COMPARISON OF A GENUS-SPECIFIC CONVENTIONAL PCR AND A SPECIES-SPECIFIC NESTED-PCR FOR MALARIA DIAGNOSIS USING FTA COLLECTED SAMPLES FROM KINGDOM OF SAUDI ARABIA

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Abstract

Molecular tools are increasingly accepted as the most sensitive and reliable techniques for malaria diagnosis and epidemiological surveys. Also, collection of finger prick blood spots onto filter papers is the most simple and affordable method for samples preservation and posterior molecular analysis, especially in rural endemic regions where malaria remains a major health problem. Two malaria molecular diagnostic tests, a *Plasmodium* genus-specific conventional PCR and a *Plasmodium* species-specific Nested PCR, were evaluated using DNA templates prepared from Whatman-FTA$^{®-}$cards' dry blood spots using both, Methanol-fixation/Heat-extraction and FTA® commercial purification kit. A total of 121 blood sam-ples were collected from six Saudi south-western endemic districts both, as thick and thin films for routine microscopic screening and onto FTA®-cards for molecular studies.

Out of the 121 samples, 75 were *P. falciparum* positive by at least one technique. No other species of *Plasmodium* were detected. *P. falciparum* parasites were identified in 69/75 (92%) samples by microscopic screening in health care centers. *P.* genus-specific PCR was able to amplify *P. falciparum* DNA in 41/75 (55%) and 59/75 (79%) samples using Methanol-fixation/Heat-extraction and FTA$^{®}$ purification kit, respectively. *P.* species-specific Nested PCR revealed 68/75 (91%) and 75/75 (100%) positive samples using DNA templates were isolated by Methanol-fixation/Heat-extraction and FTA$^{®}$ purification methods, respectively.

The species-specific Nested PCR applied to Whatman-FTA$^{®}$ preserved and processed blood samples represents the best alternative to classical microscopy for malaria diagnosis, particularly in epidemiological screening.

Key words: Malaria diagnosis, Saudi Arabia, PCR, Nested-PCR, FTA® cards.

Introduction

Malaria is considered as the most common vector-borne disease worldwide. Human malaria is caused by protozoan parasites belonging to the *Plasmodium* genus, namely: *P. falciparum, P. vivax, P. malariae, P. ovale*, and *P. knowlesi*, transmitted by *Anopheles* blood-feeding female mosquitoes (Tuteja, 2007; Daneshvar *et al*, 2009). *P. falciparum* is responsible for malignant malaria cases, being the most lethal form of the disease (Mbugi *et al*, 2006). According to the World Health Organization, 207 million cases of malaria were reported and approximately 627 000 deaths worldwide. 3.4 billion People are considered at risk of acquiring the infection in 97 countries (WHO, 2013). Furthermore, malaria is now spreading to areas previously free of the disease. Also, millions of people travel each year from non-endemic regions to areas of malaria transmission where they get exposed to the disease. Many studies indicated the high risk of transmitting malaria by blood transfusion when no approved laboratory tests are applied to screen the blood for malaria (Pomper *et al*, 2003).

In Saudi Arabia, except for the central part which is free of malaria, the rest of the country includes malarious areas that differ in their epidemiological characteristics and the mosquito vector, though malaria vector, which is extremely well adapted to desert conditions occurs in all areas. Malaria epi-

demiological aspect varies also in the same place, from one year to another, and it was affected by the control measures taken against malaria (Al-Seghayer *et al*, 1999; MOH, 2005). Malaria is endemic in the lowlands of Aseer region in the south-western province and particularly present in southern province of Jazan where *P. falciparum* accounts for about 85-90% reported cases, and where disease existence was perpetuated by continuous importation from neighbouring Yemen (Vassallo *et al*, 1985; El-Sebai and Makled, 1987; Warrell, 1993). Reported malaria cases in Saudi Arabia decreased every year. In 2003, a total of 1724 confirmed cases were reported, of which 582 were endogenous. Compared to 15,666 reported cases in 1990; the Ministry of Health took initiative for strengthening malaria control activities in the country (WHO, 2005). But, there was always a high risk for perpetuating malaria in endemic foci, as well as introduction of the disease to non-endemic areas by the important number of visitors coming for work or Ummrah and Hajj, in particular from endemic countries (Al-Hassan and Roberts, 2002; Alkhalife, 2003; Al-Tawfiq, 2006). Therefore, to prevent malaria transmission and spread, and achieved the goal of malaria elimination in KSA, new measures will be required through active detection and treatment of local and imported cases.

Malaria can be diagnosed by clinically, but the disease may be misdiagnosed because it shares signs and symptoms with other tropical illnesses (Wongsrichanalai *et al*, 2007) as typhoid fever, rheumatic fever, and bacterial meningitis. Microscopy of the Giemsa-stained thick and thin films was the standard laboratory diagnostic technique for identifying malaria infections very long age, but technique was capable of reliable diagnosis only when done by skilled microscopists using defined protocols (Avila and Ferreira, 1996; Cheesbrough, 1998). The nicroscopy-based diagnosis was tedious and suffered low sensitivity when the parasitaemia levels were few. Several diagnostic techniques have been developed for diagnosis as dark field microscopy, immunoflueorescent antibody tests, quantitative-buffy-coat (QBC), several enzyme immunoassays, and western blotting, but most of them showed complications for field applications and insufficient sensitivity (Cheesbrough, 2009). The serological tests based on detection of anti-*Plasmodium* antibodies were always hindered by relatively low specificity and inability to distinguish between past and recent infections (Gilles and Warrell, 2002). Several immunochromographic blood tests were developed for specific detection of *Plasmodium* parasites genus-specific or even species specific circulating antigens like HRP 2 and pLDH molecules. The evaluation of different rapid diagnostic tests, based either on monoclonal or polyclonal antibodies against *plasmodium* parasites antigens, showed varied specificity grades (Humar *et al*, 1997; Forney *et al*, 2001; Wongsrichanalai *et al*, 2007; Cheesbrough, 2009). The development of several molecular tools such as PCR and others derived methods like Nested-PCR and Multiplex-PCR protocols could overcome almost all handicaps presented by the former assays. Many molecular techniques showed high diagnostic indices and accuracy as confirmatory diagnostic tools in suspicious patients, especially when parasitaemia levels are very low to be detected by standard microscopic examination (Al-Harthi and Jamjoom, 2008). The frequency of asymptomatic careers detected by PCR methods were always much grea-ter than those diagnosed by conventional microscopic examination of stained thick blood films (Coura *et al*, 2006). More sophisticated molecular tools like Real-time PCR were introduced for malaria parasites detection in patient's samples; however their use for standard screening was highly limited by the high cost of reagents and needed infrastructure (AnnLee *et al*, 2002; Perandin *et al*, 2004; Mangold *et al*, 2005).

Polymerase chain reaction is becoming relevant for malaria control and elimination

strategies in endemic areas with high incidences of chronic and asymptomatic cases. Because of their important diagnostic indices, some PCR assays were regarded as the gold standard method in many settings and health facilities (Proux *et al*, 2011). Collection of finger prick blood spots onto filter papers emerged as simplest and affordable alternative to whole blood collection, transportation, and processing for the molecular analysis in particular from rural areas (Al-Harthi and Jamjoom, 2008; Ataei *et al*, 2011; Pieter *et al*, 2014).

In this study, two different molecular diagnostic techniques, a *Plasmodium* gen- us-specific conventional PCR (Cicerone *et al*, 1999) and a *Plasmodium* species-specific Nested PCR (Snounou *et al*, 1993; Singh *et al*, 1996), was assessed for diagnosis using DNA templates prepared from dry blood spots collected from endemic Saudi southwestern endemic areas on commercial FTA cards. Two DNA isolation protocols, Methanol-fixation/Heat-extraction and the FTA® commercial purification system, were compared using both PCR techniques.

Materials and Methods

Blood samples collection and storage: Samples and patients' data collection was coordinated with Jazan District Local Health Authorities, including the National Centre for Training and Research of Tropical Diseases in Jazan. Samples were collected from health care centers in south-western endemic foci of Samtah, Al-Ardah, Jazan, Tiwal, Baisha, and Fifa mainly during high transmission seasons of years 2009-2012, between January and May. A total of 121 samples were collected from different febrile patients complaining of malaria associated symptoms as considered by physicians on duty, after their consent. All blood samples were collected in quadruplicate onto Whatman-FTA® filter cards (Whatman, Florham Park, NJ), air dried, labelled and stored at room temperature in separated plastic bags. Three negative whole blood samples collected in EDTA-treated tubes & on FTA® cards

from healthy individuals living in non-endemic areas as negative controls. 3D7 *P. falciparum* reference strain cultured parasites spotted on FTA®-cards, kindly provided by Liverpool School of Tropical Medicine, was used as positive control.

Microscopic examination: For each patient, thick and thin blood films were prepared and air-dried at room temperature. Only thin blood smears were fixed in methanol before staining. Both films were stained with 1% Giemsa stain in PBS. The films were then examined for malaria parasites using oil immersion objective (x100). 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; 4+ for more than 10 parasites per single field (WHO, 1991). At least 100 thick film fields were examined before a slide was considered negative. Parasite species and stages were confirmed using thin blood films.

DNA templates preparation: Two approaches were used for DNA templates preparation from snipped dry blood spots, Methanol-fixation/Heat-extraction and the FTA® commercial purification Kit. First, one 5 mm diameter and two 2.5 mm diameter pieces were cut from dry blood spots on FTA® filter papers for each sample by using a clean punch. Methanol-fixation/Heat-extraction was done by fixation of 5mm diameter snippet in 500µl of absolute methanol for 15 minutes at room temperature. Methanol solution was discarded and the vial left open on its side until complete evaporation of remaining methanol. 300µl of PCR-grade water was added and the snippet was heated for 15 minutes at 98°C. During its heat incubation, the tube was vigorously vortexed for approximately one minute at intervals of 5 minutes. The snippet was then removed by a micropipette tip and extracted DNA concentrated in a vacuum-heat concentrator to a final volume of 40µl and stored at -20°C. 2.5 mm diameter snippets were separately treated by Whatman-FTA® commercial reagent

(Whatman, Florham Park, NJ) and TE buffer following the procedure recommended by the manufacturer. The snippets were washed three times at room temperature in 0.2ml PCR tubes by using 100µl of FTA® purification reagent by moderate manual rocking, for 5min. each. Then, the snippets were rinsed twice in 200µl of TE buffer for 5 minutes, and used directly in PCR reactions.

Molecular assays: Two PCR techniques targeting *Plasmodium* 18S SSU rRNA genes in malaria were applied to DNA templates prepared as described above; a conventional *P.* genus-specific PCR using Ssufw/Ssurv set of oligonucleotides adapted from Ciceron *et al.* (1999) and a *P.* species-specific Nested-PCR using two sets of primers, rPLU6/rPLU5 in the first reaction and (*Pff*/*Pfr* plus *Pvf*/*Pvr*) in the Nested one to detect and discriminate between *P. falciparum* and *P. vivax* adapted from Snounou *et al.* (1993) (Tab. 1).

All PCR reactions were carried out in final volumes of 25µl containing 10 µl of Methanol/heat isolated DNA or a 2.5 mm FTA®-treated snippet as initial templates, 0.2mM of each primer, 12.5µl of 2x HotStart® Taq Master Mix (Qiagen, USA). Cycling conditions for genus-specific PCR were as the following: an initial activation at 95°C/15min, 40 cycles of (94°C/30sec, 55°C/30sec, 69°C/1min) and a final extension step at 69°C/5min. Positive expected amplicons are of 291 bp. Cycling conditions for species-specific first reaction PCR were as follows: 95°C/15min, 40x(94°C/30sec, 57°C/35sec, 62°C/70 sec) and a final extension step at 65°C/5 min. Concerning the Nested reaction, 2µl taken from the first rPLU6/rPLU5-PCR reaction product were amplified using the following thermo-cycling scheme: 95°C/15min, 40x(94°C/30sec, 54°C/30sec, 66°C/40sec) and 66°C/5min producing an amplicon of 205 bp with *P. falciparum* and 120 bp with *P. vivax* species. PCR products were separated on basis of their sizes by electrophoresis onto agarose gels with appropriate concentrations; 1.2% for genus-specific PCR and 1.4% for species-specific Nested PCR products. Molecular size of visualized DNA bands when produced, were determined by extrapolation to the bands of a 100 bp scale DNA ladder.

Results

Out of 121 blood samples collected from patients who visited health care centres seeking medical care in six malaria endemic localities of south-western region in Saudi Arabia, 69 were confirmed as *P. falciparum* malaria positive by expert microscopists using Giemsa stained thin and thick blood smears. No other *Plasmodium* species were found by microscopy. Parasitaemia levels findings were reported using a 1 to 4 crosses system and distributed by localities of origin (Tab. 2). Fifteen samples were found with 1+ parasitaemia grade, 20 with 2+, 27 with 3+, and only 7 samples showed heavy parasitic load with a 4+ parasitaemia level.

The analysis of 121 FTA®-cards spotted whole blood samples treated with Methanol-fixation/Heat-extraction and FTA® purification kit separately by a *P.* genus-specific PCR and a *P.* species-specific Nested PCR showed the collected results. A representative agarose gel of separated amplification products issued from each protocol is illustrated (Fig. 1). In total, 75 out of 121 studied samples were found *P. falciparum* positive by at least one technique. These were used therefore as total positive cases to calculate diagnostic relative indices of each assay. In details, only 41/75 (55%) Methanol-fixed/Heat-extracted DNA templates and 59/75 (79%) DNA templates purified by FTA® purification kit were found positive by the *P.* genus-specific conventional PCR. *P.* species-specific Nested PCR was able to amplify *P. falciparum* DNA in 68/75 (91%) and 75/75 (100%) samples using DNA templates isolated by the methanol-fixation/Heat-extraction and FTA® purification methods, respectively.

No PCR products were obtained by both techniques using three human quantified negative control DNA templates obtained

from blood samples of healthy individuals living in non-endemic areas. 3D7 *P. falciparum* reference strain cultured parasites preserved on FTA®-cards and used herein as positive control showed positive results in all runs by both techniques.

PCR sensitivity increased with parasitaemia grades as estimated by microscopy. Using Methanol-Heat isolated DNA, *P.* genus-specific PCR was positive for 43% and 50% of blood samples with 1+ & 2+ parasitaemia grades, respectively, and species-specific Nested PCR for 81% of samples with 1+ parasitaemia level. Both PCR techniques showed a sensitivity of at least 93% using DNA templates isolated by FTA® purification reagent from samples with 3+ & 4+ parasitaemia levels.

The species-specific Nested PCR gave 100% sensitivity by using DNA templates of samples with 4+ parasitaemia grade isolated by both extraction methods. Details are given in tables (1, 2, 3 & 4) and figure (1).

Table 1: Set of oligonucleotides used in *Plasmodium* genus-specific PCR and *P. falciparum/P. vivax* species-specific-Nested-PCR (Ciceron *et al*, 1999; Snounou *et al*, 1993).

Primer	Nucleotide Sequences (5'-3')	Nts
Plasmodium Ssufw/Ssurv PCR (Ciceron *et al*, 1999)		
Ssufw	AGTTACGATTAATAGGAGTAG	21
Ssurv	CCAAAGACTTTGATTTCTCAT	21
Plasmodium species-specific Multiplex-Nested-PCR (Snounou *et al*, 1993)		
rPLU6	TTAAAATTGTTGCAGTTAAAACG	23
rPLU5	CCTGTTGTTGCCTTAAACTT	20
PfF	TTAAACTGGTTTGGGAAAACCAAATATATT	30
PfR	ACACAATGAACTCAATCATGACTACCCGTC	30
PvF	CGCTTCTAGCTTAATCCACATAACTGATAC	30
PvR	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	30

Table 2: Parasitaemia level determined by microscopic screening of malaria suspected incoming cases.

Localities	Total collected samples	Negative	1+	2+	3+	4+
Samtah	48	14	9	10	13	2
Al-Ardah	17	8	1	2	4	2
Jizan	21	14	2	1	3	1
Tiwal	8	4	0	0	2	2
Baisha	20	8	0	7	5	0
Fifa	7	4	3	0	0	0
Total	121	52	15	20	27	7

Table 3: Sensitivity, specificity, positive and negative predictive values of *Plasmodium* genus-specific PCR and *P.* species-specific nested PCR applied to DNA templates prepared from FTA® dry blood spots by Methanol-fixation/ Heat-extraction and Whatman-FTA® purification reagent, and routine microscopic screening of Giemsa stained blood films

Diagnostic technique	Specimens	Sensitivity	Specificity	+ve P.V.	-ve P.V.
Plasmodium genus-specific PCR	Methanol/Heat	41/75 (55%)	46/46 (100%)	100%	58%
	FTA® Reagent	59/75 (79%)	46/46 (100%)	100%	74%
Plasmodium species-specific-Nested-PCR	Methanol/Heat	68/75 (91%)	46/46 (100%)	100%	87%
	FTA® Reagent	75/75 (100%)	46/46 (100%)	100%	100%
Microscopic examination	Thick blood smear	69/75 (92%)	46/46 (100%)	100%	88%

Fig.1: Agarose gels of *Plasmodium* genus-specific conventional PCR (Panel A) and *Plasmodium* species-specific Nested PCR (Panel B) amplification products of malaria patients (S1, S2,..) blood DNA prepared by Methanol-fixation/Heat-extraction (lanes MH) versus Whatman-FTA® Reagent (lanes F). FTA®-cards preserved cultured 3D7 reference strain control. A 100 bp molecular weight ladder separated in lanes M.
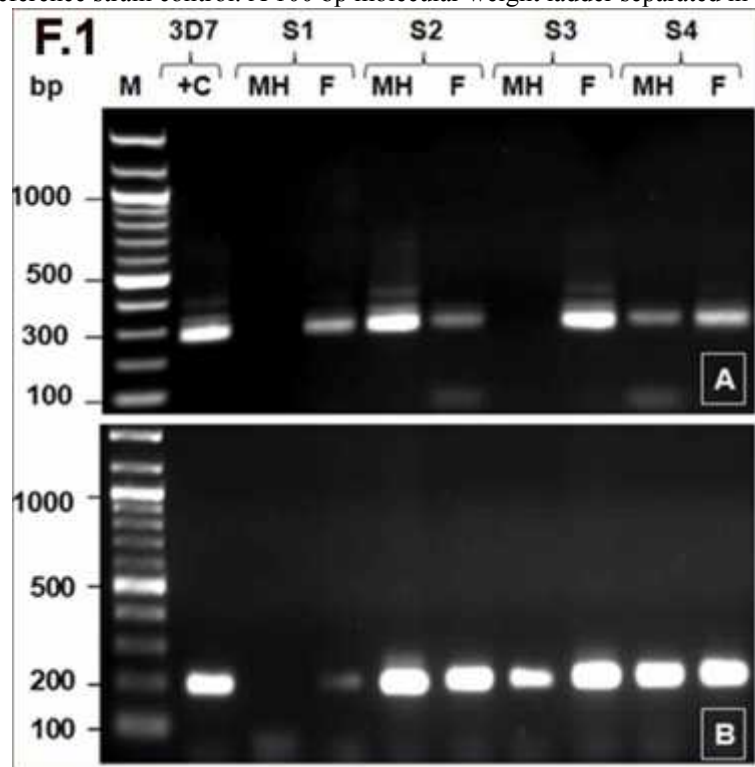

Table 4: Relative sensitivity of *P*. genus-specific PCR and *Plasmodium* species-specific Nested PCR applied to DNA templates prepared FTA® dry blood spots by Methanol-Heat and FTA® purification reagent in relation to parasitaemia level.

Parasitaemia level	Number of Samples	+ve by *P*. genus-specific PCR		+ve by *P*. species-specific-Nested-PCR		+ve by microscopy
		Methanol-Heat	FTA® Reagent	Methanol/Heat	FTA® Reagent	Thick blood smear
-ve	46	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
1+	15+6*	9 (43%)	11 (52%)	15+2* (81%)	15+6* (100%)	15 (71%)
2+	20	10 (50%)	16 (80%)	19 (95%)	20 (100%)	20 (100%)
3+	27	17 (63%)	25 (93%)	25 (93%)	27 (100%)	27 (100%)
4+	7	5 (71%)	7 (86%)	7 (100%)	7 (100%)	7(100%)

no*: number of samples negative by routine microscopic examination found positive by indicated molecular technique. These samples were estimated at minimal parasitaemia grade (1+).

Discussion

Though 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 patients with clinically suspected malaria signs are frequently encountered (Omar *et al*, 1999; Al-Harthi and Jamjoom, 2008). Also, an important number of imported malaria cases is being registered annually both, in endemic and non-endemic areas, among visitors coming for work or Ummrah and Hajj (Alkhalife, 2003; Al-Tawfiq, 2006).

The microscopic examination of Giemsa-stained blood smears remains the standard diagnostic procedure for malaria in Saudi Arabia. But, this method becomes more tedious and less sensitive in areas where malaria elimination strategies are expected to identify and treat actively all cases, especially chronic and asymptomatic carriers with

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very low parasitaemia levels (WHO, 2013). Therefore, more efficient field adapted diagnostic tools are vital to achieve more accurate results in active surveys. The only tests totally adapted to field screenings are the immunochromatographic devices, but the evaluation of these rapid diagnostic tests showed discrepant indices of specificity and the sensitivity was found to be limited by a minimum level of parasitaemia (Humar *et al*, 1997; Forney *et al*, 2001; Wongsrichanalai *et al*, 2007, Cheesbrough, 2009). Molecular techniques like PCR and some PCR-derived techniques are being regarded as the gold standard malaria diagnostic tools in many research settings and health facilities because of their extremely high sensitivity and specificity (Hanscheid *et al*, 2002). In particular the use of *ssu rRNA* genes as DNA targets in malaria diagnosis by PCR has proven highly effective (Moody, 2002). Furthermore, available Nested-PCR methods can differentiate between *Plasmodium* species and detect mixed infections (Singh *et al*, 1996, Scopel *et al*, 2004). In this study, two PCR techniques targeting 18S ssu rRNA genes were assessed for malaria diagnosis in Saudi endemic areas, a *P.* genus-specific conventional PCR adapted from Ciceron *et al.* (1999) and a *P.* species-specific Nested PCR described initially by Snounou *et al.* (1993). Classically, molecular tools applied to malaria diagnosis have been performed using nucleic acids isolated from the whole blood samples, and this impeded seriously their use for active epidemiological surveys, especially in rural areas lacking adequate facilities for processing or maintaining blood samples in ideal conditions for posterior molecular analysis. Nevertheless, collection and transportation of blood samples as dry spots onto filter papers has proven to be a very useful and the inexpensive alternative which was increasingly used (Ataei *et al*, 2011; Pieter *et al*, 2014). In this study, 121 blood samples were collected onto Whatman-FTA® cards from suspected patients in six malaria endemic districts of Saudi Arabia.

Whatman-FTA® cards were supposed to be impregnated with chemicals that stabilize DNA and RNA molecules within the fibre matrix (Ndungguru *et al*, 2005). It has been experimentally established that filter paper collected blood samples do not require immediate processing, can be preserved at room temperature, and once dried blood samples are not infectious offering the best substitute in settings with restricted access to the advanced diagnostic facilities (Li *et al*, 2004; Al-Harthi and Jamjoom, 2008). However, the success of DNA amplification with satisfactory levels by PCR depends greatly on the quality of the initial DNA template provided (Proux *et al*, 2011). Accordingly, the collected dry specimens were processed separately by the Methanol-fixation/Heat-extraction and FTA®-purification reagent to prepare DNA templates for both assessed PCR assays. Differences in the sensitivity of PCR detection of *P. falciparum* using DNA templates extracted by different methods have previously been described (Bereczky *et al*, 2005).

Seventy-five samples were found *P. falciparum* positive by at least one technique. These were used therefore, as total positive cases to calculate diagnostic relative indices of each assay. Although, the genus-specific conventional PCR was reported as 100% sensitive using DNA templates from whole blood samples by Ciceron *et al.* (1999), it showed less sensitivity (79%) with filter dry blood spots, in this study. FTA® DNA-purification kit provided better templates for the amplification, achieving 79% and 100% sensitivity compared to 55% and 91% when used in *P.* genus-specific PCR and *P.* species-specific Nested PCR, respectively. Both PCR techniques showed an increasing sensitivity in parallel with parasitaemia level; a sensitivity of at least 93% using DNA templates isolated by FTA® purification reagent from samples with 3+ and 4+ parasitaemia levels. It was reported by the manufacturer that FTA® DNA-purification kit ensures high quality of DNA templates for PCR

analysis by removing heme, inhibitors and other possible contaminants (Whatman Ltd, 2002). Other authors reported that the biological material including blood samples kept on filter-paper appeared to conserve its genomic characteristics with less risk of contamination, loss or degradation during storage and handling in adequate conditions (Long *et al*, 1995; Scopel *et al*, 2004). False negative results obtained by using Methanol/Heat extracted DNA templates might reflect trapping of DNA in the filter-paper, as previously explained (Zhong *et al*, 2001).

P. species-specific Nested PCR applied to DNA templates prepared by FTA$^{®}$ purification reagent showed the highest sensitivity (100%) detecting *P. falciparum* DNA in six samples misdiagnosed by the routine microscopic screening. The high occurrence of sub-microscopic parasitaemia levels detectable only by such sensitive PCR techniques has been reported as solid evidence that the chronic malaria cases were much more prevalent in endemic areas than previously estimated (Bottius *et al*, 1996; Okell *et al*, 2012). Also, identification of submicroscopic malaria infections by field adapted molecular protocols would be highly relevant for monitoring malaria epidemiology towards the achievement of sustainable malaria elimination in Saudi endemic areas.

Conclusion

Plasmodium species-specific Nested PCR achieved the best diagnostic performance indices with 100% sensitivity, specificity, Negative and positive predictive values when applied to dry blood spots collected onto FTA$^{®}$ cards and treated with FTA$^{®}$ purification reagent. This molecular protocol represents a viable alternative to classical microscopy for malaria diagnosis, particularly in remote rural settings and during active screening.

Only *P. falciparum* was found to be the causal agent in all collected samples both, by microscopic examination of the Giemsa stained thin blood smears and species-specific Nested PCR.

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