ORIGINAL ARTICLE

Accuracy of Blood Spots Nested PCR Method for the Detection of Malaria in Clinical Suspects of Lahore, Pakistan

SAIMA NAZ MOHSIN[1], SABA KHALIQ[2], MOHAMMAD YASEEN BAZAI[3], *ABREEN MUDDASSIR[4], MEHAK FATIMA[5], AMIR RAZI[6]*

[1]Research Officer/Centre In-charge, [5]Trainee Research Assistant, PHRC Research Centre NHRC, ShaikhZayed Medical Complex Lahore
[2]Associate Professor, Department of Physiology and Cell Biology, University of Health Sciences, Lahore
[3]Assistant Professor, Department of Biochemistry, Bolan University of Medical & Health Sciences Quetta
[4]Senior Registrar, Department of Medicine, ShaikhZayed Medical Complex Lahore
[6]Assistant Professor, Lahore Business School, The University of Lahore, Lahore
Correspondence to: Dr. Amir Razi, Email: amir.razi@lbs.uol.edu.pk, Cell: 0321-8842263

ABSTRACT

Background: In developing countries, like Pakistan,malaria surveillance and control program poses substantial challenges for health care providers due to low quality diagnosis and limited resources. There is a desperate need to highlight the actual cases of malaria in the green zones (malaria eliminated zone) of Pakistan by using a more sensitive technique for malaria detection than microscopic method.

Aim: To compare the sensitivity and specificity of microscopic method and blood spot nested PCR method for the detection of malaria in clinically suspected cases.

Study design: Cross-sectional validation study

Place and duration of study: National Health Research Complex, Shaikh Zayed Medical Complex and University of Health Sciences Lahore from 1st March 2019 to 30th November 2019.

Methods: A total of 150 blood samples and blood spots were collected from suspected malaria cases during the peak malaria season from Lahore, Pakistan, and categorized into clinical suspects and healthy individuals based on early symptoms. These samples were subjected to microscopic detection of the malaria parasite followed by nested PCR based on dried blood spots collected on filter paper. The results were compared with a standard nested PCR from whole blood samples to find out accuracy of thedried blood-spot method.

Results: Nested PCR method for malarial parasite detection from dried blood spot was found to be more sensitive and specific than the microscopic method in detection of malaria in suspected cases.

Conclusion: Our study has highlighted false negative cases of malaria from the areas of Lahore, where malaria has been declared eliminated, which raises the need for the use of sensitive techniques for detection of malaria in endemic zones to avoid the unnecessary disease burden.

Keywords: Malaria; Nested PCR; Plasmodium; Pakistan; Malaria Surveillance Program

INTRODUCTION

Malaria is one of the most prevalent vector-borne diseases of all times and poses threat to economic growth due to the higher rate of mortality associated with it, particularly in malaria-endemic developing countries with an estimated 216 million clinical cases and 445,000 deaths globally during 2016. Most of the cases in 2016 were reported in the WHO African Region (90%), followed by the WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%). Approximately, 863,000 deaths by malaria were reported in 2008 out of a total of 243 million cases, but this rate has been decreased to 459,000 deaths and 210 million reported cases throughout the world with the majority of cases from Africa in 2014. In 2015, it was estimated that 446,000 deaths had occurred due to malaria globally.A similar levels of deaths were estimated in 2016 i.e., 445,000 deaths globally of which 407,000 deaths (approximately 91%) were in only the WHO African Region[1].

Malaria is transmitted by the female species of Anopheles mosquitoes,and in Pakistan its transmission is dependent on two Anopheles species i.e., culicifacies and stephensi[2]. It is caused by Plasmodium, a unicellular parasitic organism, living within the midgut of mosquitoes. There are six different species of Plasmodium which can cause malaria i.e. Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale and Plasmodum knowlesi; while, Pknowlesi has been misinterpreted as P.malariae in the past[1,3,4]. The confirmed reported of malaria cases in Pakistan was 202,013 in 2015[5]. In Pakistan, the most prevalent Plasmodium species is vivax followed by falciparum, which has been reported from Balochistan and Sindh only. Overall, the prevalence of Plasmodium vivax compared to Plasmodium falciparum has increased over the past few years in Pakistan[6]. Pakistan reported 10% of the total worldwide cases of P. vivaxin 2015, and 11% of worldwide deaths due to P. vivax[5].

Despite the epidemic episodes and associated mortality and morbidity in endemic zones, the global incidence of malaria is declining, probably by the establishment of worldwide Malaria Control Programs, but this incidence remained unaffected by a slight change in Pakistan, India, and Bangladesh. Pakistan is still a higher risk population for malaria infection with about 80% of the population living in the endemic zone[7]. In Pakistan, approximately half million cases are still being reported annually and approximately 12% of the rural population carry malarial parasites without an apparent symptom of malaria, making it an alarming situation that must be

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resolved immediately with more rapid and sensitive diagnosis method[8].

In most of the malaria endemic zones, microscopy is the traditional method of choice for the detection of malarial parasite due to its easy access and fewer expenses. However, it is a more challenging method in terms of technical errors and expertise needed for accurate detection and reporting of positive cases.[7] Moreover, the factors like identification of individual parasite detection on the slide, expertise of microscopist, preparation of slides along with quality assurance for detection, affects the sensitivity of the microscopic method for the detection of malaria. Approximately 10 to 30 parasites per ml of blood smear can be detected accurately providing an expert examination of the slide with maximum quality assurance[9]. Unfortunately, this level is not maintained in endemic regions especially during outbreaks due to more samples and time constraints. Thus, misinterpretation at the species level is common, especially in mixed parasite cases and low parasitemia leads to under-reporting of actual cases[10,11]. Moreover, discrepancy of results/methods among laboratories led to misinterpretation of the actual results such as lack of expertise, reusing contaminated slides, artifacts and lack of quality assurance.[12] Some of these limitations have been overcome by adaptation of other methods, including immune-chromatographic identification of antigens which is an easy method but cannot detect *P.malariae*and*P.ovale.*[13]In recent decades, the availability of polymerase chain reaction (PCR) assays had led to the establishment of a rapid and sensitive test for identification of malaria parasite from the blood; even with a low parasitemia of one parasite per ml of blood.[14,15] Since, malaria is endemic in underdeveloped and often remote areas of the world, therefore, to maintain high specificity and sensitivity of PCR based assays, these methods must be coupled with simple sampling and DNA extraction methods. The nested PCR assay is developed as a method suitable to detect even low parasitic load more accurately which can be used to screen remote areas of the world when coupled with a simple sampling method of dried blood spot on filter paper, and the detection can be done even after 3 to 6 years of collection[13,16-18].

In developing countries, like Pakistan, malaria surveillance and control program poses substantial challenges for health care providers due to poor diagnosis and limited resources. A malaria control program in Pakistan has included 99 districts of the total 156 districts in the green zone (malaria eliminated zone), which has highlightedfor substantially eliminating malaria, including Lahore[19]. Despite the inclusion of several districts in the green zone by malaria control program of Pakistan, the appearance of clinical symptoms for malaria in suspects highlighted the need of validation of detection method for malaria parasites in endemic zones which are declared safe. Thus, this study was carried out to get an insight into the actual picture of disease burden in Lahore, Pakistan, due to the high number of clinical cases that have symptoms of malaria and to establish the facility of dried blood spot testing for the detection of malaria parasite through nested PCR.

METHODOLOGY

This Cross-sectional Validation Study (for the validation of Blood spot technique by PCR) was conducted at a public research center in collaboration with a public research institute of Pakistan for 15 months. For method validation study 100 paired samples (EDTA whole blood+ finger-prick blood spots on filter paper) were collected from clinically suspected cases of malaria. Fifty healthy individuals without any clinical history of malaria at the time of sample collection were also included in this method validation study. It was ensured that these healthy individuals did not get malaria during the last six months prior to sample collection.

Febrile patients (all age groups) presenting symptoms of malaria (temperature >37.5°C, headache, chills) at malaria clinics were included in the study and were termed as suspected cases of malaria, whereas febrile patients with any other co-morbid infections (HCV, HBV & HIV) and those who had received anti-malarial drugs during the past four weeks and critically ill patients unable or unwilling to provide a blood sample were excluded from the study.

After getting informed consent 5ml blood was collected through venipuncture in two EDTA vials. One vial was used for complete blood count (CBC) and for thick and thin blood films preparation. From the other vial parasite DNA was extracted to be used for nested PCR for the detection of malaria parasite from whole blood samples using DNA extraction kit (Favorgen, Taiwan).

A duplicate finger-prick blood samples from each individual were spotted onto labeled Whatman filter paper. Blood spots on filter paper were allowed to air-dry and were kept in plastic bags with desiccator and stored at room temperature until DNA extraction (Favorgen, Taiwan) followed by PCR.

Thick and thin blood films were stained with Giemsa. The blood films were then dispatched to the experienced microscopist (having more than 20 years experience as consultant hematologist) in a private lab and were screened for malaria parasites by microscopy with (100×) oil immersion lens. The microscopist was blinded to PCR result. Blood smears were considered negative if no parasite was seen in 100× oil immersion fields in a thick blood film. Thin blood smears were used to confirm the presence of malarial parasite, if any.

The DNA of each isolate was extracted from dry blood spots usingGF-1 Blood DNA Extraction Kit (Vivantis, CA, USA) according to manufacturer's protocol.Plasmodium species (*P.vivax, P. falciparum, P.ovale* and *P.malariae*) were detected using nested polymerase chain reaction (PCR) amplification of 18s ribosomal RNA gene, as described by Buppan et al[20].

Qualitative variables like gender, socioeconomic status, education and clinical presentation like (headache, lassitude, fatigue, abdominal discomfort, muscle and joint pain, fever, chills, perspiration, anorexia, vomiting, and worsening malaise) were presented as frequencies and percentages. The diagnostic performance of microscopy of thick and thin films and dried blood spot sampling technique for the detection of malaria parasite through nested PCR was evaluated in comparison with the results of the nested PCR method using whole blood samples through venipuncture.Performance indices were the

number of true positive (TP), the number of true negatives (TN), the number of false positive (FP) and the number of false negatives (FN). Sensitivity was expressed as TP/ (TP + FN) and specificity as TN/(TN + FP). The accuracy of the tests was calculated as (TP/TN) /number of all tests.

RESULTS

For this study a sum total of 150 individuals were registered, 100 cases were clinical suspects of malaria categorized on the basis of the appearance of early symptoms such as fever, chills, headache and 50 healthy individuals for method validation were selected. In all suspected malaria cases, whether they were declared as positive or negative for malaria parasite, later on, early symptoms of malaria were common, such as fever, chills, headache, malaise, and muscle & joint pain for which they were classified as clinical suspects (Table 1).

The majority of the participants of the study were male with 57% and 76% of clinical suspects and healthy individuals respectively, while females constituted 43% and 24% of clinical suspects and healthy group, respectively. These groups were further described on the basis of their socioeconomic status and educational level with overall, 41% participants from the middle class and 41% from lower class whereas 18% were from the upper-class category. Most of the individuals in both groups were classified as holding at least an intermediate level education with the majority having up to masters level education and there was a low ratio of below matric or uneducated individuals (Table 2).

For finding out the sensitivity and specificity of our method of extraction of parasite from dried blood spot and its confirmation by nested PCR method, we run a parallel standard nested PCR from whole blood samples of the individuals that were picked in duplicate as blood spots and whole blood for the microscopic slide preparation and standard nested PCR from the same individual at a single time. The results that were obtained from microscopic slides and dried blood spot based nested PCR were compared with a standard nested PCR method for finding out the sensitivity and specificity of each method using formula mentioned in Table 3.

Of total 150 study participants, 49 individuals were tested positive for malaria parasite when diagnosed at the microscopic slide and standard nested PCR, while 13 individuals were classified as false negative on the microscope that was confirmed as positive when they were subjected to standard nested PCR. There was also a single case that was reported as false positive from microscope where actually having no parasitemia. Thus, the sensitivity of the microscopic detection method for malaria parasite was found to be 79% and specificity of this method was 98.86%. On the other hand, there were comparable results obtained from our method of dried blood spot based nested PCR with about 97% sensitivity and 100% specificity. All the cases that were tested positive from dried blood spot based nested PCR were confirmed as positive from standard nested PCR except 2 which were categorized as negative from dried blood spot due to low parasitemia and a slight band for the product in the agarose gel probably attributed to a lower copy number of templates. There were no false positives detected by dried blood spot based nested PCR method as was found by microscopy. Thus, based on our results it was confirmed that dried blood spot based nested PCR method is more accurate with a higher sensitivity and specificity than microscopy and can be opted as a preferable clinical method for detection of malarial parasites in malaria endemic zones and especially where it is required to collect and transport samples from far-flung areas.Furthermore, 62 cases that were reported as positive as detected by standard and dried blood spot based nested PCR were located according to their distribution in different areas of Lahore and its vicinity (Table 4). All these cases were distributed in the main city of Lahore which indicated the possibility of other such cases that are misinterpreted in the vicinity of Lahore and other areas in Punjab.

Table 1: Clinical presentation of suspected cases of malaria from Lahore, Pakistan (n=100)

Symptoms	Clinically suspected cases		
	PCR +ve /M – ve N=13 (%)	PCR +ve/M +ve n=49 (%)	PCR –ve / M – ve N=38 (%)
Headache	11 (84.6)	15 (30.6)	22 (57.8
Lassitude	7 (53.8)	3 (6.2)	10 (26.3)
Fatigue	11 (84.6)	15 (30.6)	9 (23.6)
Abdominal discomfort	5 (38.5)	7 (14.2)	11 (28.9)
Muscle & Joint Pain	7 (53.8)	26 (53.0	16 (42.1)
Fever	13 (100)	49 (100)	38 (100)
Chills	13 (100)	45 (91.8)	27 (71.0)
Respiration	9 (69.2)	23 (46.9)	12 (31.5)
Anorexia	7 (53.8)	30 (61.2)	13 (34.2)
Vomiting	7 (53.8)	19 (38.7)	16 (42.1)
Worsening Malaise	5 (38.5)	30 (61.2)	11 (28.9)

Table 2: Demographic data of all study participants from Lahore Pakistan (n = 150)

Variable	Suspected cases of malaria (n = 100)	Healthy control (n = 50)
Gender		
Male	57 (57%)	38 (76%)
Female	43 (43%)	12 (24%)
Socioeconomic status (based n monthly income		
≤20000	42 (42%)	19 (38%)
20001-50000	44 (44%)	17(34%)
>50000	14 (14%)	14 (28%)
Education		
Masters	17 (17%)	14 (28%)
Bachelors	13 (13%)	11 (22%)
Intermediate	14 (14%)	7 (14%)
Matric	23 (23%)	7 (14%)
Primary	13 (13%)	6 (12%)
Illiterate	20 (20%)	3 (6%)

Table 3: Comparison of malaria detection by Microscopy and Filter Paper Based Nested PCR Methods with Standard Nested PCR Using Whole Blood

Microscopy	Nested PCR		Total
	+ve	-ve	
+ve	49	1	50
-ve	13	87	100
Total	62	88	150

Sensitivity =TP/(TP+FN) X 100 = 79.03%
Specificity = TN/(TN+FP) X 100 = 98.86%

Filter paper based Nested PCR	Nested PCR		Total
	+ve	-ve	
+ve	60	-	60
-ve	2	88	90
Total	62	88	150

Sensitivity =TP/(TP+FN) X 100 = 96.77%
Specificity = TN/(TN+FP) X 100 = 100%

Table 4: Distribution of malaria cases in Lahore and its vicinity

Location	PCR +ve/M +ve	PCR +ve/M –ve	Total cases
Shahdara	7	1	8
Wafaqi Colony	2	1	3
Johar Town	5	1	6
Sabzazar	6	1	7
NarangMandi	6	-	6
Sodiwal	4	-	4
Ichra	3	1	4
Other non-specific areas of Lahore	16	8	24
Total	49	13	62

DISCUSSION

For successful eradication of malaria, an optimum diagnostic method with specificity and sensitivity to avoid misinterpretation and consequent recurrence of malaria are required. To combat this socioeconomic dilemma, various malaria control programs have been established in endemic countries, including Pakistan.[1] A malaria control program in Pakistan has included 99 districts of the total 156 districts in the green zone, which was selectedfor substantially eliminating malaria, including Lahore.[19] This study was carried out to get an insight into the actual picture of disease burden in Lahore due to the high number of clinical cases that have symptoms of malaria but tested negative by the traditional detection method.

The present study showed that the detection of malaria parasites by nested PCR method is more specific and sensitive than microscopy. In our study, we have presented a simple method for blood collection on a filter paper as dried blood spot and coupled it with a nested PCR assay for diagnosis of malaria. The primary purpose of selecting this method was the collection of epidemiological data during malarial outbreaks from urban areas.Furthermore, this method was important to introduce an effective plan for a more sensitive and rapid diagnosis of malaria during routine clinical diagnosis at distantly located but established diagnostic centers in developing countries due to the stability of samples collected as dried blood spots on filter paper. Hwang et al[18] in 2012 reported successful detection of P. *falciparum* parasite from dried blood spots archived after six years of sample collection with a decreased sensitivity of only those samples which were collected ten years prior to testing. These results are also supported by Tham et al[17] who assessed malaria parasites by PCR assay immediately after blood spot collection and verified the results three years after initial assays with the same specificity and sensitivity for detection.

Microscopy being a gold standard method for the detection of the malaria parasite and with fewer expensesispreferred in laboratory settings in Pakistan, but it demands technical excellence and found responsible for false reporting in many cases.[21] In this study, PCR based method has been proved superior to microscopy, it was not only effective to confirm microscopically confirmed malaria cases, but also rectified positive cases of malaria that were microscopically overlooked as negative. Such false negative samples can lead to drastic consequences on patient health due to delay in medication and availability of carriers, which can lead to episodes of infection in healthy individuals[22].

Depending upon the results of our study, we can suggest thatnested PCR method for detection of malarial parasite combined with dried blood spots collection method is more sensitive and more specific (sensitivity 96.77%, specificity 100%) than microscopic method (sensitivity 79.03%, specificity 98.86%). Our results are consistent with prior studies conducted in Singapore[17], Greece[23], Turkey[24], Tanzania[25], Thailand[26], Saudi Arabia[27], Ethiopia[28,29], Brazil[30], Iran[31] and Sudan[32], in which a comparison of the microscopic method with PCR was done for malaria detection and had postulated the superiority of PCR in sensitivity and specificity than microscopic method. Although these false negative results are attributed to a low parasitic load oflessthan 20 parasites per microliter[33] but can generate a troublesome situation in endemic zones by leaving malaria patients without suitable treatment thus can halt the progress of the “Malaria Control Program”. This condition is particularly alarming and our findings have raised a question on the strategies of “National Malaria Control Program Pakistan” and “Roll Back Malaria program” which has declared the Province of Punjab as a green zone with substantially lower cases of malaria[19]. Our study has highlighted a number of false negative reports encountered in the vicinity of Lahore (Table 4) and there must be more in the region which should be detected by some sensitive technique rather than the traditional microscopic method to avoid false reporting which can bring further challenges to malaria control. False negative cases could serve as a reservoir for recurrence of malaria, thus misinterpretation of malarial cases would be dangerous and a challenge to malaria control programs. Our data have highlighted the question whether malaria is controlled or compromised in the developing countries, as most of the detection of malaria cases are in developing countries is carried out by traditional microscopy[21], rather than advance and sensitive detection methods. This situation,ifexists in other endemic zones, can result in the devastating growth of resistant strains of malaria, which will directly take the toll by increasing mortality.

The limitation of this study was a small sample cohort due to limited funds availability. However, we warrant a multicenter study with a nationwide cohort in the near future to further substantiate our fact that malaria detection is compromised in developing countries leading to underestimation of malaria cases instead of elimination.

CONCLUSION

In light of the findings of our study, it can be concluded that the number of malaria cases in Pakistan is underestimated. This situation is particularly alarming if exists in other endemic zones, thus such studies are required to be established in malaria-eliminating countries to avoid the misinterpretation and associated burden of the disease. Moreover, this study is an indication of the implication of rapid and sensitive techniques for the detection of malaria instead of the traditional method in clinical setups of developing countries.

RECOMMENDATIONS

1. A multicenter study must be initiated with a large cohort of malaria suspects, which must be taken into consideration from different areas of Pakistan, to validate the need of the malaria control program to be offered in green zones of Pakistan rather than declaring it as the malaria-eliminating zone.
2. Where possible the traditional microscopic method must be used carefully and combined with more sensitive and specific method of determination, such as nested PCR assay coupled with dried blood spots as has been validated in this study. To implement this strategy initially, reference labs can be established to cover the workload of urban areas. We recommend that dried blood sampling technique is best for this purpose.
3. If microscopic detection of malaria is the only method which can be offered at a particular laboratory than specific training must be given to experts to avoid false reporting to compensate the cost of misinterpretation and a higher disease burden.
4. The suspected cases that are confirmed for malaria parasite must be treated with proper medication and being followed up for completion of the course to avoid self-discontinuation of the drug by the patient and associated chances of drug resistance.

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