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LABORATORY DIAGNOSIS OF MALARIA INFECTION IN CLINICALLY SUSPECTED CASES USING MICROSCOPIC EXAMINATION, RAPID ANTIGEN TESTS & PCR

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Abstract

Accurate diagnosis of malaria is important for providing malaria burden and preventing misdiagnosis. To analyses the diagnostic performance of conventional blood film microscopic examination, OptiMAL Rapid Antigen Test, Hexagon Malaria Combi, Diaquick Malaria Pf/Pan Cassette in comparison with species-specific nested polymerase chain reaction (nest-PCR) in Makkah and Taif in Kingdom of Saudi Arabia. A total of 100 blood samples collected from febrile cases in Makkah and Taif Cities were screened for malaria by microscopic examination, rapid diagnostic tests (RDTs) using nest-PCR as standard method. The results showed differences in detection rates of nested-PCR, conventional blood film microscopic examination, OptiMAL test, Hexagon Malaria Combi, Diaquick Malaria Pf/Pan Cassette (72%, 57%, 59%, 44% & 52% respectively) in 100 clinically suspected cases. Microscopic examination, OptiMAL test, Hexagon Malaria Combi and Diaquick Malaria Pf/Pan Cassette showed sensitivity (72.2%,72.2%, 62.9%, 65.3%), specificity (82.1%, 75%, 82.1%,& 82.1%), accuracy index (75%, 73%, 62%, 70%), positive predictive value (91.2%, 88.1%, 88.6%,& 90.4%) and negative predictive value (53.5%, 51.2%, 41.1%, 47.9) using nested-PCR as the reference technique. The detection rates of nested-PCR for *Pasmodium* infection was superior than conventional blood film microscopic examination, OptiMAL test, Hexagon Malaria Combi microscopy and Diaquick Malaria Pf/Pan Cassette. The nest-PCR method was more reliable than conventional microscopic examination for the diagnosis of malaria infections, and this is particularly true in cases of mixed infections and submicroscopic infections.

Keywords: Malaria, nest-PCR, Diagnosis, Makkah, Taif, Saudi Arabia.

Introduction

Malaria remains the most important vector-transmitted parasitic disease and a leading cause of morbidity and mortality worldwide (Gilles and Warrell, 2002). Malaria in humans can be caused by one of five malaria parasites (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*). *P. falciparum* causes the most severe form of the disease. It has been increasingly recognized that *P. vivax* is also associated with severe symptoms (Rogerson and Carter, 2008).

For more than 100years, conventional bloodfilm microscopic examination remains the gold standard method for malaria diagnosis due to its ability to identify infected *Plasmodium* species and quantify parasitemia levels at a low cost, time consuming, but it needs an experienced microscopists and continuous quality control and quality assurance systems. Also, misdiagnosis can occur in low parasitemia cases, and false identification of some species (Kang *et al*, 2017).

Malaria Antigen Rapid Diagnostic Tests (mRDTs) based on antigens detection in patient blood by using a monoclonal antibody that detect a specific enzyme secreted by *Plasmodium* species, it's called *Plasmodium* lactate dehydrogenase (PLDH), it's also able to detect a specific regions of *P. falciparum* & *P. vivax*. These tests have the same problems as microscopy, in low parasitemia they may not be accurate, and gave false positives resulted from antigenemia weeks be-

yond the actual infection and deletion or mutation of parasite antigens can lead to false negative. Otherwise RDTs cannot determine parasitemia level, or differentiation of *Plasmodium* (Endeshaw *et al*, 2008).

Molecular detection based on the amplification of parasitic DNA is one of these alternative ways for diagnosis. However, this way is based on amplification of the parasite DNA. PCR based diagnostic was known as the most sensitive, specific and powerful tool to detect and differentiate mixed *Plasmodium* species infection (Mahale *et al*, 2019).

Snounou *et al.* (1993) developed the nested PCR method back in 1993 and widely used as the gold standard molecular assay for malaria parasites detection (Johnston *et al*, 2006). Over the years multiple PCR methods have been developed for the lab detection of *Plasmodium* infection including conventional, real-time PCR techniques that allow the detection and differentiation of all *Plasmodium* species infecting human with at least five parasites per microliter of blood and even probably as low as 0.002parasites/μL (Kamau *et al*, 2011). But PCR is still costly and not very useful for routine diagnosis and requires trained personnel and reagents that need to be frozen or refrigerated, and cannot be used in the field. PCR is useful for epidemiological monitoring of *Plasmodium* infections as asymptomatic and mixed infections are frequently accompanied with the malaria cases (Yin *et al*, 2018).

The present study analyzed the diagnostic performance of conventional blood film microscopic examination, OptiMAL Rapid Antigen Test, Hexagon Malaria Combi, Diaquick Malaria Pf/Pan Cassette as compared with species-specific nested polymerase chain reaction (nested PCR) in Makkah and Taif, Kingdom of Saudi Arabia.

Materials and Methods

Subjects: A total of 100 patients enrolled in this study, presented with fever of unknown origin. The patients with an average age of 18-55 years, attending the internal medicine department at Maternity and Children hospital, King Faisal hospital, Makkah, and King Faisal specialized hospital, Taif, KSA, from January 2013 to April, 2017. The patients were screened for *Plasmodium* infection by conventional blood film microscopic examination (CBFME), OptiMAL test, Hexagon Malaria Combi and Diaquick Malaria Pf/Pan Cassette then confirming the results using species-specific nested PCR.

Ethics approval: All procedures followed were in accordance with the ethical standards of human research and in accordance with the Helsinki Declaration. Ethical approval for the study was obtained from the Ethics Committee of the Faculty of Applied Medical Sciences, Umm Al-Qura University (AMSEC 1-1-2013). All patients signed acknowledgment consents to declare their participation agreement.

Specimen: EDTA-treated blood samples for microscopic examination and RDTs was collected from malaria suspected subjects for the presence of *Plasmodium* parasites using microscopic examination of thick and thin blood films, OptiMAL test, Hexagon Malaria Combi and Diaquick Malaria Pf/Pan Cassette. For PCR analysis, blood drops were spotted onto grade 1 Whatman filter paper (Whatman International Ltd., Maidstone, United Kingdom) allowed to dry at room temperature, stored in individual plastic bag until DNA extraction.

Conventional blood film microscopic examination (CBFME): Thick and thin blood films were prepared from peripheral blood, stained with fresh 10% Giemsa's solution screened for malaria parasites by microscopy with (100×) oil immersion magnification. Stained thin film preparations of positive thick films were examined to determine the species: *P. falciparum*, *P. vivax*, *P. malariae* or mixed infection (Endeshaw *et al*, 2008).

Rapid immunochromatographic test: Opti MAL test employs immunochromatography in a dipstick that contains monoclonal antibodies (Mabs) directed against the enzyme pLDH. There are two Mabs: one is specific for *P. falciparum* and located at the bottom

of the dipstick and the other is specific for *Plasmodium* sp. and located in the middle of the dipstick. The whole process was conducted following the manufacturer's instructions. Briefly, a drop of blood was added to a well in a microtiter plate and mixed with two drops of lysis buffer, which disrupts the red blood cells and releases the pLDH. The specimens were then allowed to migrate to the top of pLDH strip. After eight minutes, the strips were placed in washing buffer, which cleared the hemoglobin from the strip. The entire process took approximately 15 min, and the results were visually interpreted immediately. A positive control line must be present at the top of the strip to verify that the test strip is functional. If this is the only line that appears, the test is considered negative for malaria. Appearance of a second line, adjacent to the positive control line, indicated the presence of a non-*P. falciparum* malaria (*P. vivax*, *P. ovalae*, or *P. malariae*). When a third line was present, this indicated a positive *P. falciparum* infection (Ferro *et al*, 2002; Abdel-Wahab *et al*, 2012).

Hexagon Malaria Combi **(**Human GmbH, Germany**)** is a rapid test for the qualitative detection of Histidine Rich Protein (HRP2) released from *P. falciparum* and aldolase, a pan-*Plasmodium* antigen released from all *Plasmodium* species and is intended for the diagnosis of malaria. The test detects *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*). The test with 20µL of whole blood was performed in accordance with the manufacturers' instructions. 5µL of blood was added into the center of the sample window. Blood was transferred by holding vertically and gently touching the sample pad in the center of the sample window. Sample allowed to be aspirated by capillary action, 2 drops of diluent was added to the sample window, the result was read after 15 minutes. Negative results in case of redviolet control line appearing in the upper part of the rectangular result area while reading was invalid if no control line appears. Positive *P. falciparum* malaria was indicated by either one test line or by two test lines additionally to control line. Two positive test lines were in mixed malaria infection. Also, one red-violet test line (band at 2), indicated a positive for *P. vivax* or non-*falciparum* species (*P. malariae* or *P. ovale*) or mixed infection.

Diaquick Malaria *Pf*/Pan Cassette (DIALAB GmbH) is a rapid test for the qualitative detection of *P. falciparum* Histidin Rich Protein (HRP2) released from *P. falciparum* and pLDH (*P. falciparum*, *P. vivax*, *P.malariae*, *P. ovale*). The test with 5ul of whole blood in specimen insertion hole, and then 4 drops of buffer were added and wait for 15-30 min, and then then read the results.
A colored band was visible only in the control region indicating negative result while two colored bands are visible in the *Pf* region and control region indicate positive for *Pf*. Also, two colored bands are visible in the Pan region and control region indicate positive for Pan. Three colored bands were visible in the *Pf* region, the Pan region and control region indicate positive for *Pf* & Pan. If there was no colored line in the control region, this indicated invalid results.

Nested diagnostic PCR: DNA was extracted from dried blood spots impregnated on filter paper using the QiagenDNeasy Blood Tissue Kit after the manufacturer's recommendation. The nested PCR using primers targeting *Plasmodium* cox3 genes and PCR conditions described (Isozumi *et al*, 2014). Primary PCR reaction, 20µL of extracted DNA were used as template for the first amplification reaction in a 50µL reaction with 1µL of the genus specific primer, and 20µL of HSTaq Master Max Mix (Qiagen). Cycling conditions consisted of an initial activation at 96°C for 5min, followed by 40 cycles at 96°C for 30s & 53°C for 30s, and a final extension step at 72°C for 5min. Amplification product was analyzed by 0.8% agarose gel electrophoresis, with an expected band of 940bp. Primary PCR product was diluted 1:20 with sterile water and used as template for secondary PCRs. The secondary PCR was done individually for each of

the four *Plasmodium* spp. Each secondary PCR was carried out in a 50µL reaction containing 10µL of the diluted primary PCR product, 1µL of each species-specific primer for the four-human malaria spp. primer in separate reaction tubes, & 20µL of HSTaq Master Max Mix (Qiagen). Cycling conditions consisted of an initial denaturation at 96°C for 1min, followed by 40 cycles at 95°C for 30s & 56°C for 30s, & a final extension at 72°C for 45s. Amplification products were analyzed by 0.8% agarose gel electrophoresis, with expected bands in the range of 87 to 233bp. Diagnosis of conventional blood film microscopic examination, OptiMAL Rapid Antigen Test, Hexagon Malaria Combi, Diaquick Malaria Pf/Pan Cassette in comparison to nested PCR was evaluated with nested PCR results. Performance indices were number of true positive (TP), & true negative (TN), numbers of false positive (FP) & false negative (FN).

Sensitivity = TP/(TP+FN)×100, specificity = TN/(TN+FP)×100, positive predictive va-lue (PPV) as TP/(TP+FP)×100, & negative predictive value (NPV) as TN/ (FN+TN) ×100, accuracy index (AI) as TP+TN/ (TP+FP+ TN+FN)×100. Differences in detection rates of diagnostic tests were tested for significance using Chi-square test. A probability value of less than 0.05 was considered statistically significant (Li *et al*, 2014).

Table 1: Primer sequences used for nested PCR

Species	Name	Primer Sequence (5' to 3')	Product (bp)
Plasmodium	MtU.F	CTCGCCATTTGATAGCGGTTAACC	937-9
	MtU.R	CCTGTTATCCCCGGCGAACCTTC	
P. falciparum	MtNst_falF	GAACACAATTGTCTATTCGTACAATTATTC	201
	MtNst_falR	CTTCTACCGAATGGTTTATAAATTCTTTC	
P. vivax	MtNst_vivF	CTAGCTTTTAACACAATATTATTGTCTATAC	87
	MtNst_vivR	GTTCTTTTTCTATTCAGAATAATGAATATAT	
P. malariae	MtNst_malF	CTAGCTTTGTACACAAATTAATTCGTCTAC	233
	MtNst_malR	CTTTATAAGAATGATAGATATTTATGACATA	
P. ovale	MtNst_ovF	ATTATTGTCAAATATAAGTACTTTAATC	204
	MtNst_ovR	GGTTGAAGTTTATGATACTAATAAC	
Plasmodium (nested)	MtUnst.F	GTAAACATGCwGTCATACATGCAC	430
	MtUnst.R	CCCCGGCGAACCTTCTTACCGT	

Results

The results were shown in tables (2, 3, 4, 5 & 6) and figures (1&2).

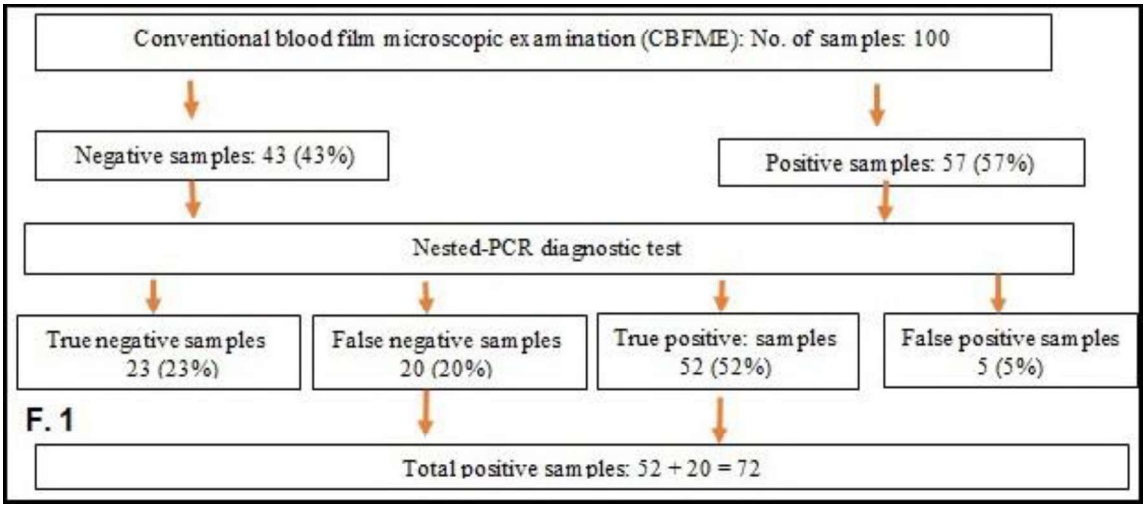

Fig. 1: Microscopic examination and nested PCR for the examined samples.

The differences in d rates of nested PCR, conventional blood film microscopic examination, OptiMAL test, Hexagon Malaria Combi, Diaquick Malaria Pf/Pan Cassette were (72%, 57%, 59%, 44% & 52% respectively) in 100 clinically suspected cases.

Table 2: Different diagnostic assays for detection of *Plasmodium* infection among suspected patients

Diagnostic tests	Nested PCR		CBFME		OptiMal		Hexagon		Diaquik	
	+ve (%)	-ve (%)	+ve (%)	-ve (%)	+ve (%)	-ve (%)	+ve (%)	-ve (%)	+ve (%)	-ve (%)
Suspected patients (100)	72 (72)	28 (28)	57 (57)	43 (43)	59 (59)	41 (41)	44 (44)	56 (56)	52 (52)	48 (48)

*CBFME: conventional blood film microscopic examination

Table 3: Matching cross tabulation of Nest-PCR assay as criterion standard test and other diagnostic assays

Diagnostic test		CBFME		OptiMal		Hexagon		Diaquik	
		+ve (%)	-ve (%)	+ve (%)	-ve (%)	+ve (%)	-ve (%)	+ve (%)	-ve (%)
Nest-PCR	Positive (72)	52 (72.2)	20(27.8)	52 (72.2)	20(27.8)	39 (54.2)	33 (45.8)	47 (65.3)	25(34.7)
	Negative (28)	5 (17.9)	23(82.1)	7 (25)	21 (75)	5 (17.9)	23 (82.1)	5 (17.9)	23 (82.1)

Using nested PCR as reference, conventional blood film microscopic examination, OptiMAL test, Hexagon Malaria Combi & Diaquick Malaria Pf/Pan Cassette showed 5, 7, 5 & 5 false-positive samples respectively that were negative by Nest-PCR, and 20, 20, 33 & 25 false-negative samples that was positive by Nest-PCR. Microscopic examination showed a sensitivity (72.2%), specificity (82.1%), positive predictive value (91.2%), negative predictive value (53.5%) and diagnostic accuracy (75%) while OptiMAL test (72.2%, 75%, 73%, 88.1% & 51.2%), Hexagon Malaria Combi (62.9%, 82.1%, 88.6 %, 41.1%, Diaquick Malaria Pf/Pan Cassette, 65.3%, 82.1%, 70%, 90.4% & 47.9% respectively.

Table 4: Evaluation of diagnostic reliability of microscopic examination assay and other rapid tests

Evaluated assays	Test Results (n)				Evaluation Parameters (%)				
	TP	FP	TN	FN	SN	SP	AI	PPV	NPV
CBFME	52	5	23	20	72.2	82.1	75	91.2	53.5
OpiMal	52	7	21	20	72.2	75	73	88.1	51.2
Hexagon	39	5	23	33	62.9	82.1	62	88.6	41.1
Diaquik	47	5	23	25	65.3	82.1	70	90.4	47.9

Of the 57 microscopically confirmed cases, 30 cases (52.63%) were *P. falciparum*, 25 cases (43.85%) were *P. vivax*, and2cases (3.5%) were *P. falciparum* and *P. vivax* mixed infections, but 43 patients were negative for *Plasmodium* infection, while 72 molecular confirmed positives for *Plasmodium* infection cases by nested PCR in 100 clinically suspected cases, 27 (27%) were infected by *P. falciparum*, 37 (37%) by *P. vivax*, and 8 (8%) were mixed infections (*P. vivax* & *P. falciparum*).

Table 5: Matching cross tabulation of identification power of nested PCR assay as criterion standard test against other diagnostic assays

Test IDs		CBFME				OptiMal				Hexagon				Diaquik			
		Pf (30)	Pv (25)	Mix. (2)	-ve (43)	Pf (0)	Pv (28)	Mix. (31)	-ve (41)	Pf (22)	Pv (18)	Mix. (4)	-ve (46)	Pf (13)	Pv (21)	Mix. (18)	-ve (48)
NEST-PCR	Pf (27)	22	0	0	5	0	0	21	6	17	0	1	9	9	0	12	6
	Pv (37)	0	22	1	14	0	23	1	13	0	15	1	21	0	18	1	18
	Mix. (8)	5	1	1	1	0	1	6	1	2	1	2	3	2	1	4	1
	-ve (28)	3	2	0	23	0	4	3	21	3	2	0	23	2	2	1	23

Table 6: Identification accuracy of evaluated diagnostic assays as compared to standard Nest-PCR identification

CBFME				OptiMal				Hexagon				Diaquik			
Pf	Pv	Mix	-ve	Pf	Pv	Mix	-ve	Pf	Pv	Mix	-ve	Pf	Pv	Mix	-ve
22/27 (81.5)	25/37 (67.6)	1/8 (12.5)	23/28 (82.1)	0/27 (0)	23/37 (62.2)	6/8 (75)	21/28 (75)	17/27 (63)	15/37 (40.5)	2/8 (25)	23/28 (82.1)	9/27 (33.3)	18/37 (48.6)	4/8 (50)	23/28 (82.1)

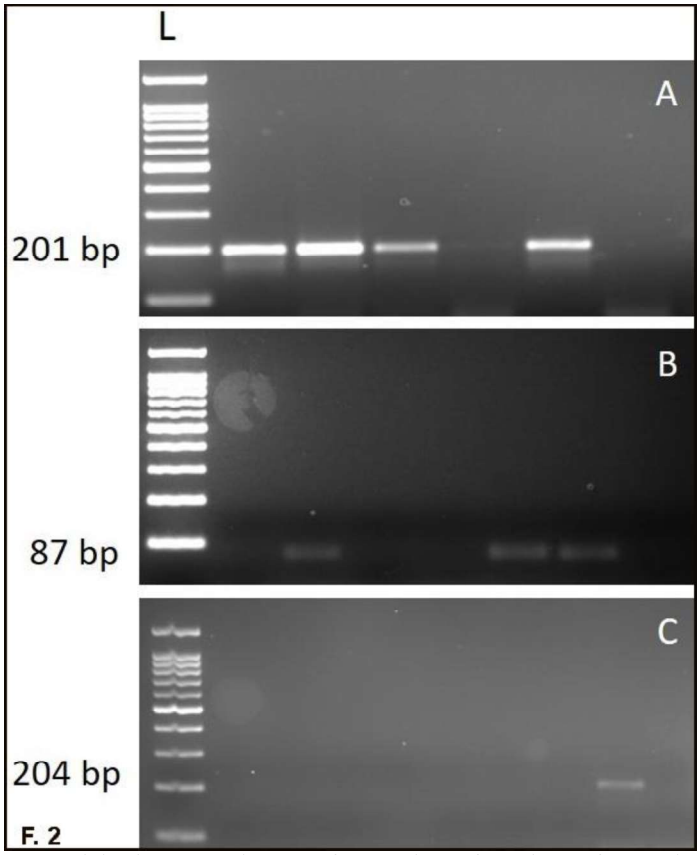

Fig. 2: Representative 2% agarose gel showing recovered patterns after second step of nested PCR of extracted DNA from investigated samples using the following primer sets: A, *P. falciparum*-specific primers; B, *P.vivax*-specific primers; C, *Plasmosium ovali*-specific primers.

Discussion

Accurate diagnosis of *Plasmodium* species is necessary to establish the correct course of therapy. Misdiagnosis could result in severe public health significance due to incorrect treatments leading to drug resistance and recrudescence occurrence (Kang *et al,* 2017).

In the current study, a total of 100 suspected patients were tested; nested PCR identified 72 (72%), conventional blood film microscopic examination identified 57 (57%) of these as positives while the OptiMAL test identified 59 (59%), Hexagon Malaria Combi identified 44 (44.00%), and Diaquick Malaria Pf/Pan Cassette identified 52 (52.00%). The possible explanations for discrepancies in test results obtained by nested PCR, microscopic examination and rapid test included (i) microscopic examination needs an experienced microscopists, continuous quality control and quality assurance systems. Also, misdiagnosis can occur in low parasitemia cases, and false identification of some species. (ii) insufficient detection of low parasitemia by rapid test (iii) Diaquick Malaria Pf/Pan Cassette and Hexagon Malaria Combi rapid test detects only live parasites producing pLDH (iv) sequestration of parasites and (v) rapid test false-positive reactions resulted from antigenemia weeks beyond the actual infection and deletion or mutation of parasite antigens could lead to false negative (Endeshaw *et al*, 2008; Kang *et al*, 2017).

PCR was used as reference standard to evaluate microscopic examination of Giemsa's stained blood smears, OptiMAL, Diaquick Malaria Pf/Pan Cassette, Hexagon Malaria Combi, PCR detected more positive cases than microscopic examination or rapid tests because it detected the parasite DNA, which could be present in either dead or live parasites, whereas rapid tests detect an enzyme produced by live parasites (Endeshaw *et al*, 2008).

Differences in *Plasmodium* infection detection rates of microscopy, OptiMAL test and PCR (45%, 42.5%, & 46.7% respectively) in 120 clinically suspected cases (Abdel-Wahab *et al*, 2002) while *Plasmodium* sp. was diagnosed in 46.9% of patients with undifferentiated fever (Neuberger *et al*, 2011). Reported studies from different countries of South Asia: Sri Lanka (Fernando *et al,* 2004), Pakistan (Iqbal *et al*, 2003) and Thailand (Pattanasin *et al*, 2003), that demonstrated 38%, 42%, & 53% malaria positive among groups using microscopy and RDTs.
Although thirty samples were diagnosed as *P. falciparum* by microscopic examination, only twenty-seven was confirmed as *P. falciparum* by nested PCR; five was mixed infections and the rest were negative. In addition, of the 25 *P. vivax* detected by microscopic examination, only 22 were *P. vivax* on PCR and the rest were negative. Moreover, of the two mixed infections detected by microscopy, only one was mixed infections on PCR and the rest was *P. vivax*. Furthermore, of the 43 negative samples detected by microscopic examination, only 23 were negative by PCR and 5 *P. falciparum*, 14 were *P. vivax* and the rest was mixed infection. In agreement, Kimura *et al*. (1995) reported that the role of microscopic examination for malaria diagnosis was questioned due to false negative results at low parasitemia levels and frequent errors in species identification in mixed infections. Also, low diagnosis reliability of microscopy for species specific and mixed-infections in endemic areas were reported (Mekonnen *et al*, 2014; Mwingira *et al*, 2014).

In the current study, positive samples by nested PCR showed higher prevalence of *P. vivax* (37%), than *P. falciparum* (27%). Mixed infections by *P. vivax* and *P. falciparum* presented 8%. On the other hand, mixed infections account for 1% of malaria cases in Colombia (Ministerio de Salud, 1999).

In the current study, *P vivax* was the most predominant species detected by nested-PCR (37%), which agreed with Santana-Morales *et al*.(2012) in Ethiopia; MacLean *et al.* (2004) in South Asia, and Fernando *et al.* (2004) in Sri Lanka.

In mixed *P. falciparum*/*P. vivax* malaria, OptiMAL presented reactions with the *P. falciparum* and *Plasmodium* sp. Mabs, a pattern which corresponds to diagnosis of *P. falciparum* infection. Thus, the actual format of OptiMAL favors the diagnosis of *P. falciparum* but masks co-infection by *P. vivax* or other species of *Plasmodium* and hence constituted a limitation of the current configuration of the test in settings of co-endemicity (Srinivasan *et al*, 2000).

Accurate identification of malaria parasites to the species level is imperative so that the patient receives appropriate therapy, particularly when the patient has relapsing malaria caused by *P. vivax* and *P. ovale*. It is also important because of the severe morbidity and mortality associated with *P. falciparum* and growing resistance to antimalarial therapy. Furthermore, it was vital to have follow-up specimens from malaria-positive patients to monitor therapy outcome and detect drug failure (Palmer *et al*, 2003). *P. falciparum* and *P. vivax* were the two predominant malaria species, accounting for 60% & 40% of cases, respectively in Ethiopia (Ministry of Health, 2001).

In Colombia, about 55% of malaria cases were caused by *P. vivax*, 45% by *P. falciparum* and less than 0.1% overall by *P. malariae* (Ministerio de Salud 1999; Vergara*et al*, 2001). Treatment regimens for *P. falciparum* differ from those for other species of *Plasmodium* because of the high frequency of resistance to chloroquine (Osorio *et al*, 1999) the absence of hipnozoite liver stages and the risk of lethal complications of infection. Therefore, diagnostic tests that both detect and distinguish *P. falciparum* and *Plasmodium* sp. infection are essential to appropriate treatment and management of malaria in Colombia.

Species differentiation in positive samples by microscopic examination in the present study showed higher prevalence of *P. falcip-*

arum (30%) than *P. vivax* (25%). Traditionally, *P. falciparum* inhibited the *P. vivax* parasitemia (Garnham, 1966). But, several lines of evidence suggested that *P. vivax* may have a suppressive effect on *P. falciparum*. The preponderance of one malaria species over the other at a particular period might vary from one area to another, not only depending on climatic and seasonal factors but also owing to variation in geographical localities (Checchi *et al*, 2006).

The results of microscopic examination of Giemsa-stained blood smears, OptiMal test, Diaquick Malaria Pf/Pan Cassette and Hexagon Malaria Combi were 20, 20, 33 and 25 false negative samples respectively that were positive by nested PCR. Also, in microscopic examination, OptiMal test, Diaquick Malaria Pf/Pan Cassette and Hexagon Malaria Combi indicated 5, 7, 5 & 5 false positive samples respectively that were negative by nested-PCR that was compatible with Iqbal *et al*. (1999; 2003). Berzosa *et al*. (2018) stated that false-negative results delayed the treatment. False negative results of RDTs were attributed to possible genetic heterogeneity of HRP2 or LDH expression, deletion or mutation of HRP2 or LDH gene, presence of blocking antibodies, or immune-complex formation (Chaijaroenkul, 2011). HRP2 assays detect *P. falciparum* only, but 'Pan-specific' aldolase has low sensitivity for *P. vivax* detection as well as 'Pan-specific' aldolase and pLDH has poor detection of *P. ovale*/*malariae* (Hanscheid, 2003). Also, parasite density during *P. vivax* infections was often low (Luz *et al*, 2006). The presence of low parasitemias, especially below the detection level established by the manufacturer as 100-200 parasites/µL was associated with false negative results. Gametocytes produce pLDH, but since gametocyte parasitemias are usually low, they are not detected efficiently by OptiMAL (Moody *et al*, 2000).

In the present study, microscopic examination gave a sensitivity (72.2%), specificity (82.1%), positive predictive value (91.2%), negative predictive value (53.5%) and diagnostic accuracy (75%) using nested-PCR as the reference technique. In agreement, Ugah *et al*. (2017) stated that microscopy has low sensitivity when performed by poorly trained personnel in endemic areas, especially in primary and secondary health-care facilities. Besides, Hexagon Malaria Combi rapid test showed a sensitivity (62.9%), specificity (82.1%), positive predictive value (88.6%), negative predictive value (41.1%) and diagnostic accuracy (62%). On the other hand, Wanji *et al*. (2008) found that Hexagon Malaria Combi rapid test sensitivity of 85.33%, specificity of 95.5% and accuracy (91.4%) among children with asymptomatic malaria living in the Mount Cameroon region. But, Wanji*et al*, (2008) reported that Hexagon Malaria Combi was sensitive (87.5%) and specific (98%) for detecting *P. falciparum*.

Misdiagnosis could lead to inappropriate or delayed treatment that has been implicated in malaria-associated deaths in developed countries (Kain, 1998). In explanation of false positivity, reasons include the incomplete treatment, delayed clearance of circulating antigen (free or in antigen-antibody complexes), and cross reaction with non-*falciparum* malaria, rheumatoid factor (Chaijaroenkul, 2011) or heterophile antibodies (Moody and Chiodini, 2002). In HRP2 assays, antigen could persist for up to 2-3 weeks after cure. So, it was recommended to use microscopy to monitor antimalarial therapy (Hanscheid, 2003). Nested PCR proved to be a sensitive and specific method for detecting malaria parasites especially in epidemiological surveys. But, preparation of DNA for PCR is often time-consuming and costly (Li *et al*, 2014). In this study, only one patient presented *P. ovale* infection by nested PCR was positive. The Optimal was less efficient in detection of *P. malariae* and *P. ovale* (Srinivasan *et al*, 2000).

Conclusion

The nested-PCR for malaria was superior than conventional blood film microscopic examination, OptiMAL test, Hexagon Malaria Combi microscopy, Diaquick Malaria

Pf/Pan Cassette in 100 clinically suspected cases. PCR produced conflicting results, particularly in mixed *Plasmodium* infections and submicroscopic infections. The molecular diagnosis was more reliable than microscopic examination for diagnosis of *Plasmodium* species as a part of the malaria surveillance programs in KSA.

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