



Research Article

## Evaluating the efficacy of a *Plasmodium* species-specific Multiplex-Nest-PCR in malaria diagnosis using different DNA isolation methods

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**Abstract:** Malaria diagnosis and speciation still rely on microscopic identification in many settings. But, microscopy is tedious and lack sensitivity, particularly in areas under advanced eradication programs where low-density infections are increasingly reported. Species-specific molecular techniques are highly sensitive and reliable alternatives for *Plasmodium* parasites detection and speciation. Nevertheless, the efficacy of molecular techniques is directly affected by the purity and quality of isolated DNA templates. A *Plasmodium* species-specific multiplex-nested-PCR was assessed using DNA templates prepared separately by phenol-chloroform method, a DNA-precipitation commercial kit, and a chromatographic commercial kit from 126 EDTA-preserved whole blood samples. 115 samples were collected from malaria suspicious febrile patients in endemic southern region and 11 malaria positive samples from foreign and national visitors of non-endemic western region of Saudi Arabia. In total, 89 specimens were found malaria positive by at least one diagnostic method, out of which 82 (92%) were detected by microscopy. *P. multiplex-N-PCR* revealed 89 (100%), 77 (86.5%), and 85 (95.5%) positive samples using DNA templates extracted by the chromatographic kit, the DNA-precipitation kit, and phenol-chloroform standard method, respectively. *P. falciparum* parasites were detected in 86 samples and *P. vivax* in three samples from foreign visitors. Thus, *P. multiplex-N-PCR* applied to DNA templates isolated by chromatographic method achieved the highest sensitivity and was particularly useful for submicroscopic malaria cases in the endemic area where intensive elimination efforts are being deployed.

**Keywords:** Malaria diagnosis, *Plasmodium* speciation, Multiplex-nested-PCR, EDTA-Blood, DNA templates.

### 1. Introduction

Malaria is the most important vector-borne disease in the world, particularly prevalent in the tropics and subtropics presenting a major impact on public health and economy [1]. Malaria is caused by five species of protozoan parasites belonging to the genus of *Plasmodium*; namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. The first four species are vector-transmitted between humans but *P. knowlesi* is a confirmed zoonotic form typically transmitted to humans from macaque monkeys [2,3]. About 70 species of blood-feeding *Anopheles* female mosquitoes are recognized as vectors of *Plasmodium* parasites to humans [4]. In 2015, according to estimates of the World Health Organization, over 212 million people were infected with malaria parasites and 429 thousand deaths occurred globally, particularly from *P. falciparum* malaria from [5]. Approximately, 1.6

million people live in areas where malaria is transmitted in Saudi Arabia. Autochthonous malaria cases are mostly registered in southern region of the country, where the disease is mainly caused by *P. falciparum*, accounting for about 90% of reported cases [6]. Cases of *P. vivax* and *P. malariae* have also been reported in the country [7]. Although significant decrease of locally transmitted malaria was achieved during the last decades after remarkable malaria control efforts, there is always a major risk for reintroduction of parasites by millions of visitors coming from endemic countries for work or pilgrimage to holy places, in particular to Makkah city [8,9]. It has been reported that a considerable number of pilgrims carrying malaria parasites visits annually the country [10].

Accurate malaria diagnosis is acknowledged as a key element for the success of malaria control and elimination programs [11,12]. Although inappropriate use of antimalarials is contributing seriously to malaria

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drug resistance development worldwide, it is still a common practice to rely on clinical signs only for treatment prescription, particularly during transmission seasons [13]. By another hand, microscopic examination of Giemsa-stained blood films remain the gold standard laboratory technique for diagnosis of malaria playing a key role almost worldwide [14]. But, this tedious method is reliable only in hands of well-trained microscopists and is very limited by the level of parasites in circulating blood [15]. The important impact of asymptomatic and submicroscopic malaria cases is of great concern as it is being ever more recognised worldwide [16-18]. More progress will be achieved in malaria control more will be the need of highly sensitive diagnostic tools to detect individuals with low parasitaemia levels who may sustain the transmission [19].

Molecular tools, namely PCR and PCR-modified techniques like multiplex, nested, and real-time PCR are shown to be the most sensitive and trustworthy alternatives to classical diagnostic methods such as microscopy and antigen detection tests, especially in regions with high incidence of asymptomatic submicroscopic cases [20]. Several PCR-derived techniques had proven during experimental and field investigation to be highly effective for detection of asymptomatic and submicroscopic cases [21-23]. In this study, we investigated a *Plasmodium* species-specific multiplex-nest-PCR targeting specific regions onto *ssrRNA* genes of all human malaria species except *P. knowlesi*, previously reported [24]. In parallel, three different methods for DNA templates preparation from whole EDTA-preserved blood samples were assessed.

## 2. Materials and Methods

### 2.1. Blood sample collection

A total of 115 blood specimens were collected from febrile patients complaining of malaria associated symptoms, as considered by physicians, in health care centres of five localities in Saudi Arabia malaria endemic South region between 2010 and 2015, after their consent. 11 malaria positive samples were collected from foreign and national visitors of Holy Makkah city in western Saudi Arabia considered malaria non-endemic area, after their consent. Specimens were collected and transported in EDTA-treated tubes. Three microscopically confirmed negative blood samples from healthy individuals living in a non-endemic area were used as negative controls. Stored DNA obtained from human *Plasmodium* species reference strains, kindly provided by Liverpool School of Tropical Medicine, were used as positive controls.

### 2.2. Microscopic examination

Thick and thin blood smears were routinely prepared for malaria diagnosis for febrile patients in South endemic regions on arrival to health care centres, particularly during high transmission season. Only thin

blood films were fixed in methanol. Both films were stained using 1% Giemsa solution. Stained smears were then examined twice by two expert microscopists using x100 objective. Parasitaemia level was assessed on thick smears and determined as 1+ for 1-10 parasites per 100 fields; 2+ for 11-100 parasites per 100 fields; 3+ for 1-10 parasites per a single field; and 4+ for more than 10 parasites per single field according to WHO standards [25]. At least 100 thick film fields were examined by each microscopist before a slide was considered negative. *Plasmodium* parasite species were determined using thin blood films.

### 2.3. DNA templates preparation

Genomic DNA was extracted from each EDTA-preserved whole blood sample by three different methods;

- (i) QIAamp<sup>®</sup> DNA Blood chromatographic commercial kit (Qiagen, Hilden, Germany) using 200µl of blood,
- (ii) Jena-Biosciences<sup>®</sup> DNA isolation/precipitation kit (Jena Bioscience GmbH, Germany) using 300µl of blood, and
- (iii) Phenol-chloroform-isoamyl alcohol standard method using 200µl of blood.

Extracted DNA templates were eluted in final volumes equivalent to the original blood samples used in each method, 200µl for QIAamp<sup>®</sup> and PCI, and 300µl for JB<sup>®</sup> extractions, in recommended buffers.

### 2.4. *Plasmodium* species-specific multiplex-nested-PCR

Two-steps multiplex-nested-PCRs were used for the amplification of specific DNA regions of *Plasmodium* parasites *ssrRNA* genes [24]. DNA amplifications were carried out in two reactions; a first conventional *Plasmodium* genus-specific PCR reaction using rPLU6: TTA AAA TTG TTG CAG TTA AAA CG and rPLU5: CCT GTT GTT GCC TTA AAC TT primers, followed by two different multiplex-nested reactions, one for the detection and differentiation of *P. falciparum* and *P. vivax* species using two pairs of primers: (*PfF*: TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT / *PfR*: ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC) specific to *P. falciparum* and (*PvF*: CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC / *PvR*: ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA) specific to *P. vivax*, and a separate reaction using (*PoF*: ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA / *PoR*: GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG) specific to *P. ovale* and (*PmF*: ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC / *PmR*: AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA) specific to *P. malariae*, for detection and differentiation of these two *Plasmodium* species. The rPLU6/rPLU5 first PCR reaction was carried out in a final volume of 25µl

containing 1.5µl template DNA obtained by QIAamp<sup>®</sup>, JB<sup>®</sup> kits, or PCI methods, 2mM of each primer, and 12.5µl of 2x HotStart<sup>®</sup> Taq Master-Mix (Qiagen, USA). The following thermocycling scheme was used in the first reaction: 95°C/15min, 40 cycles of (94°C/30sec, 57°C/35sec, 62°C/70sec) and a final extension step at 65°C/5min. The multiplex-nested reactions were performed using 2µl of the first PCR products in a total volume of 25µl under the following conditions: 95°C/15min, 40 cycles of (94°C/30sec, 54°C/30sec, 66°C/40sec) and 66°C/5min for final extension. Amplicons of 205bp, 120bp, 800bp, and 144bp are expected if specific DNA of *P. falciparum*, *P. vivax*, *P. ovale*, or *P. malariae* is present in analysed specimens. PCR products were separated by electrophoresis on 1.4% agarose gels alongside a 100bp scale DNA ladder and visualized using EtBr staining.

### 3. Results

In total, 82 out of the 115 blood samples collected from febrile patients in five Saudi southern malaria endemic localities during high transmission seasons between 2010 and 2015 were confirmed on site as *P. falciparum* malaria positive by expert microscopists. No other *Plasmodium* species were detected among all

Saudi and foreigner patients in these localities. Out of 11 blood samples obtained from visitors in Makkah hospitals, 8 samples showed *P. falciparum* and three samples *P. vivax* by microscopy. Variable parasitaemia levels, ranging from 1+ to 4+, were reported by examination of positive thick blood smears in this study (Table 1).

Agarose gels of separated *P.* genus-specific rPLU6/rPLU5 PCR and the two following multiplex-N-PCR products obtained using DNA templates extracted separately by QIAamp<sup>®</sup>, JB<sup>®</sup>, and PCI are presented herein as a model (Fig. 1). No PCR products were seen with DNA templates isolated by the three methods from blood samples of healthy controls. Seven samples collected from malaria clinically suspicious people, but negative by microscopy, were found *P. falciparum* positive by Multiplex-N-PCR. In total, 89/126 samples were found positive by at least one method. 79 were determined both, by microscopic examination of thin blood smears and multiplex-N-PCR, as *P. falciparum* and 3 as *P. vivax*. The three vivax positive samples were from three foreigner visitors of Makkah holy city. Sensitivity, specificity, and predictive values of each diagnostic procedure were calculated relative to these findings (Table 2).

**Table 1. Results of microscopic examination carried out by health care laboratory specialists. Parasites were identified as *P. falciparum* except for 3 Makkah visitors identified as *P. vivax* (#v).**

Study Area	Localities	Negative	Parasitaemia				Total Positive
			1+	2+	3+	4+	
Endemic	Samtah	12	12	13	7	3	35
	Al-Ardah	7	2	3	4	2	11
	Baisha	6	4	4	2	1	11
	Jizan	13	1	1	1	2	5
	Tiwal	3	1	0	2	2	5
	Fifa	3	2	0	1	1	4
Non endemic	Makkah	*NA	1	3 (1v)	4 (2v)	3	11 (3v*)
<b>Total</b>		44	23	24	21	14	82

\*NA: not applicable

**Table 2. Relative sensitivity, specificity, positive and negative predictive values of *Plasmodium* species-specific multiplex-nest-PCR using DNA templates prepared by chromatography (QIAamp<sup>®</sup>), precipitation (JB<sup>®</sup>), and phenol-chloroform-isoamyl alcohol (PCI) versus microscopic findings in health care centers.**

Diagnostic technique	Specimens	Sensitivity (%)	Specificity (%)	+ve P.V. (%)	-ve P.V. (%)
Microscopic examination	Thick blood smear	82/89 (92.1%)	37/37 (100%)	82/82 (100%)	37/44 (84.1%)
	QIAamp <sup>®</sup>	89/89 (100%)	37/37 (100%)	89/89 (100%)	37/37 (100%)
<i>P. species-specific</i> multiplex-nest-PCR	JB <sup>®</sup>	77/89 (86.5%)	37/37 (100%)	77/77 (100%)	37/49 (75.5%)
	PCI	85/89 (95.5%)	37/37 (100%)	85/85 (100%)	37/41 (90.2%)

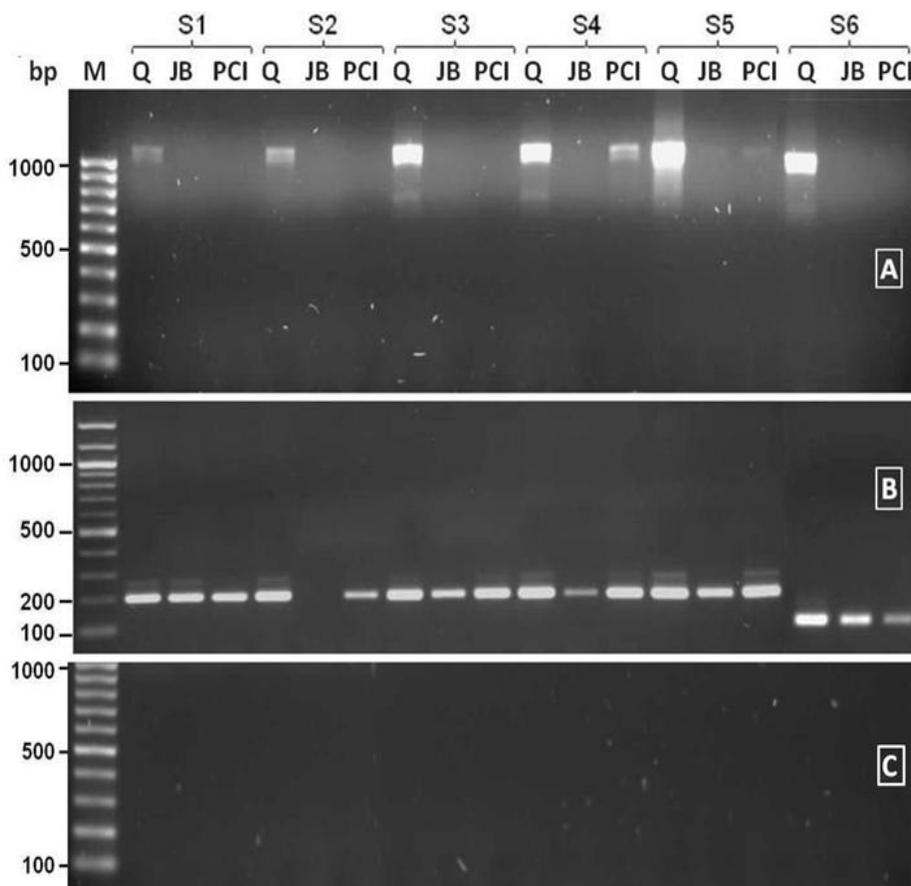


Fig. 1. Representative agarose gels of *Plasmodium* genus-specific rPLU6/rPLU5 first PCR (panel A), *Pf/PfR+PvF/PvR* multiplex-nested-PCR (panel B), and *PoF/PoR+PmF/PmR* multiplex-nested-PCR (panel C) products of blood samples' DNA templates isolated by QIAamp® (lanes Q), JB® (lanes JB), and phenol-chloroform-isoamyl alcohol (lanes PCI). Samples (S1 to S5) are *P. falciparum* positive and sample (S6) is *P. vivax* positive. A 100bp molecular weight marker was separated into lanes M.

#### 4. Discussion

Despite the significant progress achieved towards malaria elimination in Saudi Arabia, the disease is still occurring in endemic southern region where *P. falciparum* is predominant [26]. In addition, imported malaria cases are being increasingly reported, including, among pilgrims in Makkah holy city [8,9]. Like elsewhere, malaria laboratory confirmation and *Plasmodium* species identification still rely on microscopic examination in Saudi health care settings despite its low sensitivity with asymptomatic chronic carriers whom identification is crucial for the achievement of sustainable malaria elimination [27]. It has been well established that more progress is being achieved towards the elimination of malaria more the need of highly sensitive diagnostic tools to detect submicroscopic cases for their prompt treatment [28]. Molecular diagnostic tools, namely PCR techniques had proven highly sensitive for *Plasmodium* parasites identification and speciation in many health and research settings [21,29].

In this study, we evaluated a 2-steps *Plasmodium* species-specific multiplex-nested-PCR targeting

specific DNA regions onto *ssrRNA* genes as a possible alternative to microscopy in Saudi Arabia [24]. The usefulness of specific DNA sequences of *ssrRNA* genes in malaria diagnosis, speciation, and detection of mixed infections was confirmed experimentally [30]. Since the performance of such techniques is directly affected by the quality of isolated DNA templates [23], we used DNA templates from 126 collected blood samples prepared by three different methods, QIAamp®, JB®, and PCI. In total 89 samples were found *P. falciparum* positive by at least one diagnostic procedure, of which 82 were positive by microscopy and 7 were negative by microscopy, but *falciparum* positive by multiplex-N-PCR and were considered then as submicroscopic clinical cases. It has been previously reported that submicroscopic cases in endemic areas are much more prevalent than previously estimated and can be detected only by sensitive molecular tools [31,32]. As shown in Fig. 1 and Table 3, QIAamp® chromatographic kit provided DNA templates with the highest quality for PCR amplification by multiplex-nested-PCR achieving a 100% relative sensitivity compared to 95.5%, and 86.5% obtained with DNA templates isolated by PCI and JB® methods, respectively. QIAamp® platform has

been reported as one of the most efficient tools for humans and pathogens DNA recovery from different biological specimens [33-36]. Two JB<sup>®</sup> and one PCI DNA templates isolated from blood samples with parasitaemia levels greater than 2+ could not be amplified after three trials, this can be due to the presence of strong PCR inhibitors or an advanced degradation of extracted genomic DNA because of the nature of chemical precipitation process used in these methods.

## 5. Conclusion

*Plasmodium* species-specific multiplex-nested-PCR proved to be more sensitive than classical microscopy for malaria diagnosis and parasite species identification, particularly for submicroscopic cases. Multiplex-nested-PCR achieved the best performance with DNA templates extracted from EDTA-preserved blood specimens by QIAamp<sup>®</sup> chromatographic method in comparison to JB<sup>®</sup> and PCI DNA precipitation methods, making it a molecular tool very useful for diagnosis of submicroscopic malaria infections, especially in endemic regions ongoing a malaria elimination program.

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