ORIGINAL ARTICLE

Amplification of Mitochondrial DNA for detection of Plasmodiumvivax in Balochistan

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Abstract

Objective: To access a new step using PCR to amplify the targeted mtDNA sequence for detecting specifically Plasmodium vivax and its co-infections, false positive and false negative results with Plasmodium falciparum.

Methods: In this study we have standardized a new technical approach in which the target mitochondrial DNA sequence (mtDNA) was amplified by using a PCR technique as a tool to detect Plasmodium spp. Species specific primers were designed to hybridize with cytochrome c oxidase gene of P. vivax (cox I) and P. falciparum (cox III). Two hundred blood samples were collected on the basis of clinical symptoms which were initially examined through microscopic analysis after preparing Giemsa stained thick and thin blood smears. Afterwards genomic DNA was extracted from all samples and was then subjected to PCR amplification by using species specific primers and amplified segments were sequenced for confirmation of results.

Results: One-hundred and thirty-two blood samples were detected as positive for malaria by PCR, out of which 64 were found to be positive by PCR and 53 by both microscopy and PCR for P.vivax infection. Nine samples were found to be false negative, one P.vivax mono infection was declared as co infection by PCR and 3 samples identified as having P.falciparum gametes were confirmed as P.vivax by PCR amplification. Sensitivity and specificity were found to be 85% and 92% respectively.

Conclusion: Results obtained through PCR method were comparatively better and reliable than microscopy.

Keywords: Malaria diagnosis, Plasmodium vivax, Polymerase chain reaction, mtDNA, cox I gene. (JPMA 67: 677; 2017)

Introduction

Malaria, a terrifying earthly disease, is sometimes regarded as the ''King of diseases" which is majorly caused by a protozoan of genus Plasmodium.[1] It is often regarded as one of the most important parasitic infection especially in tropical and subtropical regions of the World.[2,3] Because of the suitable temperature, rainfall required for the development of the Anopheles mosquitos and unhygienic conditions, poor population of these regions are at a greater risk of malaria.[4] The world's most malaria endemic regions comprised of Asia, Africa, Central and South America.[5] The four Plasmodium species responsible for causing malaria in humans include Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae and Plasmodiumovale. The occurrence of these species depends on the geographical location and climatic conditions of the area.[6] Pakistan is highly endemic to plasmodiumfalciparum and plasmodiumvivax. Being a tropical country, Pakistan holds a vast irrigation system, large number of stagnant water bodies which provide the mosquitoes standard climatic conditions for their breeding.[7] The aptitude of malaria has also risen due to monsoon rains in the country. In Pakistan, lower incidence of malaria was restricted to Punjab and Azad Kashmir (AJK) areas while Balochistan and Federally Administered Tribal Areas (FATA) were found with higher malarial incidences during 2004.[8] P.falciparum is thought to be the cause of most serious and sometime the fatal kind of disease and causes most of the mortality cases. P.vivax can also cause severe illness and morbidity but the mortality tolls are lower than P.falciparum whereas P.malariae and P.ovale infections are rare across the world.[2,3]

On a global level, Plasmodiumvivax threatens approximately 2.8 billion people and its eradication could be more difficult than P.falciparum due to its complicated biological characteristics such as presence of liver hypnozoites which causes numerous relapses.[9-13]

Accurate treatment of malaria is solely dependent upon the early and accurate diagnosis.[8] Microscopic screening of Giemse-stained thick and thin blood smears is considered to be the gold standard and is used as a conventional method to diagnose malaria in various industrialized countries.[14,15] Despite the fact that this technique is economical, quick and

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can recognize the infection at parasitemia level (usually at a level 50 to 500 parasites/µl) and can distinguish between species,[16] however, this due to its inability to sense low parasitemia level and co-infections drops its reliability.[17,18] It also requires skillful technicians to identify the species accurately and unskilled technicians may lead to misdiagnose and ultimately invalid treatment.[14,15]

The molecular method Polymerase Chain Reaction (PCR) based on DNA amplification has shown great specificity and sensitivity in contrast to microscopy.[19,20] It is a remarkable addition to microscopy for identification and conformation of specimens. It serves as a radiant detector of harmful agents in the blood and for the detection of different Plasmodium species around the world. PCR has the ability of detecting the malarial parasite even in mixed infections and low parasitemia level.[21-23]

At a population level studies in a variety of organisms, partial or complete mitochondrial genome sequences have proved to be a useful marker and specifically in case of Plasmodium genus, mitochondrial genome is predominantly well-suited for studies. The mitochondrial genome contains genes encoding many ribosomal RNAs and three components of cellular respiration (cytochrome c oxidase gene I, cytochrome c oxidase gene III and cytochrome b). All these 3 genes are essential for a range for cellular processes; like membrane potential maintenance, heme and coenzyme Q biosynthesis, and oxidative phosphorylation.[24]

At present, detection of plasmodium is done through amplification of a sequence based small subunit ribosomal RNA (ssRNA) genes. But due to limited number of copies of parasite in the infected cells amplification of mitochondrial DNA is preferred for the detection of parasite. This study concentrates on the utilization of PCR as a standardized method for the detection of malarial parasite by amplification of mitochondrial DNA as it has greater copy number in the contaminated cell. The objective was to standardize a protocol through which PCR is utilized to amplify the targeted mtDNA sequence for distinguishing particularly Plasmodium vivax among different locale of Balochistan area and the outcomes be affirmed by sequencing.

Materials and Methods

The study was conducted from August, 2013 to October, 2014. A total of 200 venous blood samples were collected in tubes with EDTA from various districts of Balochistan province. Sample collection was based on the clinical signs and symptoms including nausea, vomiting, fever, muscular cramps etc. Both thick and thin blood films were prepared, stained with Giemsa stain, dried and observed under microscope for the identification of plasmodium species.

For confirmation of microscopic results PCR method was used to amplify the targeted mtDNA sequence for detecting specifically Plasmodium vivax and its co-infections, false positive and false negative results with Plasmodium falciparum. DNA was extracted from 200µl of thawed EDTA blood samples through extraction kit (Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Kit #K0721) according to the manufacturer's instructions. The molecular method PCR was used for amplification of DNA. Specific primers were designed for Plasmodium vivax and Plasmodium falciparum in order to hybridize cytochrome c oxidase genes of the mitochondrial genomes based on the sequence of P. vivax cox I and P. falciparumcox III genes. The sequences used for P. vivax were PvmF (5-AAGTGTTGTATGGGCTCATCATATG-3) and PvmR (5-CAAAATGGAAATGAGCGATTACAT-3) and for P. falciparum were PfmF (5-CCTGCATTAACATCATTATATGGTACATCT-3) and PfmR (5-GATTAACATTCTTGATGAAGTAATGATAATACCTT-3). The fragments obtained after amplification were of 273 bp and 290 bp. The PCR reaction was performed in a mix with a final volume of 20µl containing: 10µl of PCR buffer, 4µl of PCR water, 0.5µl of Primer and 5µl of DNA template in an Eppendorf thermal cycler.

The amplified products were resolved by 2% agarose gel electrophoresis and were visualized by Ethidium Bromide staining and were photographed under UV light.

A total of 5 PCR products were sent to MACROGEN laboratory, South Korea for sequencing purpose and the results of sequencing were analyzed with the help of NCBI nucleotide Basic Local Alignment Search Tool.

Results

A total of 200 blood samples were collected from patients of varying age groups from different districts of Balochistan province, Pakistan. All the samples were collected on the basis of clinical signs and symptoms and blood smears were prepared for microscopic examination.

A total of 125 patients were identified as being infected with malaria by the analysis of prepared blood smears by microscopy. Out of these, 68 were identified as positive for Plasmodiumvivax and 53 for Plasmodiumfalciparum infection; four were found positive for both P.vivax and P. falciparum infections and none was found positive for P.Ovale and P. malariae (Table-1).

All microscopy positive and negative samples were run independently through PCR for the detection of plasmodium species by the use of species specific primers for 100% identification of P.vivax by the amplification of mitochondrial DNA. The amplification results revealed that out of 200 samples, 132 (66 %) were found positive for malarial infection

Table-1: Numbers and percentages of POSITIVE, negative and co-infections as described by microscopy and PCR for P.vivax.

Results	Microscopy	PCR
Positive	53 (42%)	64 (48%)
Negative	68 (54%)	62 (47%)
Co-infections	4 (3%)	6 (5%)
Total	125	132

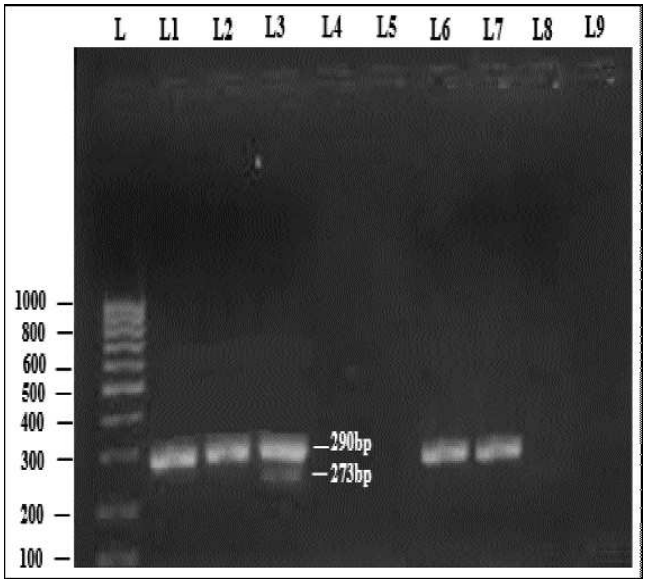

Figure-1: L:100bp DNA ladder. L1, L2, L6, L7: Products amplified by specific primers for P. vivax. L3: Mixed infection (290 bp showing P.vivax and 273 bp showing P.falciparum). L4, L5, L8, L9: Both microscopy and PCR negative samples for P.vivax.

including 6 cases of mixed infections. Among them, 64 (48 %) were positive for P.vivax malaria (Table-1).

From 132 positive samples, 47 were considered as true positive as they were identified as positive by both microscopy and PCR. Six were identified as positive by microscopy but confirmed as negative by PCR therefore, were considered as false positive. Nine samples were wrongly identified as negative by microscopy for P.vivax but were declared as positive by PCR amplification and were considered as false negative. Two of the samples that revealed co-infections by PCR did not confirm results of microscopy. One of these was negative on microscopy for malarial parasite and the other had only mono infection. These results indicate that PCR was able to detect 9 more cases which were declared as negative by microscopic analysis (Table-2).

When samples were run through gel electrophoresis, product with 290 bp was observed for P.vivax and 273 bp for P.falciparum (Figure-1).

To confirm the specificity for Plasmodiumvivax five samples were sent for sequencing. The sequencing data for P.vivax were 100% homologous to the sequences previously deposited in Gene bank. The sensitivity and specificity was found to be 85% and 92% respectively according to following formula:

Sensitivity = True positive / True positive+ false negative

Specificity = True negative / True negative+ false positive

Discussion

Malaria, a deadly mosquito-borne disease affects approximately 300 million people and causes loss of more than a million of precious lives each year.[25] An appropriate and early diagnosis is the key to successful treatment of malaria and to avoid unnecessary anti-malarial treatments. Therefore, proper testing and identification procedures must be implemented. In Pakistan, the disease mainly affects people who are inhabiting in the rural areas of the country because of having poor health and sanitary conditions and the country is estimated to have a burden of 1.6 million cases.[25] Plasmodiumvivax is found to be a dominant species in Pakistan.[26]

The present study focuses on the use of simple and sensitive molecular method PCR in order to detect P.vivax by the amplification of mitochondrial DNA (cytochrom c oxidase gene), cox I gene of P.vivax and cox III gene of P.falciparumjust for cross checking co-infections of both species in samples collected from various districts of Balochistan province. Similar studies have been conducted in Brazil for the detection of P.falciparum and P.vivax by the amplification of mtDNA as the copy number of mitochondrial genome is high in the infected cell.[27,28]

Table-2: Comparison of the results of microscopy and PCR indicating true positive, true negative, false positive and false negative cases.

True Results Obtained by pcr and microscopy	PCR	Microscopy	False Results obtained by microscopy	Microscopy	Total of Microscopy
Positive	64	47	Positive	6	53
Negative	62	59	Negative	9	68
Coinfections	6	2	Co-infections	2	4
Total	132	108	Total	17	125

In this study sampling was done purely on the basis of clinical symptoms. Most of the samples were collected during seasons (August to October and April to July) in which malarial infections were found to be more prevalent. Contemplates have demonstrated that malarial species show regular variations in Balochistan like in different parts of the country where it's additionally considered as occasional.[29] According to WHO report, P.vivax is the most prevalent Plasmodium specie throughout the country including Balochistan.[30] Contrary to WHO, our microscopic data revealed 73 (59%) of P.falciparum and 57 (46%) of P.vivax infections which shows high prevalence of P.falciparum in our study and such occurrence was also observed earlier in another study.[31] The reason behind this could be that our sampling was mostly done during the season when P.falciparum was at its peak, therefore, it was found to be more prevalent. P.vivax shows its peak infections between April to September compared to, P.falciparum which peak infection has been observed between August to December and regional variations in high infection peaks were also found in mountainous areas of Balochistan.[32,33] Four samples comprising (3%) of total samples were observed as mixed infections and no positive results were found for P.Ovale and P malariae, which has been reported in other studies previously.[34]

According to these results, out of 200 blood samples 132 were found to be positive for malaria parasite by PCR and 64 (48%) infections were identified for having P. vivax DNA. Nine cases were declared to be negative for infection through microscopic examination but PCR detected them as positive. This proves that detection methods where PCR is used are more sensitive than microscopy and this finding are in line with earlier studies.[20,35] Only four mixed infections were identified by microscopy while PCR confirmed 6 mixed infections in which one was declared to be negative for malaria by microscopy while the others were found to be positive with mono-infection for P.falciparum. Similar cases have been reported earlier by workers.[35-37]

In this study three cases were found as positive for P. falciparum by microscopy, while PCR diagnosed them as positive for P. vivax. Such misdiagnosis of P. vivax creates severe problems when contrasted with P. falciparum because of the way that P. vivax could bring about backslide and bad results. Due to such wrong conclusion the patients stay untreated and turn as transporters of parasite and may grow new disconnects (counting drug safe parasites). In this way revised species findings are amazing and have a great significance for keeping in mind the ultimate goal to treat and control jungle fever.[12] Likewise, it has been demonstrated that at low parasitemia level when a microscopist missed a parasite, PCR recognized the DNA as it is observed to have the capacity to distinguish as low as 0.01 parasites/µL of blood.[11]

Alternatively, 53 microscopy positive samples were also detected as positive by PCR. This shows the specificity of PCR as reported previously.[37,38] The sensitivity and specificity of PCR in this study were found to be 85% and 92% respectively. Similar type of study was conducted in Turkey where samples were collected on the basis of clinical signs and symptoms and showed a sensitivity and specificity of 76% and 100% respectively.[39]

In conclusion, the method adopted in this study by using PCR is an effective method to diagnose and identify the species of plasmodia in blood samples. Although, microscopic diagnosis is a simple and cheap test but on the other hand, to examine a blood smear slide to acceptable accuracy, lengthy training sessions and a lot of experience are required. PCR test for the detection and differentiation of malaria parasites requires more time and cost yet it has been shown to be a more sensitive assay with the ability to differentiate plasmodial species and detect mixed infections.

Molecular techniques are costly, including the cost of labour and access to reagents, compared with examination of blood smears. This represents a true impairment for its implementation in reference laboratories located in poor regions of the world, where malaria is endemic.

As Pakistan is a developing country and due to lack of resources microscopy is preferred for the diagnosis of malaria for being an inexpensive method. Therefore, the probability of incorrect and miss-diagnosis increases which leads to an increase in morbidity and mortality rate.

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