Journal of Rawalpindi Medical College (JRMC); 2018;22(2): 156-160

Original Article

Species-Specific 18S rRNA Gene Amplification to Detect the Sequence Variation in Plasmodium Vivax Parasite

Rizwana Yasmin [1] , Syeda Mariam [2], Mahadev Harani [1], Mehwish Kalam [2], Mumtaz Khan Muhammadee [3], Abid Ali Hashmi [4], Mustafa Kamal [2]

1.Department of Pathology, Jinnah Medical and Dental College, Karachi;2. Department of Biotechnology, University of Karachi; 3. Department of Microbiology, University of Haripur, Haripur, KPK; 4. HEJ Research Institute of Chemistry, University of Karachi

Abstract

Background: **To ascertain species-specific 18S rRNA gene amplification, in order to detect the sequence variation in Plasmodium Vivax parasite.**

Methods: **Blood samples from 150 suspected malaria cases were collected . Microscopically diagnosed cases of malaria were subjected to nested PCR amplification for genus and species confirmation. The 18S rRNA gene was amplified from 34 P.vivax positive cases. The obtained PCR products were subjected to sequence analysis, phylogenetic study and multiple sequence alignment.**

Results: **The genus specific-positive malaria cases along with the mixed species done by nested PCR were 40.7 % (61 out of 150) while microscopy results revealed malaria positive cases to be 32.6 % (49 out of 150). The difference in detection of malaria parasite by PCR was more sensitive as compared to microscopy (two-tailed p.value = 0.0015).Among the nested PCR malaria-positive cases, P. vivax, was isolated in 55.7% (34 of 61) .Samples and P. falciparum were isolated in 34.4% (21 of 61) samples and mixed infection were detected in 9.8% (6 of 61) cases. While among the microscopy -positive malaria cases, P. vivax, was isolated in 57.1% (28 of 49) . P. falciparum were isolated in 32.7% (16 of 49) samples and mixed infection were detected in 10.2% (5 of 49) cases. Phylogenetic analysis and multiple alignment of the 18S rRNA was done on 13 selected cases of P.vivax isolates which revealed vast genetic diversity and variation in Single Nucleotide Proteins (SNPs) including deletion, substitution and insertions. All isolates appeared to be highly polymorphic.**

Conclusion: **Detection of Plasmodium species with nested PCR is effective way to identify the individual Plasmodium species and mix infection in low parasitemia for proper treatment option. The phylogenic studies and bio informative tools are reliable methods to identify the relatedness of human malarial parasites and zoonotic e.g. P. knowlesi.**

Key Words: **Malaria diagnosis, P. vivax, Sequencing, 18S rRNA.**

Introduction

The magnitude of Plasmodium vivax (P.vivax) infection is high and it is more prevalent in various cities of Pakistan. A distinctive feature of P. vivax infection is the overall low parasite density in peripheral blood. So to eradicate the malaria proper diagnosis of specific species in low parasitemia and mix infection is crucial . It is estimated that majority of the world people are at risk for malaria caused by P. vivax As this parasite has distinct features like bearing the cold weather and prolonged remission, it leads to remarkable morbidity with this speicies.[1, 2] Five plasmodium species are responsible for human malaria P. vivax, P. falciparum, P. ovale, P. malariae and P. knowlesi. These five have characteristics symptoms, different morphological identity, nature of immunity and drug susceptibility. People think that malaria caused by P.vivax is benign but now severe cases of this species have been reported in several regions [3] P.vivax mostly infect young reticulocytes, and its infection has low parasite density. [4] Recently data showed that prevalence of P. vivax is more in Pakistan. The meticulous data on P.vivax biological character, genetic diversity and pattern of antimalarial resistance is yet not established properly.[5] To determine the distinctive nature of 18S rRNA genes that discloses significant species-specific diversity is a potential diagnostic marker.[6] The considerable initial molecular marker of Plasmodium was the 18SrRNA that depicts diverse type of multiple copies and exhibit great variability during development and infective stages.[7] It is assumed that P. vivax causes mild infection but genetic polymorphism, emergence of drug resistance and hypnozoid stage lead to severe complicated malaria so identification of specific species by 18S rRNA is validate tool for species

identification.[8] The 18S rRNA is complementary to species specific region. The species of plasmodium can be best detected by magnifying this particular gene .[9,10] Since early 1990,implementation of PCR based recognition for species specific 18S rRNA which has both conserved and variable but it is absent in the human genome. It is primarily a good technique for plasmodium identification 18S rRNA as it can detect even few parasites in blood. Some species have three; distinct rRNA genes that diversely appear during the parasite's development in infective stages.[11, 12] The RNA is more preferable for targeting this geneas it is massively available .In contrast the amount of DNA is less.In present study microscopically confirmed malaria parasite samples were processed for accurate diagnosis of the P. vivax and amplification portion of the 18S rRNA gene was performed via nested PCR by using species specific oligonucleotide primers. Along with this obtained result were verified by sequencing and bioinformatics tools .Phylogenic analysis as well as genetic variation were assessed via multiple sequence alignment.

Patients and Methods

This descriptive study was conducted in the Department of Biotechnology, University of Karachi ,from 2012 to 2014. Blood samples were collected from the various hospitals and clinics. Light microscopy was performed in pathological lab of Medicare Hospital. DNA extraction and nested PCR based molecular method was done in the Dept. of Biotechnology and Central Science Lab of University of Karachi. One hundred and fifty patients irrespective of age and gender who had clinical suspicion of malaria were recruited. Two ml venous blood was collected in Ethylenediaminetetraacetic Acid (EDTA) tubes. Thick and thin film was prepared on glass slides and stained with Giemsa solution to examine the slides in light microscope for plasmodium species identification and differentiation of individual species on morphological basis. [14, 15] Microscopically confirmed plasmodium species were selected for DNA extraction by using QiAamp DNA Mini Kit (QIAGEN, USA)The extracted DNA were then stored at -20°C.[16] The 0.8% concentration of agarose in gel was used to make 40 ml of 1X TAE buffer. The 40µl of 0.5mg/ml stock solution was made. After melting the ethidium bromide was added init to get the working concentration of 0.5µg/ml. 10µl of each sample premixed with 2µl of 6X sample loading buffer was loaded in wells. In 1X TAE buffer pH 8.0 was used for electrophoresis at 80 volts for 45 minutes to observe the DNA band in ultraviolet light.[17] Two sets of particular primers which were genus and species specific were designed as previously described .[18] The extracted DNA was amplified via nested PCR so specific species identification can be achieved for any sequence variations. The 18S rRNA gene was amplified from 34 P.vivax positive cases to detect genetic diversity.

Gel electrophoresis of PCR products was conducted according to standard methodology.[19] For gel electphoresis of obtained DNA 40 ml of 1X TAE buffer solution made by adding 2% agarose gel. In molten gel 40µl of stock solution (0.5mg/ml) of ethidium bromide was added to made 0.5µg/ml final concentration.10µl of each amplified product was loaded in each wells and electrophoresis in1X TAE buffer was carried out at 80V for 30 min.The DNA bands were visualized by placing the gel on UV transilluminator, and photographs were taken by digital camera. Amplified fragment size of P.vivax 18S rRNA was estimated by using DNA ladder as markers. The product size was 117bp. For sequencing analysis the PCR substances were sent Macrogen Inc.(Seoul Korea) for sequence analysis in18S rRNA gene. The received results r were evaluated for phylogenetic and sequence variations in18S rRNA . Particular frame was picked with the help of NCBI BLAST method. Multiple sequence alignment were performed by choosing Clustal X 2.1 and phylogenic relationship among isolates was represented by constructing phylogenetic tree according gene bank database. Data was analyzed by SPSS version 21.

Results

Amplification of genus and species-specific primers was performed on all 150 samples collected. The genus specific-positive malaria cases along with the mixed species done by nested PCR were 40.7 % (61 out of 150) while microscopy results revealed malaria positive cases to be 32.6 % (49 out of 150) (Table 1).

Table 1: Detection of Malarial Parasites by Microscopy and PCR (n= 150)

		Nested PCR		
Microscopic analysis	Positive cases	Positive cases	Negative cases	Total cases
		49	0	49
	Negative cases	12	89	101
	Total cases	61	89	150

p.value = 0.0015 (significant < 0.05)

Table 2: Distribution of species in Malaria parasite-positive cases by Microscopy (n= 49) and nested PCR (n= 61)

Species detected	Microscopic positive cases (49/150)	Nested PCR positive cases(61/150)
P. Vivax	28/49 (57.1%)	34/61 (55.7%)
P. Falciparum	16/49 (32.7%)	21/61 (34.4%)
Mixed infection	5/49 (10.2%)	6/61 (9.8%)

McNemar's test with the continuity correction was applied. It was observed that detection of malaria parasite by PCR is more sensitive as compared to microscopy and p.value was significant (two-tailed p.value = 0.0015).Among the nested PCR malaria-positive cases, P. vivax, was isolated in 55.7% (34 of 61) samples and P. falciparum were isolated in 34.4% (21 of 61) samples and mixed infection were detected in 9.8% (6 of 61). Among the microscopy -positive malaria cases, P. vivax, was isolated in 57.1% (28 of 49) samples, P. falciparum were isolated in 32.7% (16 of 49) samples and mixed infection were detected in 10.2% (5 of 49)(Table 2). DNA amplification of 61 positive cases showed 1640 bp band size (Fig 1). PCR amplification for 18S rRNA gene for P. vivax strains was done on 61 positive cases and 34 positive were positive and product size of 117 bp was obtained (Fig 2).Phylogenetic analysis of 18S rRNA gene of 13 P. vivax cases was done by using Clustal X 2.1 and viewed by Tree View X (Figure 3) . 18S rRNA of P.vivax local isolates were aligned with published 18S rRNA gene sequence of P. vivax accession no.KT 991239.1. Phylogenic analysis showed that 13 isolates with reference sequence formed 3 main sub clusters (Figure 4 and 5).First sub cluster was subdivided into two more sub clusters. Reference sequence

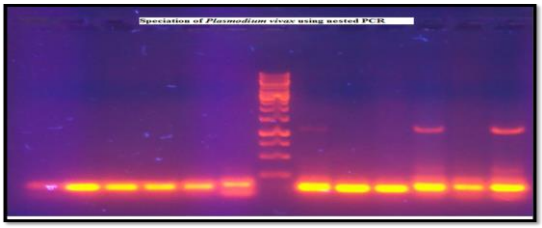

Fig 1: Gel Electrophoresis of Genomic DNA

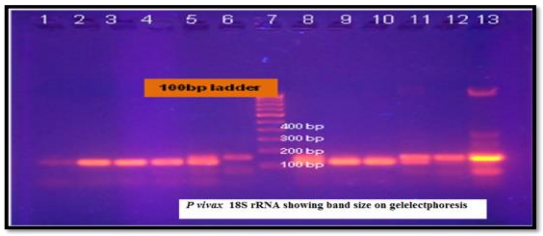

Fig 2: P.vivax 18S rRNA band size estimation

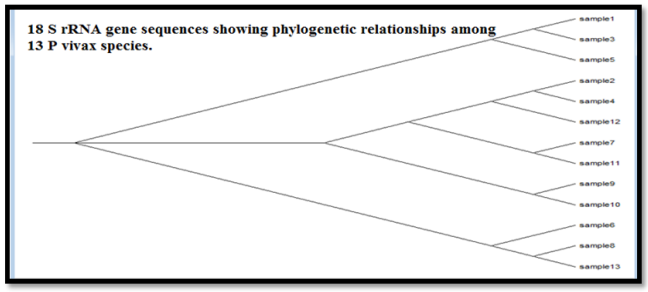

Figure 3: phylogenic relationship in 18SrRNA gene sequencing

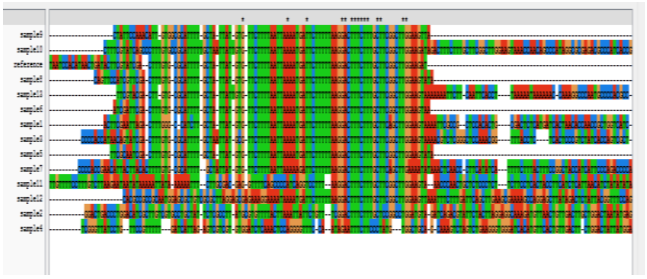

Fig 4 : Multiple sequence alignment in 18S rRNA gene

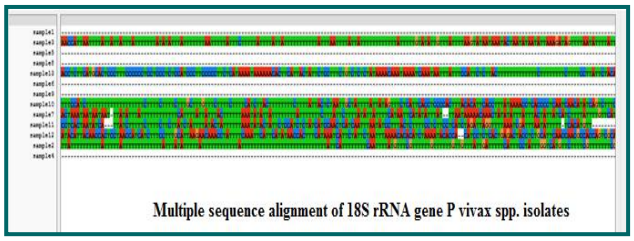

Fig 5: Multiple sequence alignment in 18S rRNA gene

KT991239.1 resided in 1st subdivision in sample 9 and 10.The second subgroup of 1st sub cluster is a larger one and has 5 isolates , clustered together. Sample 2/4/12/7/11 were present in this set, out of which sample 7 and 11 were more alike while 2/4/12 were similar having a little distance in between. The 2nd and 3rd sub clusters had 3 isolates in each 2nd sub clusters consisting of sample 1, 3 and 5 in which sample 5 which diverged in a separate cluster.3rd subcluster consisted of sample 6, 8 and 13 in which sample 6 diverged separately while sample 8 and 13 were linked and alike. Multiple alignments showed vast diversity in the obtained sequences. Various SNPs observed in the sequences including deletion, substitution and insertions.

Discussion

All isolates appeared to be highly polymorphic. The reason of the diversity may be the mixed infection of different plasmodium species in same host as well as transmitting of non human form of malaria to humans. This great genetic diversity may be a key reason of increasing drug resistance and incidence of malaria again. PCR based diagnosis may also fail because of

sequence variation in the primer regions. Phylogentic studies are important to identify the relatedness of human malaria parasite with zoonotic parasites.

Episodes of vector-born diseasees like dengue, Chikungunya, Crimean-Congo hemorrhagic fever and leishmaniasis are reported off and on in Pakistan but among them malaria is a constant and leading cause of morbidity. Rapid, reliable and species-specific validated tools are crucial for malaria diagnosis to catch unvisualised malarial parasite .[20] In this study we want to emphasize that application of various bioinformative tools are helpful to evaluate genomic dynamics on target gene for accurate malaria diagnosis, management and eradication.

P. vivax is regularly reported in Pakistan especially in Karachi. Discrimination of specific species and mixed infection are mandatory for proper treatment. No doubt light microscopy and rapid immunochromatographic assay are most used diagnostic tests for malaria infection but in mixed infections and low level of parasitemia, it is difficult to exactly interpret the results.[21] To overcome these difficulties, in present study the genus and species specific primer were designed and nested PCR technology was implemented for 1640 bp band size and 18S rRNA gene for distinguishing the genus and specific species.

In our study detection of malaria parasite by nested PCR was more sensitive than microscopy (p=0.0015).The sequencing of the P.vivax 18S rRNA provides undisputable confirmation of specific species in prevailing infections and any diversity is noted. Several studies done on national and international levels reported that this gene is validated marker for species recognition. [2, 9, 10, 17, 20- 25.]Therefore we chose the 18 S rRNA genes for species identification and genetic variations. There are many published studies which reported that most widely used target gene is 18S rRNA gene and all of them are in agreement with our study. [2, 9, 10, 17, 20- 25.] In present study we first amplified P. vivax 18S rRNA gene by nested PCR and obtained 117pb fragment. Later the sequence analysis of this gene was performed for detection of sequential variation. The data were analyzed by use of bioinformative tools like multiple sequence alignment and phylogenetic analysis that revealed vast diversity in the obtained sequences as well as various single nucleotide proteins (SNP) observed in the sequences including deletion, substitution and insertions. All isolates appeared to be highly polymorphic. Earlier studies also reported genetic diversity in this gene.[26, 27, 28, 29.] A recent study target the same gene and observed deletions and substitutions via sequencing.[30] Another study detected polymorphisms in 18S r RNA gene.[31.] Yet another study reported that genetic variation escapes the parasite from immune surveillance Whereas several other studies reported that this gene has unusual properties so they studied this gene with different angles and mostly observed various copy numbers and stages.[7, 32.] Other studies detected existence of multiple copies in this gene while some suggested that plasmodium qualitative assessment can made by detecting highly conserved regions and variable zones allowing species identification.[33, 24.] Whereas we only focused on species specification, phylogenetic analysis & genetic variation in 18S rRNA gene.

Conclusion

1.PCR detection of malaria parasite is more sensitive method for diagnosis as compared to Microscopy. 18S rRNA is a robust for evolutionary changes and use of bioinformatics tools and phylogenic relationship pave the way to assess genetic variation. So 18S rRNA is a validated diagnostic approach for precise species identification and epidemiologic studies.

2.Mix infection creates a dilemma when overlapping infection is detected in same patient. So for proper remedy molecular identification is best option. The molecular techniques need to be extended to identify additional target from Plasmodium species.

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