

## ASSESSMENT OF THREE BLOOD GENOMIC-DNA PREPARATION METHODS FOR MALARIA MOLECULAR DIAGNOSIS

By

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### Abstract

Species-specific PCR techniques are highly sensitive and reliable alternatives to classical methods for malaria diagnosis and speciation, especially in endemic regions under advanced control or elimination programs where asymptomatic and low-density infections are increasingly reported. Nevertheless, the performance of these techniques is directly affected by the quality of isolated DNA templates. A *Plasmodium falciparum/vivax*-specific diagnostic Nested-PCR (*Pf/Pv* N-PCR) was used to assess three DNA preparation methods, Qiagen® Mini-Chromatographic kit (QIAmp®) and Jena-Biosciences® DNA isolation kit (JB®) for genomic DNA extraction from EDTA-preserved whole blood samples, and Whatman-FTA® purification reagent (FTA®) for DNA preparation from dry blood spots (DBS) collected onto FTA®-cards.

A total of 84 out of 137 blood specimens collected from malaria suspicious febrile patients who visited five health care centres in south-western endemic localities of Saudi Arabia were found *P. falciparum* positive by at least one method. Among these, only 76 (90%) were reported *P. falciparum* malaria positive by two expert microscopists. No other species of *Plasmodium* were detected. *Pf/Pv* N-PCR revealed 84/84 (100%), 75/84 (89%), and 81 (96%) *P. falciparum* positive samples using DNA templates prepared by QIAmp®, JB®, and FTA® purification methods, respectively. Therefore, *Pf/Pv* N-PCR, when applied to QIAmp® DNA templates showed to be a highly sensitive diagnostic method, particularly useful for sub-microscopic specimens from clinically malaria suspicious patients in endemic areas. On the other hand, *Pf/Pv* N-PCR of FTA®-DBS DNA templates revealed 5 positive cases missed by microscopy, encouraging its use as an affordable field semi-adapted protocol for malaria active screening, especially in remote rural regions with limited laboratory infrastructure.

**Key words:** Malaria diagnosis, Nested-PCR, DNA Extraction, EDTA-Blood, FTA®-Blood Spots.

### Introduction

Malaria continues as the most important vector-transmitted infection in the world, particularly present in the tropics and subtropics. Five species of protozoan parasites belonging to the genus of *Plasmodium* are known to cause human malaria; *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*, the only zoonotic form (WHO, 2014; Wesolowski *et al*, 2015). *Plasmodium* parasites are transmitted to humans by about 70 species of blood-feeding *Anopheles* female mosquitoes (Sinka *et al*, 2012). In 2013, according to estimates of the World Health Organization, over 3 billion people were considered at risk of acquiring

the infection, 198 million cases of malaria occurred and 584000 died from the disease worldwide, particularly from *P. falciparum* malaria, the malignant form (WHO, 2014). In Saudi Arabia, autochthonous malaria cases are mostly registered in the south-western region, where *P. falciparum* accounts for about 90% of reported cases, and where the disease existence is perpetuated by continuous importation from neighbouring Yemen (Alkhalife, 2003; MOH, 2005). Although significant decrease of locally transmitted malaria was achieved during the last decades after remarkable malaria control efforts (WHO, 2005), there is always a major risk for re-introduction of the

disease by millions of visitors coming for work or Ummrah and Hajj, in particular from the endemic areas (Alkhalife, 2003; Al-Tawfiq, 2006; Al-Harhi, 2015). It has already been reported that a considerable number of pilgrims carrying malaria parasites visits annually the country (Khan *et al.*, 2002).

In many endemic regions worldwide, it is still a common practice to rely on clinical signs solely for malaria diagnosis and consequent treatment, especially during the transmission pick seasons (White, 2004). El-Bahnasawy *et al.* (2013) stated that in spite of the great technological progress achieved worldwide, still arthropod borne infectious diseases is a puzzle disturbing the health authorities and that malaria tops the prevalent human mosquito-borne diseases. Classically, laboratory confirmation of the clinically suspected malaria cases is done by the microscopic examination of Giemsa-stained thick and thin blood films, but this centennial technique is capable of reliable diagnosis only when performed by skilled microscopists and is limited by the parasitaemia level (Avila and Ferreira, 1996; Cheesbrough, 1998). Different rapid diagnostic tests (RDTs) were also developed for diagnosis of malaria by detection of genus-specific or even species-specific circulating antigens, namely HRP2 and pLDH molecules circulating in blood. But, since their introduction, the RDTs showed very varied grades of specificity and their sensitivity depends on parasitaemia level (Forney *et al.*, 2001; Cheesbrough, 2009; Wongsrichanalai *et al.*, 2007). Then, molecular tools, namely PCR and PCR-modified techniques like Multiplex, Nested, and Real Time-PCR had become the most sensitive alternative to classical diagnostic methods, especially in regions with high incidence of asymptomatic chronic cases (Proux *et al.*, 2011). Several experimental investigations of different molecular tests confirmed their high sensitivity as confirmatory malaria diagnostic techniques for

the sub-microscopic cases (Coura *et al.*, 2006; Al-Harhi and Jamjoom, 2008). More progress will be achieved towards the elimination of malaria; more must be the need of highly sensitive diagnostic tools to detect sub-microscopic cases that may sustain transmission (Ouedraogo *et al.*, 2009). A *Plasmodium falciparum/vivax* species-specific Nested PCR (*Pf/Pv* N-PCR) targeting *Plasmodium* 18S SSU rRNA genes able to detect and differentiate between both species was adapted from Snounou *et al.* (1993) and Singh *et al.* (1996) to carry out the present study. *Pf/Pv* N-PCR was used to assess three commercial tools for genomic DNA templates preparation from whole blood samples collected from *P. falciparum/vivax* endemic Saudi south-western localities (Al-Harhi, 2015).

### **Subjects, Material and Methods**

**Collection of blood samples:** A total of 137 samples were collected from febrile patients complaining of malaria associated symptoms, as considered by physicians on duty in five health care centres of Saudi south-western province of Jazan between 2009 and 2013, after their consent. Specimens were collected and transported in EDTA-treated tubes and onto Whatman-FTA-cards (Whatman, Florham Park, NJ) as dry blood spots (DBS). Two negative whole blood samples from healthy individuals living in non-endemic areas and the *P. falciparum* cultured reference strain 3D7, kindly provided by Liverpool School of Tropical Medicine, were used as negative and positive controls, respectively.

**Microscopic examination:** For each patient, on arrival, thick and thin blood smears were routinely prepared for malaria diagnosis. Only thin blood films were fixed in methanol and both films were stained using 1% Giemsa solution. Stained smears were then examined twice by two expert microscopists using x100 objective. Parasitaemia level was determined on thick smears 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 standards of WHO (1991). At least 100 thick film fields were examined by each microscopist before a slide was considered negative. *Plasmodium* parasites species were determined using thin blood films.

Genomic DNA templates preparation: DNA templates were extracted from the EDTA-preserved whole blood samples using two kits following the manufacturers' protocols, QIAmp<sup>®</sup> DNA Blood Minicolumns Kit (Qiagen, Hilden, Germany) using 200 µl of blood samples, and Jena-Biosciences<sup>®</sup> DNA isolation/precipitation kit (Jena Bioscience GmbH, Germany) using 300 µl of blood samples, both through centrifugation method. Extracted DNA samples were adjusted to 200 µl for QIAmp<sup>®</sup> isolates and 300 µl for JB<sup>®</sup> extracts in recommended elution or dilution buffer. For DNA templates preparation from FTA<sup>®</sup>-cards DBS, a 2.5mm diameter piece was cut using a clean punch from each DBS. The snippets were separately treated by Whatman-FTA<sup>®</sup> commercial reagent (Whatman, Florham Park, NJ) and TE buffer. They were washed three times at room temperature in 0.2 ml PCR tubes using 100 µl of FTA<sup>®</sup> reagent by moderate manual rocking, for 5 minutes each, and rinsed twice in 200 µl of TE buffer for 5 minutes. The snippets were used directly as template in first PCR reactions.

*Pf/Pv* N-PCR: A diagnostic Nested PCR targeting *Plasmodium* 18S SSU rRNA genes was adapted from Snounou *et al.* (1993). The assay consists in a first *Plasmodium* genus-specific PCR reaction using rPLU6: TTA AAA TTG TTG CAG TTA AAA CG and rPLU5: CCT GTT GTT GCC TTA AAC TT oligonucleotides, followed by a *P. falciparum* and *vivax* species-specific Multiplex Nested-PCR using *PfF*: TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT and *PfR*: ACA CAA

TGA ACT CAA TCA TGA CTA CCC GTC oligonucleotides specific to *P. falciparum*, and *PvF*: CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC and *PvR*: ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA specific to *P. vivax*. PCR reactions were carried out in final volumes of 25 µl containing 1.5 µl template DNA obtained by QIAmp<sup>®</sup> and JB<sup>®</sup> kits, and 2.5 mm diameter FTA<sup>®</sup>-cards treated snippets, 0.2 µM of each primer, and 12.5 µl of 2x HotStart<sup>®</sup> Taq Master-Mix (Qiagen, USA). The following thermo-cycling scheme was used in the first reaction: 95°C/15min, 40x(94°C/30s, 57°C/35s, 62°C/70s) and a final extension step at 65°C/5min. Nested reactions were performed using 2 µl of rPLU6/rPLU5-PCR products under the following conditions: 95°C/15min, 40x(94°C/30s, 54°C/30s, 66°C/40s) and 66°C/5min, producing an amplicon of 205 bp if *P. falciparum* DNA is present and 120 bp if *P. vivax* specific DNA was present. PCR products were separated by electrophoresis onto 1.4% agarose gels alongside a 100 bp scale DNA ladder and visualized using EtBr staining.

## Results

A total of 76 out of 137 specimens collected from febrile patients in Jazan province during high transmission seasons between 2009 and 2013 were confirmed on site as *P. falciparum* malaria positive by expert microscopists. No other *Plasmodium* species were detected among all patients. Reported parasitaemia levels were estimated using Giemsa-stained thick blood smears as 1+ to 4+ (Tab. 1).

Results of *Pf/Pv* N-PCR analysis of the 137 DNA templates prepared separately by QIAmp<sup>®</sup>, JB<sup>®</sup>, FTA<sup>®</sup> are collected relatively to the samples' parasitaemia grades as determined by microscopy (Tab. 2). The first agarose gel of separated *Pf/Pv* N-PCR products is presented herein as a model (Fig. 1). No PCR products were obtained with two negative control DNA templates obtained from blood samples of healthy

individuals living in non-endemic areas and prepared by the three studied methods. 3D7 *P. falciparum* reference strain DNA samples showed positive results in all runs. 8 samples of malaria clinically suspicious, but negative by microscopy, were found positive by Nested-PCR.

In total, 84/137 included specimens were

found *P. falciparum* positive by at least one method. Thus, sensitivity, specificity, and predictive values of each method were calculated relatively to these findings. Details of diagnostic indices calculated for each molecular protocol are collected alongside microscopic findings (Tab. 3).

Table1: Microscopic examination performed by two expert microscopists.

Microscopy	Parasitaemia level					Total samples
	Neg.	1+	2+	3+	4+	
Microscopic examination	61	18	22	29	7	137
Total samples	61	76				

Table 2: Sensitivity of *Pf/Pv* N-PCR using DNA templates prepared by QIAmp<sup>®</sup>, JB<sup>®</sup>, and FTA<sup>®</sup> methods relatively to parasitaemia level.

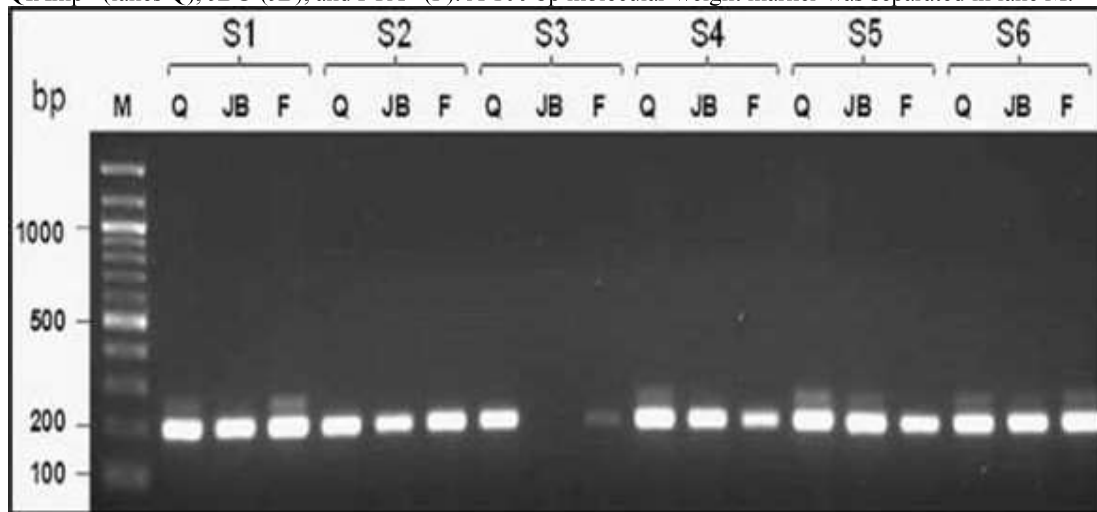
Parasitaemia level	Number of Samples	+ve <i>falciparum</i> -specific Nested-PCR		
		QIAmp <sup>®</sup>	JB <sup>®</sup>	FTA <sup>®</sup>
-ve	53	0	0	0
1+	18 + 8*	26 (100%)	19 (73%)	23 (88%)
2+	22	22 (100%)	21 (95%)	22 (100%)
3+	29	29 (100%)	28 (97%)	29 (100%)
4+	7	7 (100%)	7 (100%)	7 (100%)

8\*: negative samples by microscopic examination, but malaria positive by at least one molecular technique estimated at minimal parasitaemia level (1+).

Table 3: Relative sensitivity, specificity, positive and negative predictive values of *Pf/Pv* N-PCR using DNA templates prepared by QIAmp<sup>®</sup>, JB<sup>®</sup>, and FTA<sup>®</sup> versus microscopic examination.

Technique	Specimens	Sensitivity	Specificity	+ve P.V.	-ve P.V.
Microscopy	Thick blood smear	76/84 (90%)	53/53 (100%)	84/84 (100%)	53/61 (87%)
<i>Pf/Pv</i> N-PCR	QIAmp <sup>®</sup>	84/84 (100%)	53/53 (100%)	84/84 (100%)	53/53 (100%)
	JB <sup>®</sup>	75/84 (89%)	53/53 (100%)	84/84 (100%)	53/62 (85%)
	FTA <sup>®</sup>	81/84 (96%)	53/53 (100%)	84/84 (100%)	53/56 (95%)

Fig. 1: Agarose gel of *Pf/Pv* N-PCR products of malaria patients (S1, S2,) blood DNA templates prepared by QIAmp<sup>®</sup> (lanes Q), JB<sup>®</sup> (JB), and FTA<sup>®</sup> (F). A 100 bp molecular weight marker was separated in lane M.



## Discussion

Despite the significant progress achieved by malaria control program in Saudi Arabia, the disease remains a serious health issue, particularly in endemic south-western regions where *P. falciparum* is the most predominant species accounting for over 90% of autochthonous cases and *P. vivax* for the remaining ones, and where patients with clinically suspected malaria signs are frequently encountered (Omar *et al.*, 1999; Al-Harhi and Jamjoom, 2008). Furthermore, important numbers of imported malaria cases are being reported annually, particularly from neighbouring endemic bordering areas of Yemen (Al-khalife, 2003; Al-Tawfiq, 2006; Al-Harhi, 2015, Bamaga *et al.*, 2015). Laboratory confirmation of malaria cases rely basically on microscopic examination of Giemsa-stained thick and thin blood smears, a laborious and highly subjective method showing low sensitivity with chronic and asymptomatic carriers (WHO, 2013). As more progress will be achieved towards the elimination of malaria, more will be the need of highly sensitive diagnostic tools to confirm sub-microscopic clinical cases for a prompt and correct treatment to avoid overtreatment and consequent origination of malaria resistant strains (Schneider *et al.*, 2007; Ouedraogo *et al.*, 2009). Besides, the implementation of more efficient diagnostic tools is vital in active surveys, especially for detection of chronic asymptomatic infections that may sustain transmission hindering any malaria elimination expectations.

Currently, the only diagnostic tools totally adapted to field screenings are the rapid diagnostic tests (RDTs), which extensive evaluations showed discrepant levels of sensitivity and specificity with limitations in detecting parasitaemia levels lower than 200 parasites/ $\mu$ L in *P. falciparum* infections, and even higher with *P. vivax* (Forney *et al.*, 2001; Branch *et al.*, 2005; Wong-

srichanalai *et al.*, 2007; Bharti *et al.*, 2008; Cheesbrough, 2009). This impede the possible use of RDTs as single diagnostic tool for monitoring malaria elimination programs, where probably more than a detection tool will be needed in combination to achieve effective results in health care centers and surveillance activities (McMorrow *et al.*, 2011). PCR and PCR-modified methods had proven highly sensitive for malaria diagnosis and differentiation, and are being considered as the gold standard method in many health and research settings (Rubio *et al.*, 2002; Hanscheid and Grobusch, 2002; Moody, 2002). In this study, we used a SSU rRNA genes based *Pf/Pv* N-PCR (Snounou *et al.*, 1993) to assess the suitability of DNA templates prepared from 137 collected samples by QIAmp<sup>®</sup> and JB<sup>®</sup> isolation kits from whole EDTA-preserved blood samples and FTA<sup>®</sup> specific reagent for DBS.

A total of 84 samples were found *P. falciparum* positive by at least one method, while only 76 among these were positive by microscopic examination showing different parasitaemia levels escalating from 1+ to 4+. The use of SSU rRNA genes as DNA targets in malaria molecular diagnosis, parasites speciation, and mixed infections identification has proven to be highly effective (Singh *et al.*, 1996). The *Pf/Pv* N-PCR applied to QIAmp<sup>®</sup>-DNA templates has been able to detect *P. falciparum* specific DNA in eight samples misdiagnosed by microscopic examination in hands of two experts. It has been reported in several studies that sub-microscopic cases detectable only by sensitive PCR techniques are much more prevalent in endemic areas than previously estimated (Bottius *et al.*, 1996; Okell *et al.*, 2012). In summary, QIAmp<sup>®</sup> extraction method provided better DNA templates for amplification by *Pf/Pv* N-PCR, achieving 100% relative sensitivity compared to 96%, and 89% achieved using DNA templates prepared by FTA<sup>®</sup> and JB<sup>®</sup> methods, respectively. Curiously,

JB<sup>®</sup> isolated DNA templates from two blood samples with relatively significant parasitaemia levels, 2+ and 3+, did not yield positive *Pf/Pv* N-PCR results in three different trials, this can be explained by the presence of strong PCR inhibitors due to the nature of chemical precipitation process used in this purification kit.

It has been experimentally well established that filter matrices impregnated with chemical stabilizers offer an excellent support for blood samples collection and preservation as a source of DNA material for posterior molecular investigations (Li *et al*, 2004; Ndunguru *et al*, 2005). In the present study, *Pf/Pv* N-PCR amplifications of FTA<sup>®</sup> stored and prepared DNA templates showed a sensitivity of 96% (81/84) revealing 5 more positive cases missed by microscopy. And, the 3 FTA<sup>®</sup>-false negative samples were all sub-microscopic cases with minimal parasitaemia levels detected only by N-PCR when QIAmp<sup>®</sup> purified DNAs were used. It has been reported by the manufacturer and other experimental studies that FTA<sup>®</sup> blood DNA storage and purification system provides good quality DNA templates for PCR runs free of heme, contaminants, and other possible inhibitors, with less risk of loss or degradation (Long *et al*, 1995; Whatman Ltd, 2002). Although, *Pf/Pv* N-PCR using FTA<sup>®</sup>-DNAs showed relatively less sensitive than when using QIAmp<sup>®</sup>-DNAs, the ability of this method to identify a considerable number of sub-microscopic malaria infections together with its advantage of collecting and transporting finger prick DBS encourages its implementation in active epidemiological surveys, especially in remote rural areas lacking adequate facilities for collecting and maintaining intravenous whole blood samples in ideal conditions.

### Conclusion

The highest diagnostic indices were achieved by *Pf/Pv* N-PCR when applied to QIAmp<sup>®</sup> isolated DNA templates, making

it a molecular tool very useful for diagnosis of sub-microscopic malaria infections, especially in endemic localities. Yet, the significant performance (96%) obtained by *Pf/Pv* N-PCR using FTA<sup>®</sup> treated DBS, encourages its use as a field semi-adapted alternative for malaria active surveys during control and elimination programs in remote rural regions.

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