Development and evaluation of a 28S rRNA gene-based nested PCR assay for *P. falciparum* and *P. vivax*

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The 28S rRNA gene was amplified and sequenced from *P. falciparum* and *P. vivax* isolates collected from northwest India. Based upon the sequence diversity of the *Plasmodium* 28SrRNA gene in comparison with its human counterpart, various nested polymerase chain reaction (PCR) primers were designed from the 3R region of the 28SrRNA gene and evaluated on field isolates. This is the first report demonstrating the utility of this gene for species-specific diagnosis of malaria for these two species, prevalent in India. The initial evaluation on 363 clinical isolates indicated that, in comparison with microscopy, which showed sensitivity and specificity of 85.39% and 100% respectively, the sensitivity and specificity of the nested PCR assay was found to be 99.08% and 100% respectively. This assay was also successful in detecting mixed infections that are undetected by microscopy. Our results demonstrate the utility of the 28S rRNA gene as a diagnostic target for the detection of the major plasmodial species infecting humans.

Keywords: 28S rRNA gene, Nested PCR, *Plasmodium* detection, Malaria diagnosis, Surveillance tools

Introduction

Malaria is a vector-borne disease, widespread in tropical and subtropical regions. An estimated 243 million cases resulted in nearly 781 000 (approx. 1 million) deaths in 2009.¹ About half of the world's population is at risk of malaria. Emerging parasite resistance to antimalarials, increased burden of the disease, and mosquito resistance to insecticides are the major threats to achieving global malarial control. The manifestation of severe infection, involving multiorgan dysfunction caused by *P. falciparum*² and *P. vivax*³ warrant the formulation of suitable therapeutic interventions and enhancement of targeted research.

Till date, microscopy (Giemsa stained smears) remains the 'gold standard' for malaria diagnosis. It is relatively simple and economical for use at the field level. But, it is time consuming, labor intensive, and requires highly trained technicians to differentiate the species of the parasite involved.⁴ Rapid diagnostic tests (RDTs) were developed as an alternative to

microscopy. These are very simple, quick, easy to perform, and do not require specific equipment or resources. These antigen-based tests relied on histidine-rich protein2 (HRP2)⁵ or on *Plasmodium* lactate dehydrogenase (pLDH).⁶ Although devised for the field level as an adjunct to microscopy, it had varying sensitivities in different geographical distributions.

With the advent of polymerase chain reaction (PCR), various assays were developed, targeting the genes, *SSUrRNA*,⁷ *STEVR*,⁸ mitochondrial *COXI* and *COXIII*,⁹ *DHFR*,¹⁰ and conserved repeat sequences (CRS).¹¹ These were developed in various assay formats like nested PCR,^{7,10} multiplex PCR,^{11,12} LAMP,¹³ QT-NASBA,¹⁴ and real-time PCR.^{15,16} Polymerase chain reactions are highly sensitive and were used for unambiguous species identification. Being stable, highly conserved, less prone to mutations, and possessing a mosaic of conserved and variable regions, SSU rRNA gene has been a widely used molecule for the determination of the malarial species.

Although rRNAs are reportedly conserved in nature, there are regions, which are variable, and which can be used for the detection of malarial parasites ranging

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from genus to different species. At present, only a few species-specific molecules can be used as an alternative for the detection of malarial parasites. In this study, we describe the development and utilization of the 28S rRNA gene as a target for nested PCR-based species-specific detection of *P. falciparum* and *P. vivax*.

Materials and Methods

Patient samples

About 500 µl of blood samples was collected in EDT from patients of the SP Medical College, Bikaner (Rajasthan), India, and was shipped in cold chain to BITS-Pilani, Rajasthan. Written informed consent was obtained from the patients (parents/guardians of children) before inclusion in the study. The samples of patients who refused consent were omitted from this study. This study was approved by the Institutional Ethical Committee of BITS-Pilani and SP Medical College, Bikaner.

DNA purification

Complete DNA was isolated¹⁷ from this crude 500 µl of blood samples by treating it with lysis buffers containing NaCl, Tris-HCl (pH 8.0), EDTA, and SDS (1%) at 37°C for 45 minutes, and with Proteinase K (Sigma) at 50°C for 2 hours, followed by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and overnight ethanol precipitation at -20°C. The precipitated DNA was washed with 70% ethanol and the pellet was air dried and suspended in TE buffer (pH 8.0) and stored at -20°C. This was used directly for PCR reactions.

Microscopy

Thick and thin smears were made and the latter were fixed with methanol. Both the smears were Giemsa stained at room temperature and examined by expert

microscopists at SP Medical College, Bikaner. At least 100 fields were examined at 1000× magnification before a slide was considered negative.

Target characterization

Amplification and sequencing of the 28S rRNA gene of *P. falciparum* and *P. vivax*

The primers (Table 1) were designed based upon the comparative differences between *P. falciparum* and the human 28S rRNA gene. The whole gene was divided into three regions (1R, 2R, and 3R) and amplified separately with various primer combinations, out of which the successful ones were used to amplify *P. vivax* (without any human DNA amplification). The primers and conditions were similar for both *Pf* and *Pv* except the region R1, which was amplified by different primers. The reagents used were similar to those described in the Nested PCR Amplification section except that primer concentrations were 250 ng. The 28S rRNA gene from six isolates for *P. vivax* and three isolates of *P. falciparum* were partially sequenced (GenBank: JQ627143–JQ627148, JQ684657–JQ684659).

Analysis of sequences

The sequences were compared to the GenBank sequences using the NCBI/BLAST program (<http://www.ncbi.nlm.nih.gov/>). The multiple sequence alignments were performed using CLUSTALW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Target validation

Primer design and development of nested PCR

After careful analysis of the conserved and variable regions, the region 3R was found to be appropriate for the nested PCR assay and various primers were tested for specific amplifications. Out of these, three

Table 1 Primers and polymerase chain reaction (PCR) conditions used for the amplification of the 28S rRNA gene of *P. falciparum* and *P. vivax* from the clinical isolates. All the reactions were run for 30 cycles after the initial denaturation at 94°C for 3 minutes. R1, R2, and R3 indicate the regions of 28S rRNA gene amplified.

Gene region	Primer pair	Primer sequence	PCR Conditions			
			Denaturation (94°C) minutes	Annealing		Expected amplicon size
				Temperature (°C)/ Time (minutes)	Extension (72°C) minutes	
1R (<i>Pf</i>)	281	5' GTTTGATTGATGT TAGGAGGGC 3'	2	57.2/1.15	2	~1.2 Kb
	282	5' TAACTCAACTTGTA TTTCACTCATAT 3'				
1R1 (<i>Pv</i>)	28SPvF3	5' ATCCGCCGAATTTA AGCAT 3'	1	47/2	2	~1.2 Kb
	28SPvR3	5' CTGGTAAAATTATAT TAATTCAACT 3'				
2R (<i>Pf</i> and <i>Pv</i>)	AKD283	5' CATACATCCGGTAA GCAATTATGC 3'	1	53.4/1.30	2	~1.2 Kb
	AKD 284	5' GTTGGTACGCTCAT ACAAGTATTAA 3'				
3R (<i>Pf</i> and <i>Pv</i>)	DCAKD 285	5' TTATGAATTATCCGA AGTGGTAAGG 3'	1.15	52.7/1.30	2	~1.8 Kb
	DCAKD 286	5' AAACACTTTACCACT AGGGATAAGC 3'				

pairs of primers that specifically amplified the corresponding gene fragment were chosen and used for evaluation on clinical isolates.

Nested PCR amplification

Polymerase chain reaction amplifications were performed in a BioRad MJ Cycler. First reaction incorporated 4 µl of purified DNA, 200 µM each of dNTPs (dATP, dTTP, dGTP, dCTP) (Finnzymes, Vantaa, Finland), 160 ng of each primer NUF (5'GAT TTC TGC CCA GTG CTT TGA ATG T 3') and NUR (5'AAT GAT AGG AAG AGC CGA CAT CGA A 3'), 1 U of Taq DNA polymerase (Bangalore Genei, Bengaluru, KA, India), and 1 × of Taq Buffer (Bangalore Genei) for a 50 µl reaction. One cycle of PCR consisted of initial denaturation at 94°C for 3 minutes, followed by 30 cycles, comprising denaturation at 95°C for 30 seconds, annealing at 50.7°C for 45 seconds, and extension at 72°C for 40 seconds for 30 cycles. The expected amplicon sizes are 771 bp for *Pf*, 775 bp for *Pv*, and 770 bp for human 3R region of the part of 28S rRNA gene amplified. The resulting amplicons were further used as templates for the nested amplifications.

Nested amplifications were performed in separate reaction tubes for the species-specific primers, using 0.75 µl of the PCR-amplified template from the first reaction, 200 µM each of the dNTPs (dATP, dTTP, dGTP, dCTP), 250 ng of each primer (NPF1-5'TAT CCT TCG GGA AGG CAT TCT G 3' and NF2-5'CTA TAT GCA CAG TAG TAA GTA ATT TA 3' for *P. falciparum* and NPv2-5'TCG GTT CGC CGG GTA TTC ATA TT 3' and NPvR-5'CAC AGT AGG AAG ATA AAT TCC 3' for *P. vivax*), 1 U of Taq DNA Polymerase, and 1 × of Taq Buffer for a 50 µl reaction. For *P. falciparum*, one cycle of PCR consisted of initial denaturation at 94°C for 3 minutes, followed by 30 cycles, comprising of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 40 seconds. For *P. vivax*, one cycle of PCR consisted of initial denaturation of 3 minutes, followed by 30 cycles, comprising of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds. The expected sizes are 297 bp for *Pf* and 294 bp for *Pv*. The resulting amplicons were analyzed on 1.5% Agarose gel. Sterile MilliQ water and uninfected human DNA were used as negative controls.

Limits of detection

For *P. vivax*, the recombinant plasmid (pBS SK+), harboring the 3R region of the 28S rRNA gene was diluted from 100 000 copies to 0.1 copies (10 fold serial dilution) per reaction (which was calculated to be equivalent to 25 000 to 0.025 parasites respectively per 50 µl reaction) and spiked with 2 µg of uninfected

human DNA before use. For *P. falciparum*, the templates included the dilutions of 3D7 DNA from 100 pg to 0.001 pg/50 µl reaction, (which was calculated to be equivalent to 5 000 to 0.05 parasites/50 µl reaction respectively), mixed with 2 µg of uninfected human DNA. The limits were assessed in triplicates separately.

Data analysis

Diagnostic performance characteristics of microscopy and nested PCR were calculated.¹⁸ The test indices measured were the number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). Sensitivity was calculated as TP/(TP+FN), specificity as TN/(TN+FP), positive predictive value (PPV) as TP/(TP+FP), and negative predictive value (NPV) as TN/(TN+FN). The test efficiency was determined using the formula $1 - (FN + FP / \text{Total}) \times 100$. Also, the 95% CI (confidence interval) for sensitivity and specificity was calculated according to the equation: $p \pm 1.96 \times \sqrt{p(1-p)/n}$, where p =point estimate of sensitivity (or specificity) and n =number positive or negative with the standard. The data was analyzed in Microsoft Excel 2007.

Results

Amplification and sequence analysis of the 28S rRNA gene

Various primers were designed (Table 1) and used to amplify the three regions (1R, 2R, and 3R) of the 28S rRNA gene from *P. falciparum* and *P. vivax*. The sizes of the amplicons were ~1.2 Kb, ~1.2 Kb, and ~1.8 Kb for the three regions respectively (Fig. 1). These were then sequenced and analyzed for species-specific regions, amenable for the design of Nested PCR primers. BLAST analysis revealed the presence of four copies of the gene sequence amplified by this nested PCR for *P. vivax*, located on chromosomes 2, 3, 5, and 10, whereas six copies of the gene sequence for *P. falciparum* located on chromosomes 1, 5, 7, 8, 11, and 13 were revealed. Sequence similarity analysis of the 28S rRNA genes also suggests that the gene on chromosomes 5 and 7 are expressed during the blood stages (A-type) and 11 and 13 during the mosquito stage of the parasite (S-type). The A-type gene differs from the S-type gene by 17% in *P. falciparum*. Similarly, for *P. vivax*, the 28S rRNA gene on chromosomes 3 and 10 are expressed during the blood stages (A-type) and 2 and 5 during the mosquito stage of the parasite (S-type). The A-type gene differs from the S-type gene by 20% in *P. vivax*. Comparative analysis with the sequences available in the GenBank revealed the size of the 28S rRNA gene as 3788 bp for *P. falciparum*, 3892 bp for *P. vivax*, 3785 bp for *P. yoelii*, and 3789 bp for *P. berghei*. All these were found to harbor ~60% AT and ~40% GC nucleotides. About 96% similarity was observed

Figure 1 Multiple sequence alignment of ~770 bp region of *Pv* (acc no: JQ627143), *Pf* (acc no: U21939), and human (acc no: M11167) R3 region of the 28S rRNA gene, trimmed to region. The boxes represent the primers used in the study. Asterisk (*) indicates the similarities and dash (-) indicates the absence of base relative to another sequence. The primers sequences used are denoted as boxed.

between *P. yoelii* and *P. berghei* 28S rRNA A-type genes. The *P. falciparum* 28S rRNA gene was 85% similar to *P. vivax* and approximately 40% similarity was observed between all the plasmodial spp and their human counterparts.

Nested PCR assay design and usage on field isolates

The *Pf* and *Pv* 28S rRNA genes were found to have 85% similarity with each other while the human counterparts had only 42% similarity with the two species under comparison. Based upon the region 3R of the 28S rRNA gene, various primers were then designed and used in combinations for the nested PCR assay. The primers, with which non-specific amplification or cross reactivity was observed (i.e., amplifying human DNA with *Pf* primers or *Pf* sample with *Pv* primers or vice versa, even at relatively high stringencies), were excluded from this study. This was initially done based on multiple sequence alignments and subsequently, the primers were evaluated with parasite DNA and patient material. Only the primers that showed proper amplification were chosen for the assay. The assay using initial primers NUF and NUR followed by the nested primers NPF1 and NF2 for *P. falciparum*, and NPV2 and NPVR for *P. vivax* (Fig. 2), gave excellent sensitivity. The schemata are illustrated in Fig. 3 and the location of the working primers in Fig. 4.

Detection limits of the nested PCR

When tested on the *P. falciparum* 3D7 cultured isolate DNA, the nested PCR reproducibly amplified till 0.01 pg or 0.5 parasites/reaction whereas, with the

Figure 2 Schematic representation of the arrangement of rRNA genes and the nested polymerase chain reaction (PCR) target. The rRNA operon represented as present in the plasmodial species is shown with the relative locations of the primers used in this study. The NUF and NUR (gene-specific and universal, i.e., human and plasmodial spp) primers target a part of the third region (3R) of the 28S rRNA gene and produce ~770 bp amplicon in the first round of PCR. Further, the nested amplifications (species-specific) produce a 297 bp amplicon for *P. falciparum* and 294 bp for *P. vivax* with their species-specific primers. The orientations of the primers were indicated by an arrow head and the sizes of the products were denoted as base pairs.

Figure 3 Agarose gel (1.0%) image of the 28S rRNA gene amplicons. 1=*Plasmodium vivax* 28S rRNA gene R1 amplicon (~1.2 Kb), 2=R2 amplicon (1.2 Kb), and 3=R3 amplicon (~1.8 Kb bp). M=1 Kb DNA Ladder Mix (MBI Fermentas #SM0331).

clone 3R, a limit of 0.1 copies or 0.25 parasites/reaction was attained for *P. vivax* (Fig. 6).

Evaluation of the nested PCR assay on field derived samples

Microscopy

Out of the 363 samples tested, microscopy identified 29.47% (107/363) as *P. falciparum*, 26.44% (96/363) as *P. vivax*, 3.03% (11/363) as mixed infections (*Pf*+*Pv*), and 41.04% (149/363) as negative (uninfected).

Nested PCR

With nested PCR, 25.89% (94/363) of the samples were identified as *P. falciparum* infected, 23.41% (85/363) were identified as *P. vivax* infected, and 10.46% (38/363) of the samples were identified as mixed infections (*Pf*+*Pv*). Out of the samples, 40.22% (146/363) were identified as uninfected. Ninety *Pf* and 83 *Pv* were found to be true mono infections by the two methods used. The ^a15/105 results of *Pf* and ^b12/95 of *Pv*, identified by microscopy as *Pf* and *Pv* mono-infections respectively (Table 2), were in fact mixed infections as diagnosed by the nested PCR. Out of this, 11 *Pf* and 9 *Pv* (diagnosed as monoinfections by microscopy) were also confirmed as mixed infections by the multiplex PCR.¹² The remaining seven samples

Figure 4 Agarose gel (1.5%) image of the nested PCR amplicons. 1=Universal first round PCR amplicon (~790 bp), 2=Nested *P. falciparum* amplicon (286 bp) and 3=Nested *P. vivax* amplicon (294 bp) 4=Negative control (uninfected sample), M=1 Kb DNA Ladder Mix (MBI Fermentas #SM0331).

were visible as very faint amplicons (with the *P. falciparum* and *P. vivax* specific nested primers) and this was assumed to be due to low parasitemia. These were considered to be TP for the mixed infections by nested PCR and FN for the species concerned in the case of microscopy. Further, three *Pf* and two *Pv* samples were unidentified by microscopy. This was also assumed to be due to low parasitemia in the samples. One *Pf* and one *Pv* sample each were identified as uninfected by nested PCR, which tested positive by microscopy. A faint band in the gel would be expected in the case of low parasitemia but this couldn't be visualized and therefore, these two samples were treated as FN by nested PCR. No amplification was observed with the uninfected DNA and water, which were used as negative controls. Thus, the nested PCR was taken as a standard and performance indices were further calculated (Table 3)

Cross reactivity with other species

No cross reactivity was observed when tested with *P. malariae* and *P. berghei* DNA. Although the universal primers produce an amplicon, for the 28S rRNA gene, the species-specific primers for *P. falciparum* and *P. vivax* showed no amplification with the species tested (data not shown). Apart from *Pf* and *Pv*, *Pm* was reported to be sporadic in different parts of India and *P. ovale* is reported to be rare. *P. knowlesi* has not been reported to date. So, this assay was limited to the two species predominant in India (*Pf* and *Pv*).

Table 2 Test results for the 363 samples as identified by microscopy and nested polymerase chain reaction (PCR). The 15^a and 12^b isolates, identified by microscopy as *Pf* and *Pv* monoinfections were identified as mixed infections by nested PCR

Microscopy	Nested PCR				
	<i>P. falciparum</i>	<i>P. vivax</i>	Mixed (<i>Pf</i> + <i>Pv</i>)	Negative	Total
<i>P. falciparum</i>	91	—	15 ^a	1	107
<i>P. vivax</i>	—	83	12 ^b	1	96
Mixed (<i>Pf</i> + <i>Pv</i>)	—	—	11	—	11
Negative	3	2	—	144	149
Total	94	85	38	146	363

Figure 5 (A) Initial polymerase chain reaction (PCR) assay on various field isolates showing ~770 bp amplicons with universal and gene specific first round primers as viewed on 1% agarose gel. M=1 Kb DNA Ladder Mix (MBI Fermentas #SM0331). All the samples were positive by the initial PCR, which serves as a DNA control for the PCR. (B) and (C) indicate nested *P. falciparum* and *P. vivax* amplicons respectively. The lanes 4 and 8 were positive for *P. falciparum*, 1, 2, 3, 5, 6, 12, 13, 15, 16, and 17 were positive for *P. vivax* and 9 was a mixed infection (*Pf*+*Pv*). The lanes 7,10,11,14, and 18 were true negatives.

Discussion

In light of the ongoing increase in the prevalence of antimalarial drug resistance^{19–21} and the severity of the disease caused by the *P. falciparum*^{2,22} and *P. vivax*,^{23–25} accurate diagnosis is a prerequisite for prompt therapy, control, and efficient epidemiological surveillance.

Till date, peripheral blood smear microscopy has been considered to be the reliable and most practical means for the detection of malarial parasites in human blood samples,⁴ mostly because of its usability under field conditions and economic viability.

But, it is time consuming and also requires a well trained and experienced microscopist. Furthermore, its sensitivity in low parasitemia detections in the samples is limited.²⁶ Polymerase chain reaction assays overcame this limitation by the amplification of Plasmodial DNA in the blood samples in various assay formats. Various molecular gene targets (like 18S rRNA, cytochrome b, DHFR, COX III, CRS) have been investigated for parasite detection. As the rRNA genes are found to be highly conserved within species and less prone to recombination or mutations, the 18S rRNA gene-based assays have been widely used till date for the detection of malarial parasites.

In this study, the 28S rRNA gene was PCR-amplified (Fig. 4) and sequenced from Indian isolates (Rajasthan) and a nested PCR assay was developed (Fig. 3 and Fig. 5). This assay was also evaluated (Fig. 6) for the detection of *P. falciparum* and *P. vivax*, which are predominantly found in most parts of India. The malaria parasite is reported to optimize protein synthesis during growth in different environments of the host and mosquito vector by maintaining different ribosome types.²⁷ Earlier studies on the 18S rRNA report that rDNA of the parasite encodes two structurally distinct rRNAs that occur in different developmental stages. The C gene transcripts represent the predominant rRNA species in the mosquito stage of the parasite, whereas the A gene defines the predominant transcripts in liver and

Figure 6 Limits of detection of the *P. falciparum* and *P. vivax* specific nested polymerase chain reaction (PCR). The 10 fold dilutions of the 100 pg 3D7 DNA till 0.001pg for *P. falciparum* (A) and 100 000 to 0.1 copies of the 3R clone of *P. vivax* (B). M indicates 1 Kb DNA ladder mix.

blood stage parasites in the vertebrate host.²⁸ The third O type rRNA (18S) was also demonstrated only in *P. vivax*.²⁹

Upon comparison, three *Pf* and two *Pv* mono infections were unidentified by microscopy, the nested PCR assay could not identify two samples that were positive by microscopy (Table 2). Microscopy identified 11/38 (28.94%) as mixed infections with *Pf* and *Pv*. Significantly, 27 of the 38 mixed infections were not identified by microscopy. As a whole, 32 FN were observed with microscopy. Such discrepancies have also been reported earlier where the diagnostic assay being evaluated was found to be far more sensitive than the reference method.³⁰ The detection limits for this nested PCR was comparable to other protocols using 18S rRNA gene. Due to this reason, the discrepant samples were also analyzed by a multiplex PCR, thus corroborating the high sensitivity of the PCRs. The detection limit for *Pf* was observed till 0.01 pg (or 0.5 par) and single copy for *Pv* (or 0.25 par). The variation in the detection limit could be attributed to the templates taken for the PCR (3D7 cultured DNA for *Pf* and 3R clone for *Pv*). This was not assessed on the blood samples due to the unavailability of the parasitic samples with confirmed parasite density. Although lesser than the cytB PCR,³¹ which had a limit of 0.75 parasites per 1 µl of DNA taken, it was found to be higher than a multiplex PCR,³² which detected upto four copies of the target gene. A real-time PCR³³ showed the lowest limit till 0.002 parasites, which was advantageous due to the fluorescent output that outnumbers the human interpretation on agarose gel. This was also comparable with other 18S rRNA-based PCR,⁷ which detected upto 0.4 parasites per 1 µl. It has also been reported earlier that the detection threshold of microscopy was 5–50 parasites per 1 µl depending on the microscopist's experience. But an average microscopist is most likely to achieve a sensitivity of upto 100 parasites per 1 µl or higher.^{34–35} Many reports incorporating PCR results also suggest the drawback of microscopy in detecting low parasitemic samples.

Mixed infections in malaria are often underestimated due to various reasons when microscopy alone is used as a screening method.^{36,37} Varying reports are available related to mixed infections with severe malaria. Series of reports from India,³⁸ Indonesia,³⁹ Malaysia,⁴⁰ and Papua New Guinea³⁷ reported higher frequencies of severe malaria cases with

mixed infections. However, earlier reports from Thailand^{41,42} summarized lower frequencies of severe malaria with mixed (*Pf*+*Pv*) infections where the severity of *P. falciparum* was decreased on co-infection with *P. vivax*. A high proportion of mixed infections (45% of the total infected samples) have also been identified from various parts of India (south and northeast)⁴³ as detected by Snounou's nested PCR assay.⁷

In this assay, outer primers that can amplify both humans and *Plasmodial* 28S rRNA genes were used. Excessive and competitive amplifications of human DNA could be expected to interfere with sensitivity and probably specificity of malarial species-specific amplification in the secondary PCR assay. But, no such spurious amplifications were observed in this study. Mixed infections are the most challenging for diagnosing malarial infections, especially for a microscopist. A physician requires accurate and sensitive diagnostic tools for prescribing the optimal therapeutic regimen. During PCR amplification of mixed-species infections, the species, present in relatively higher concentrations will frequently dominate over another that is present in relatively low concentrations,^{44,45} with primer binding energies contributing to maximum extent of amplification. The initial amplification targets the human and also plasmodial 28S rRNA gene. This forms an inbuilt positive control for PCR failures. The second reaction (nested) is species-specific (*Pf* and *Pv*). This nested PCR assay surpassed microscopy with 99.08% sensitivity and 99.45% efficiency when tested on the samples isolated from Bikaner, in the northwestern part of India where serial episodes of *Pf* and *Pv* with mixed infections are routinely seen.³⁸ With the emergence of drug resistance, the ability to differentiate mixed infections with minor species is crucial as the first line drugs used for the other species vary according to the geographical location and resistance pattern. The nested PCR described here successfully detected all the mixed infections, even more than multiplex PCR.

Although the 18S rRNA gene was widely used as a prototype for many PCR assays targeting plasmodium till date, this is the first study investigating the use of 28S rRNA gene for the detection of *P. falciparum* and *P. vivax*. Being present in all the plasmodia, this would be an excellent candidate for various nucleic acid-based assays. This target offers a promising alternative to the current targets available to

Table 3 Comparison of sensitivities, specificities, positive predictive values (PPVs), negative predictive value (NPVs), and efficiencies, of microscopy and nested polymerase chain reaction (PCR) for the detection of *P. falciparum* and *P. vivax* in the 363 samples tested. The 95% confidence interval (CI) are represented in the parenthesis

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)
Microscopy	85.39 (81.75–89.02)	100 (100–100)	100 (100–100)	81.82 (77.85–85.79)	91.18
Nested PCR	99.08 (98.11–100)	100 (100–100)	100 (100–100)	98.63 (97.43–99.83)	99.45

detect malarial species. The novel primers and optimized reaction conditions of this assay enables significantly higher sensitivity for the detection of *P. falciparum* and *P. vivax* DNA in tissues suspected of harboring the respective parasites and is thus helpful in detecting mixed infections. The high sensitivity of this method, targeting the 28S rRNA gene provides a valuable addition for the epidemiological studies routinely done for malaria eradication programs in areas where only *P. falciparum* and *P. vivax* coexist like in India.

Conclusion

This study demonstrates the use of the 28S rRNA gene as a new target for the detection of *P. falciparum* and *P. vivax* from clinical isolates by nested PCR, till the detection limit of 0.5 parasites for *P. falciparum* and 0.25 parasites for *P. vivax*. This could also be further extended to other species once the genome information is available. Further studies using larger cohorts from diverse geographical locations will be useful to establish this assay.

Conflict of Interest

We declare no conflicts of interest.

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