

A PCR method based on 18S rRNA gene for detection of malaria parasite in Balochistan

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Abstract

Objective: To establish a polymerase chain reaction method based on 18S ribosomal ribonucleic acid gene for the detection of plasmodium deoxyribonucleic acid in patients suffering from malaria symptoms.

Methods: This cross-sectional study was conducted from September 2013 to October 2014 in district Quetta of Pakistan's Balochistan province. Blood samples were collected from patients suffering from general symptoms of malaria. A polymerase chain reaction-based technique was applied for the diagnosis of malaria and detection of responsible species in the patients who were suspected to carry the parasite. Performance of this polymerase chain reaction method was compared against the microscopy results. Parasite number was also calculated for microscopy positive samples. All samples after the genomic deoxyribonucleic acid isolation were subjected to polymerase chain reaction amplification and agarose gel electrophoresis.

Results: Of the 200 samples, 114(57%) were confirmed as positive and 86(43%) as negative for malaria by microscopy. Polymerase chain reaction identified 124(62%) samples as positive and 76(38%) as negative for malaria. The comparative analysis of both diagnostic methods confirmed 109(54.5%) samples as positive by both techniques. Besides, 5(6.58%) samples were identified as false positive and 15(12.1%) samples as false negative by polymerase chain reaction. Sensitivity, specificity and positive predictive values for polymerase chain reaction in comparison to microscopy were 87.98%, 93.42% and 96%, respectively.

Conclusion: Polymerase chain reaction-based methods in malaria diagnosis and species identification were found to be more effective than other techniques.

Keywords: Microscopy, Malaria, Polymerase chain reaction (PCR), Plasmodium vivax, Plasmodium falciparum. (JPMA 66: 1587; 2016)

Introduction

The parasitic malaria disease is transmitted through mosquito bites. The common malaria symptoms are fever, chills and flu-like sickness. This might lead to severe complication and death if not properly treated. Approximately 198 million cases of malaria arose globally in 2013, resulting in 500,000 deaths. An estimated 3.4 billion people in the world are still living at risk of malaria. Being a major threat to the life of human, this deadliest vector-borne infection is considered as the king of diseases.¹

Four major species of unicellular Apicomplexan parasite belonging to genus Plasmodium are responsible for causing malaria in humans. They include Plasmodium falciparum, P. vivax, P. ovale and P. malariae. Among all species, P.falciparum is the deadliest one.^{1,2} Malaria is also one of the major causes of morbidity and mortality in

Pakistan. It is prevalent in all provinces; the highest prevalence was seen in Balochistan with 37% verified cases near areas bordering Iran and Afghanistan.³ Seasonal variation in the incidence of malaria pattern is seen with high infection rates during summer season while mild to moderate rate is seen during winter season.^{4,5} P.vivax was found highly prevalent species in the country with a history of 64% infections and P.falciparum as the second-most prevalent species having 36% infections. Furthermore, recent findings indicate an alarming situation with increase in incidence and mortality rates due to P.falciparum.^{3,5} No malaria case with P.ovale and P.malariae has been recorded yet.^{4,6}

Malaria still remains a foremost and emergent threat to the public health and development of the country despite efforts to control and eradicate it.⁷ Moreover, lack of accurate diagnosis, failure in disease management and vector control are main factors that contribute in malaria transmission.⁵ However, precise and timely diagnosis is the key for the effective management and control of malaria. In resource-limited countries and also in developed countries which are facing malaria as an extensive burden, there is a need to develop efficient

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diagnostic strategies to deal with this problem.⁸

Microscopy is a conventional method for the diagnosis of malaria. Plasmodium presence is detected from thick smears while species are identified from thin smears.^{9,10} Although microscopy is recognised as the gold standard of malaria diagnostic procedure, but it is time-consuming, laborious and requires highly trained and expert healthcare workers. The most important shortcoming of microscopy is low sensitivity in identification of species accurately at low parasitaemia level.¹¹ Rapid diagnostic test (RDT) is an immunochromatographic assay that is used to detect malaria antigen in a small amount of blood.¹² This diagnostic assay also has its limitations like false identification and low sensitivity. Malaria misdiagnosis and false identification can lead to problems like lengthened and deteriorative illness. Patients are exposed to unwanted side effects of drugs and true cause of disease remains hidden that may have a potential life threat.¹³

Several polymerase chain reaction (PCR)-based techniques have been developed for routine clinical malaria diagnosis, e.g. conventional and real-time assays, which not only sufficiently discriminate all four species of Plasmodium but sensitive enough to detect even one parasite / μ l of patient's blood. Therefore, PCR has proved to be more accurate and sensitive than routine microscopic detection which can detect 10-30 parasites / μ l of blood.^{14,15} The 18S ribosomal ribonucleic acid (rRNA) gene is considered to be an ideal target for PCR-based identification of Plasmodium and its species. This gene is a combination of conserved and variable regions that are totally absent in the human genome.¹⁶ In Balochistan, malaria has become a life-threatening issue and there is a need for the development of advanced techniques for its diagnosis. Only non-molecular techniques like RDT and microscopy are used for routine malaria diagnosis in Balochistan. Limitations in these techniques have been proven from studies all around the world. Molecular techniques like PCR-based assays are used all around the world and are considered more accurate and sensitive for the diagnosis of malaria.^{14,15}

The current study was planned to assess and evaluate the accuracy of the PCR assay in the malarial parasite detection and related species identification in the province. This study can prove to be a milestone for the establishment of modern techniques for accurate diagnosis of malaria and can be useful in the management and control of malaria in Balochistan.

Materials and Methods

This cross-sectional study was conducted in district

Quetta, Pakistan's Balochistan province, from September 2013 to October 2014. Approval was obtained from the ethical committee of the Balochistan University of Information Technology, Engineering and Management Sciences (BUITEMS). Convenient sampling technique was used. Whole blood samples were collected from patients who were suffering from malaria symptoms like chills, fever, vomiting, and body and muscle pain. These patients were sent by medical practitioners to different diagnostic laboratories and hospitals in Quetta. Data regarding age, gender and symptoms of the patients was collected with the help of a proforma. Blood obtained from healthy patients with no history of plasmodium infection was used as negative control.

For each sample, 3-5 ml of venous blood was collected in a sterile tube and it was mixed gently with 0.5 mM of ethylenediaminetetraacetic acid (EDTA) to prevent blood coagulation. This blood was stored at -20°C for further analysis. Freshly extracted blood was used to prepare thick and thin Giemsa-stained smears which were analysed by expert microscopist for detection of plasmodium and relative species.

Thick Giemsa-stained blood smears were used to find out parasite/ μ l. Parasite in a thick smear field was calculated for standard 8,000 white blood cells (WBCs)/ μ l for which 200 WBCs were analysed for presence of parasite in thick smear field and it was multiplied by 40 to get the number of parasite / μ l.

Genomic deoxyribonucleic acid (DNA) was extracted from microscopy negative and positive samples using Thermo Scientific GeneJET Genomic DNA Purification Kit according to the device's protocol. Blood sample was briefly thawed at room temperature. Then, in a clean micro-centrifuge tube the required amount was mixed with proteinase K along with lysis buffer and incubated in a water bath at 56°C for 10 minutes to completely lyse the cells. After incubation, absolute ethanol was added to it and mixed by vortexing. This mixture was then transferred to a spin column and centrifuged for one minute at $6000\times g$. Wash buffer 1 was added to this solution and the spin column was centrifuged at $8000\times g$. Wash buffer 2 was added and again it was centrifuged at $12000\times g$. A one-minute centrifugation was repeated to remove all wash buffer from it. After one-minute incubation at room temperature with elution buffer, the spin column was again centrifuged at $8,000\times g$ to elute the DNA from the spin column, and DNA was used for further analysis.

PCR was performed for all samples, whether microscopy positive or negative. For this purpose all PCR reactions were carried out in a Thermo HybridPCR Express Thermal

Cycler with a total 20µl reaction mixture which was prepared by mixing 5µl of genomic DNA with 4µl of nuclease-free water and 10µl of thermo scientific 2X master mix supplied with 0.05 U/µl Taq DNA Polymerase, 4 m Mmagnesium chloride (MgCl₂), 0.4Mm of each deoxyribose-containing nucleoside triphosphate (dNTPs), reaction buffer and primers which are 0.5µl (10 pMol /µl) Plasmodium ribosomal primer forward F (5'-TTAAAATTGTTGCAGTTAAAACG-3') and 0.5µl (10 pMol /µl) Plasmodium ribosomal primer reverse R (5'-CCAGACAAATCATATTCACG-3').^{17,18} These primers amplify a 803 base pair target sequence for P.vivax and a 808 base pair target sequence from P.falciparum's 18S rRNA gene.

Gel electrophoresis was performed with a 1.5% agarose gel to analyse the PCR products were visualised on ultraviolet (UV) transilluminator and compared with 100 base pair Norgen Biotech DNA marker. Sequencing for all PCR-positive samples was performed at Macrogen Laboratories, South Korea.

Results

Of the 200 blood samples, 114(57%) were found to be microscopy positive and 86(43%) as negative for Plasmodium. The range of parasitaemia level was calculated at 412 parasites /µl to 214,200 parasites /µl by

Table-1: Comparative analysis of microscopy and PCR.

	PCR	Total	
		Positive	Negative
Microscopy	Positive	109	5
	Negative	15	71
Total		124	76

PCR: Polymerase chain reaction.

Table-2: Sensitivity, specificity and positive predictive value of PCR in comparative analysis with microscopy.

		PCR-Sequencing			Total Microscopy
		P.f	P.v	Neg	
Microscopy	P.f	54	4	3	61
	P.v	1	50	2	53
	Neg	7	8	71	86
Total	PCR-Sequencing	62	62	76	200

P.f: Plasmodium falciparum

P.v: Plasmodium vivax

Neg: Negative

Sensitivity =87.98%,

Specificity =93.42%,

Positive predictive value (PPV) = 96 %

Negative predictive value (NPV) =82.55%

PCR: Polymerase chain reaction.

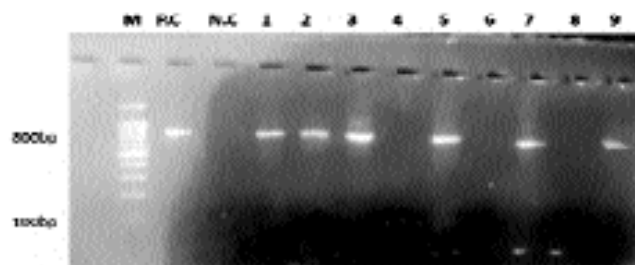


Figure: PCR amplified products from microscopy positive or negative samples of patients suffering from malaria symptoms.(M : 100 base pair marker, P.C: positive control, N.C: negative control, samples: 1,2,3, 9: PCR positive samples : 5 microscopy positive but PCR negative samples: 4, 6, 8 microscopy positive but PCR negative).

microscopy. PCR confirmed 124(62%) cases as positive, of which 15(12.1%) cases were missed by microscopy. Furthermore, 76(38%) cases were confirmed negative for malaria by PCR, of which 5(6.58%) were found to be positive by microscopy. Confirmation of all PCR-positive samples was done through sequencing and alignment with National Centre for Biotechnology Information's Basic Local Alignment Search Tool (NCBI BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Figure, Table-1).

Of the PCR-positive malaria cases, 62(50%) cases were confirmed as P.falciparum and 62(50%) as P.vivax. Of the microscopy-positive cases, 61(54%) samples were detected as P.falciparum while 53(46%) as P.vivax infection. Sensitivity, specificity and positive predictive value (PPV) were 88%, 93.42% and 96%, respectively, for PCR results with comparison to microscopy (Table-2).

Discussion

For every disease, including infectious disease like malaria, one of the key steps is accurate, prompt and cheap diagnosis which aids in better management and correct treatment. Several techniques are available for diagnosis of Plasmodium and related species from patients' blood. However, not a single technique can be considered as the best suited for malaria diagnosis in all its aspects. Different features and complexity of parasites inside the host body make malaria diagnosis a tough job.¹⁹ Two basic techniques, microscopy and RDT, are used for malaria diagnosis. Microscopy is considered as the gold standard of malaria diagnostic method all around the world, including Pakistan. Unfortunately, errors in microscopy make it a defective diagnostic practice. Moreover, interruption in precise diagnosis often leads to disruption in the disease control and treatment.²⁰ Another technique is RDT, which is United States Food and Drug Administration's (FDA) approved malaria diagnostic technique. But it also suffers from several limitations like failure in identification of all

Plasmodium species, detection at very low parasitaemia levels, and failure in detection of gametocytes. In case of negative RDT results, microscopy is always required.^{21,22} According to studies, PCR has proven to be more sensitive and specific as compared to microscopic detection and species identification of plasmodium.^{19,23} Several studies were conducted to amplify target from small subunit (SSU) rRNA gene with genus and species specific primers for plasmodium detection and its species identification. These assays were efficiently capable of identifying all four species of plasmodium including *P.falciparum*, *P.vivax*, *P.ovale* and *P.malariae*. Their specificity and sensitivity have been confirmed to be higher than RDT and microscopy.¹⁶ In the current study, 114 of the 200 samples were identified as positive for plasmodium by microscopic examination. PCR, on the other hand, proved more sensitive as it detected 15 more cases that had been identified as negative by microscopy, maybe because of low parasitaemia level. Moreover, five microscopy positive samples with considerable parasitaemia levels were declared negative by PCR. This may be due to poor slide preparation, wrong diagnosis, slide contamination or DNA degradation of parasite during sample collection. Overall, PCR was more specific and sensitive as it detected 124 samples for plasmodium presence. Sequencing of those PCR products further revealed flaws in microscopy as for five samples species were wrongly identified by microscopy. But molecular technique like PCR is considered as less applicable method in routine clinical diagnosis due to high cost and maintenance. However, superiority of this technique cannot be neglected for parasite species identification and quantification at low parasitaemia levels.²⁴ These problems are far smaller than the consequence caused due to wrong diagnosis by conventional methods. Cost of this method can be effectively reduced by applying it in diagnostic laboratories only for the microscopy negative samples that are strong clinical suspect.²⁵

The studies have specified highest prevalence of malaria in Balochistan and its capital Quetta. This alarming situation has shown a possible threat to the life and health of people living in the malaria-prevalent areas of Balochistan. This threat needs to be addressed on an urgent basis and crucial steps are required to control and eradicate this infection from the province.^{3,4} As a confirmatory diagnostic test, this technique can bring a substantial improvement in Plasmodium detection and identification of species of Plasmodium in the blood of patients and can play a significant role in control and management of malaria in the province of Balochistan.

Conclusion

Molecular method like PCR was found to be more efficient in malaria diagnosis and species identification. Further studies should be conducted to investigate the effectiveness of this molecular method as a laboratory technique for malaria diagnosis.

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