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Original Article

Molecular Detection of Malaria in South Punjab with Higher Proportion of Mixed Infections

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Abstract

Background: Malaria is well known for its fatalities worldwide, *Plasmodium vivax* and the *Plasmodium falciparum* are the two important species of malaria reported from Pakistan and creating lots of morbidities across the country.

Method: Study was conducted to determine the Surveillance of malaria in South Punjab by microscopy and Polymerase chain reaction (PCR).

Result: 40 samples out of 100 patients were found positive for malarial parasites. One patient was found with mixed infection, whereas *P. falciparum* and *P. vivax* infections were detected in 17 and 22 patients respectively. In nested PCR, genus-specific primers for *Plasmodium* species. in round 1 and species-specific primers for *P. falciparum* and *P. vivax* in round 2 were used. By the application of PCR 41% were found to be infected by *Plasmodium spp.* Among *Plasmodium* positive patients: mixed, *P. falciparum* and *P. vivax* infection were detected in 10, 15 and 16 patients respectively. Thirty nine microscopically positive patients confirmed to have Plasmodium spp. One negative by PCR, 2 microscopically negative patients had shown *Plasmodium* spp. infection (*P. falciparum* and *P. vivax*) by PCR. In total samples, *P. falciparum, P. vivax* and mixed infection accounted for 36.6%, 39.0% and 24.3% respectively.

Conclusion: Microscopy was found deficient for interpretation of mixed infections, low parasitaemia, and species specific diagnosis. The sensitivity, specificity and efficacy of nested PCR was calculated 95%, 98% and 97% respectively showing PCR as a more effective and efficient diagnostic tool for malaria.

Introduction

Mankind is still struggling against the parasites, *Plasmodium* is considered as cause of malaria since ancient times and is the leading cause of mortality worldwide, infecting approximately 3.3 billion people were at risk globally in 2011 (1-3). *P. falciparum* is responsible for most of the mortality (4, 5). Both treatment and control of malaria are hampered by the spread of resistance to common antimalarial drugs, especially against *P. falciparum* (6, 7). In Pakistan, malaria threatens millions of people, due to poor conditions; and it remains endemic in most parts of country (8). Five different species of *Plasmodium: P. falciparum, P. vivax, P. malariae* and *P. ovale*, *P. knowlesi* cause human malaria (9, 10). Two species of *Plasmodium* have reported in Pakistan: *P. vivax* (75%) and *P. falciparum* (25%) (8). As there is no vaccine available for malaria and current treatments suffer from several limitations (11), hence the emphasis falls on accurate diagnosis of malaria to provide novel drugs to treat different types of malaria, especially for *P. falciparum* the most fatal infection (12-15). Polymerase chain reaction (PCR) has proved to be an efficient, sensitive and specific method for diagnosis of mixed infections, low parasitaemia and species detection for malaria (10, 13, 16-20). PCR has the potential to overcome all the limitations of the traditional diagnostic method, but their high cost limits their clinical implication for malaria diagnosis (3, 21). The present study was aimed to determine the epidemiology of malaria by comparison of microscopy and nested PCR. Genus-specific and species-specific primers for 18s rRNA gene of *Plasmodium* species were used for two *Plasmodium* species (*P. falciparum* and *P.vivax*) infection by nested PCR. Then, the results of both methods were also compared.

Materials and Methods

Sampling

Blood samples of malaria patients were collected from South Punjab in 2010. Sampling was performed after permission of patients and their relatives. Whole blood (5 ml) was drawn by sterilized syringes and collected in EDTA vacutainer tubes. Negative Controls' blood was obtained from students of department of Zoology, university of the Punjab, Lahore. All the samples were stored at -20 °C.

Microscopy

The blood smears were stained with 1% giemsa stain in phosphate-buffered saline (ph 7.0) and examined under the microscope at a magnification of 1,000x for the presence of malaria parasites.

DNA extraction

DNA was extracted from 200 µl of EDTA blood using QIAamp DNA blood mini kit (QIAGEN, Germany) according to given protocol. The extracted DNA was stored at -20 ^{0}C.

Nested PCR

The purified DNA templates were used for amplification of 18s rRNA gene using primers (Table 1), as described by (16), in nested PCR. All the oligonucleotides were prepared from CEMB (Center for Excellence in Molecular Biology), Lahore. The PCR master mix for 50 µl reaction was prepared by mixing 5.0 µl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.8 at 25°C]) (Fermentas, EU), 4.0 µl of 10 µM deoxyribonucleoside triphosphate (dNTPs) (Fermentas, EU), 5.0 µl of 2.5 mM $MgCl_2$ (Fermentas, EU), 1.5 µl of each primer (10 µM), 0.5 µl *Taq* DNA polymerase (1 U/µl; Fermentas, EU) and 22.5 µl nuclease-free water. Fifty µl reactions using 40 µl of master mix and 10 µl of DNA template were performed. The reaction conditions used for PCR1 were: hold at 95 °C (10 min); 35 cycles of: denaturation at 94 °C (1 min), annealing at 60 °C (2 min), and extension at 72 °C (2 min); hold for final extension at 72 °C (10 min) and hold for indefinite period at 4 °C.

Table 1: Primers sequences and product size

Species	Primer	Sequence (5/ - 3/)	Size (bp) of PCR product
Plasmodium sp.	rPLU5	CCTGTTGTTGCCTTAAACTTC	1,100
	rPLU6	TTAAAATTGTTGCAGTTAAAACG	
P. falciparum	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	
P. vivax	rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	120
	rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	

The PCR2 reactions used same conditions except annealing temperature was 55 °C. The amplified products were visualized on 1.5% agarose gel.

Calculations

The sensitivity, specificity, and efficacy of nested PCR were calculated by using these formulas respectively: [true positives / (true positives + false negatives) x 100%]; [true negatives / (true negatives + false positives) x 100%]; and [1 – (false negatives + false positives / total) x 100].

Results

Microscopy is a conventional method for detection of malaria, but PCR has been developed for the rapid and correct diagnosis of malaria. From total of 100 clinically positive samples, 60 patients were negative and 40 patients were positive for malaria by microscopy; whereas by nested PCR, 41 specimens were positive and 59 specimens were negative for Plasmodium spp. The comparison of malaria epidemiology in South Punjab observed by clinical symptoms, microscopy, and nested PCR in present study was presented in Table 2.

According to Table 3, one specimen was found to be infected by *P.vivax* by microscopy but it was confirmed to be negative for *Plasmodium* spp. by nested PCR. 1 mixed infection (*P. falciparum* and *P. vivax*) was diagnosed by microscopy; rather nested PCR determined mixed infections (*P. falciparum* and *P. vivax*) in 10 specimens. *Plasmodium* was detected in 41% samples by nested PCR as compared to 40% by microscopy. Species identification by nested PCR was done for all *Plasmodium* positive samples (41%). Out of which 15% were having *P. falciparum* infection, 16 were having *P. vivax* infection, and 10 were mixed infections (*P. falciparum* and *P. vivax*). Figure 1 is showing Nested PCR result of three samples with *P. falciparum, P. vivax* and mixed infections. Incorrect speciation of *P. falciparum* and *P. vivax* was resolved by nested PCR in 8 samples; *P. falciparum* was identified in 4 clinically and microscopically positive specimens that showed *P. vivax* infection by nested PCR; and 4 *P. vivax* positive specimens were shown to be *P. falciparum* infection by nested PCR.

Table 2: Comparison of diagnostic methods for Malaria cases in South Punjab by Clinical Symptoms, Microscopy and Polymerase Chain Reaction (PCR)

***Plasmodium* infection**	**Clinically symptomatic**	**Microscopic examination**	**PCR**
Positive Samples	100	40 *P. falciparum* [17] *P.vivax* [22] *P. falciparum* + *P.vivax* [1]	41 *P.falciparum* [15] *P.vivax* [16] *P.falciparum* + *P.vivax* [10]
Negative Samples	0	60	59
Total	100	100	100

Table 3: Comparison of microscopy and PCR results for diagnosis of malaria

Malaria-causing species	Parasites detected by both methods	
	Microscopy	PCR
P. falciparun	*P. falciparum* [17]	*P. falciparum* [10] *P. vivax* [4] *P. falciparum* + *P. vivax* [3]
Non-*P. falciparum*	*P. vivax* [22]	*P. vivax* [11] *P. falciparum* [4] *P. falciparum* + *P. vivax* [6] Negatives (1)
Mixed infection	*P. falciparum* + *P.vivax* [1]	*P. falciparum* + *P. vivax* [1]
Negatives	Negative [60]	Negative [58] *P. falciparum* [1] *P. vivax* [1]
Total	100	100

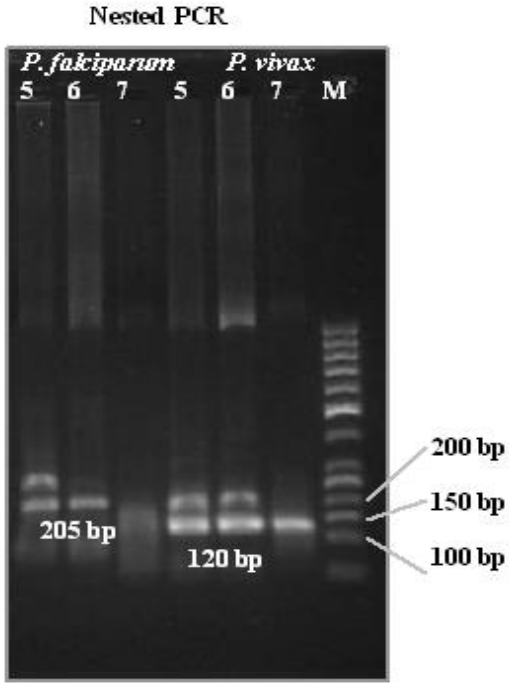

Fig. 1: Nested PCR results for *P. falciparum* and *P.vivax* from 3 malaria patients. M: 50 bp ladder, Lane 1-3 (5, 6, 7 (*P. falciparum*)); lane 4-6 (5, 6, 7 (*P. vivax*)) results

All concordant results for parasite identification were resolved by nested PCR; nested PCR was able to determine plasmodium DNA in 2 specimens that were depicted to be negative for malaria by microscopy. The sensitivity, specificity, and efficacy of nested PCR were calculated to be 95%, 98%, and 97% respectively. The specificity and sensitivity of nested PCR, calculated from present study was better than that of microscopy.

Discussion

Malaria is a life-threatening infection impacting most of the developed countries of the world. The WHO recommended method and the gold standard for routine laboratory diagnosis of malaria is microscopy, despite its decreased sensitivity and specificity in situations of low parasite density and mixed infections. Compared to microscopy, molecular methods (PCR) has achieved much higher detection sensitivities and specificities, especially in cases of low parasitaemia or mixed infections and differential diagnosis of *Plasmodium* species (17, 21-23). They are more important due to the automation of the process and have objective of reading the results by machines. This makes them a valuable option for large-scale epidemiological studies (24). Especially, nested PCR has proven to be a sensitive method for diagnosis of all species of *Plasmodium* and has expected to exceed the sensitivity of microscopic examination (25).

Moreover, nested PCR has appeared to be effective in correcting wrong diagnosis, identified as *Plasmodium* species by microscopist. This was obvious in the present study with the misdiagnosed *Plasmodium* negative specimens (1%). The non concordant smear positive/PCR negative cases can also be attributed to either degradation of parasite DNA or low parasitemia combined with degradation of parasite DNA (26). In certain cases, parasite morphology is damaged due to exposition to prophylactic medication or auto-medication, making malaria diagnosis by microscopy difficult (27), which may lead to the death of the patient by improper medication.

PCR was appeared to be effective in specific diagnosis of *Plasmodium* in present study. It was found that 8 specimens were microscopically misdiagnosed as *P. falciparum or P. vivax* infection and they were diagnosed correctly by PCR. It is suggested that it can be due to chemoprophylactic effect on the shapes of the parasites (22). In 2% of samples, the parasite could not be determined by microscopy; by PCR, the parasite was detected even in a very low quantity. One was *P. falciparum* and one was *P.vivax* infection.

It has been found that the PCR assay is usually effective in detecting malarial mixed infections than microscopy (16, 21, 23) but not in all situations (28). 24.3% of *Plasmodium* spp. infected samples were confirmed to be mixed infection (*P. falciparum* and *P. vivax*) by the PCR but only 2.4% was identified by microscopy. In case of mixed infections, it was suggested that one species has the ability to dominate over other species; as a result, one may be overlooked in microscopic examination (29). In present study, 6 samples, microscopically diagnosed *P. vivax* infection were determined as mixed infection (*P. falciparum and P. vivax*) by PCR and 3 samples microscopically detected *P. falciparum*, were depicted as mixed infection (*P. falciparum* and *P. vivax*) by PCR. It is clear from the present study that *P. vivax* have higher tendency to dominate over *P. falciparum*. Detection of mixed infection may be of clinical importance because interactions between different species simultaneously infecting the same individual could result in significant changes in the course of the infection and disease. It may also be helpful in the effective treatment of malaria because the treatment of malaria depends on the correct diagnosis of the species (15). As *P. falciparum* and *P. vivax* have developed resistance against specific drugs and there are many drugs which are effective for *P. vivax* but not for *P. falciparum*. For example, mefloquine is an effective drug for treatment of *P. vivax* malaria but not effective on *P. falciparum* malaria. Therefore, PCR is also helpful in differential treatment of malaria. PCR detected a high number of mixed infections in the samples analyzed, but its routine clinical use for diagnosing malaria is still under consideration because of its high cost and resource requirements (18, 21). The high prevalence of *P. vivax* (39%) may lead to serious complications like cerebral malaria but the comparatively less prevalent (36.6%) *P. falciparum* also poses a significant health hazard.

Conclusion

Compared to microscopy, the nested-PCR is a rapid, sensitive, and specific method for the detection of malaria.

The primary goal of the present study was to assess the value of a PCR-based method for the routine diagnosis of malaria at species level and study was conducted to evaluate the epidemiology of malaria in South Punjab. Although the number of samples used here are small, but the high degree of both sensitivity and specificity is encouraging. Larger studies of both *P. falciparum* and *P. vivax* malaria in endemic regions will enhance the generalizability of the present findings.

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