detection of plasmodium falciparum infections among primary schoolchildren in kibiti district, eastern Tanzania: An Area with Moderate Malaria Transmission. Am J Trop Med Hyg 2019; 101(4): 809–11.
25. Mukhtar MM, Eisawi OA, Amanfo SA, Elamin EM, Imam ZS, Osman FM, et al. Plasmodium vivax cerebral malaria in an adult patient in Sudan. 2019; 18(1): 316.