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Detection of Plasmodium Falciparum Using Nested PCR Among Suspected Patients in Wad Medani City, Gezira State, Sudan

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Abstract

Introduction:

Plasmodium falciparum is a virulent species and causes most severe and pathogenic form of malaria, characterized by high parasitic burdens and vital organ dysfunctions. The diagnosis of falciparum malaria in Sudan depends on microscopy and Rapid diagnostic test (RDTs). Nested PCR targeted 18S rRNA gene provides an alternative technique for identification of parasite in thick and blood film.

Objective:

The study aims to estimate the diagnostic performance of 18S rRNA gene analysis by nPCR for detection of P. falciparum versus microscopic detection.

Method:

This is a cross sectional hospital based study. Two hundred and twenty blood samples and relevant data were collected from patients with falciparum malaria attending Wad Medani Teaching Hospitals and 26 samples from healthy participants. Parasite count was counted using stained thick blood film. The DNA extraction was done using TE buffer. Moreover, Nested PCR for 18S rRNA gene was done using specific primers. Data were analyzed using MedCalc programs (V. 16).

Results:

The microscopic examination of blood films showed that all patients (220/100%) were positive for P. falciparum among patients. one sample (3.8%) was positive among healthy participants (1/26). When using nPCR for 18S rRNA gene 219 samples were positive (99.5%) form patients and one sample was positive (3.8%) among healthy participants. The area under curve (AUC),

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sensitivity, specificity, positive predictive value, negative predictive sensitivity and Weighted Kappa agreement for the nPCR were 0.935, 98.6%, 88.6%, 60.1%, 99.7% and 0.871(95% CI:0.770 - 0.972), respectively.

Conclusion:
Nested PCR for 18S rRNA gene is sensitive, specific, reliable technique for diagnosis of Plasmodium falciparum in the endemic study area and can be introduced as a confirmatory diagnostic tool.

Keywords: 18S rRNA gene, nPCR, falciparum malaria, Microscopy, Sudan.

I. INTRODUCTION:

Malaria is a serious, major public health problem worldwide. *Plasmodium* species infect more than 219 million people worldwide, 92% of them in African region and causes about 435,000 cases of death (WHO, 2018). In Sudan, 75% of population at risk for acquiring malaria. The main plasmodium species of malaria in Sudan is *P. falciparum* which representing 87.6% and 5% mixed infection with *P. vivax* (Federal Ministry of Health, 2017; Mohamedahmed *et al*., 2019). The Giemsa blood stained smears followed by Microscopy, remains the gold standard method for diagnosis of malaria parasites. The technique has a potential for detection and confirmation of parasite morphology, differentiation between parasite species, estimation the level of parasitemia, in addition to its easiness to be perform and cheap. However, detection limit is about 10–100 parasites/μL, require good trainee personnel and good quality of smear preparation (Wongsrichanalai *et al.,* 2007; Okell *et al*., 2009; Cheng *et al*., 2015).

Estimating parasite density using white blood cells is useful procedure in management and drug efficacy studies (Planche *et al*., 2001). As stated by WHO, PCR is most sensitive and specific compared with other diagnostic techniques but requires special and expensive reagents, apparatus and lab conditions that may be difficult to be present in field (Payne, 1988; WHO, 2015). The sensitivity of nPCR appear clear in detection limit that reported which approximately 0.4 and 0.05 parasites/μL (Hänscheid and Grobusch, 2002; Mixson-Hayden *et al*., 2010; Polley *et al*., 2012). The 18S rRNA gene considered a biological marker which used in malaria controls (Seilie *et al*., 2019). The gene is highly expressed on red blood cells by asexual stage of the parasites (Waters *et al*., 1989). In Sudan need for reliable test with high specificity and sensitivity for diagnosis of *P. falciparum* especially in endemic area with falciparum malaria which help in disease detection and subsequently eradication. This study was designed to evaluate the performance of 18S rRNA nested PCR in detection of *P. falciparum*.

II. RESEARCH METHODOLOGY:

Study design:

Study was conducted in Wad Medani city, capital of the Gezira State in East-Central Sudan. The city lies on the West bank of the Blue Nile, nearly 85 miles (136 km) Southeast of Khartoum. A hospital based cross-sectional study was conducted among 220 patients known diagnosis with falciparum malaria according to clinical and laboratory diagnosis (blood film) and 26 healthy

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participants. The study cases from all ages and both sexes attending Wad Medani Teaching Hospitals during the period between November 2018 to January 2019.

Samples collection:
Data of all participants were collected using a questionnaire. Three venous blood sample from each participant was collected into EDTA container. The parasite count was counted from thick blood (Cheesbrough, 2009).

DNA extraction:
The TE buffer DNA extraction from dry blood spot (DBS) was done according to method described by Bereczky *et al*., 2005, total circle of DBS were punched by a sterile surgical plate and placed into a sterile, clean 1.5 eppendorf. About 100 µl of TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA in distilled water) was transferred into tube and incubated at 50°C for 30 minutes. The punches were pushed several times with different pipette tips toward the bottom of the tube and incubated at 97°C for 15 min. The tube was centrifuged at high speed for 30 seconds and the supernatant was used for nested PCR (Bereczky *et al*., 2005).

Nested polymerase chain reaction (nPCR) for 18S rRNA gene:
The 18 small-subunit rRNA gene contains both genus and species specific sequences that detect *P. falciparum* through nested PCR (Table 1). The nested PCR based on the principle described by Snounou *et al*., (1993) which used rPLU5 and rPLU6 for outer PCR which detect all *Plasmodium* species followed by rFAL1 and rFAL2 for detection of species specific of *Plamodium falciparum* by using a primers as shown in Table (1). The outer PCR include 10 µl of master mix (APSLABS, India), 0.5 µl from each rPLU5 and rPLU6 primers, 5 µl of DNA template and complete volume to 25 µl by nuclease free water. The protocol modified by Johnston *et al*., (2006) by using outer PCR cycling conditions (Denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min (30 cycles). In nested PCR, 10 µl of master mix (APSLAB, India), 0.5 µl from each rFAL1 and rFAL2 primers, 1 µl from outer PCR product and complete volume to 25 µl using nuclease free water. The cycling conditions (Denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min (30 cycles). The amplified DNA was then run in 1.5% Agarose gel electrophoresis after stained by Ethedium Bromide (Sigma, USA) in 100 Amp for 30 minutes (Figure 2) (Snounou *et al*., 1993).

Table (1): Primers for nested PCR of 18S rRNA gene in malaria parasites.

Species	Primer	Sequence (5´–3´)	Size (bp) of PCR product
***Plasmodium* sp.**	rPLU5 rPLU6	CCTGTTGTTGCCTTAAACTTC TTAAAATTGTTGCAGTTAAAACG	1,100
P. falciparum	rFAL1 rFAL2	TTAAACTGGTTTGGGAAAACC AAATATATT ACACAATGAACTCAATCATGA CTACCCGTC	205

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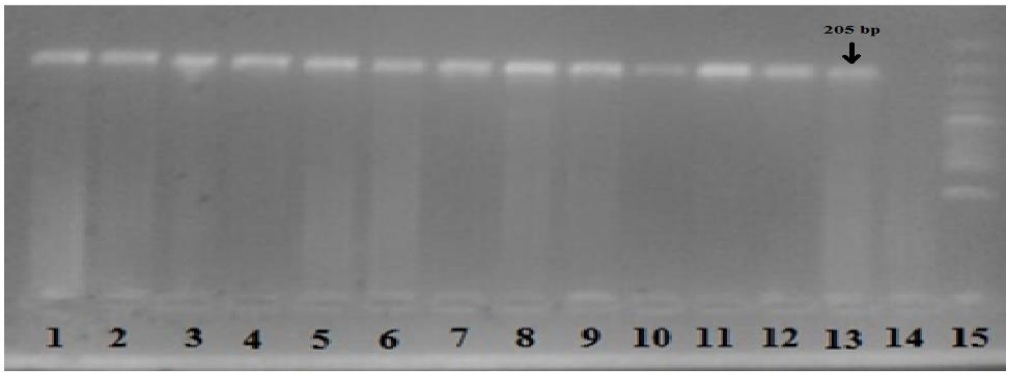

Figure 2. Species specific nPCR product. Note: lines1-13 is positive, line 14 is negative and line 15 is DNA ladder (100 bp)

Ethical considerations:
The Ethical approval and permission obtained from Ministry of Health, Gezira State and informal consent from each participant.

Data analysis:
Data was analyzed using Med Calc program (V. 16). Sociodemographic data and test validation was obtained. The validation of nPCR was done using receiving operating characteristics (ROC) curve by estimating sensitivity, specificity, predictive values and area under the curve (AUC). Also agreement between two methods calculated using Weighted Kappa.

II. RESEARCH RESULTS:

Two hundred and twenty patients and 26 healthy controls were participated in the study. The distribution of the males (133; 60.4%) was higher than the female (87; 39.6%). The mean age was 21.4 ± 17.5 year, in which 38 (17.3%) of cases were observed in children under 5 years, while 64 (29.1%) of cases were observed in children above 5 years. The mean of parasite count was 59096.75 parasite/µl (SE=924.097) (range between 79 – 549,333 parasite/µl). The common symptoms were fever (99%), fatigue (88.6%) and headache (87.7%) (Table 1).

Table 1. Socio-demographic characteristics of study participants:

Characteristics		**Cases (N=220) No (%)**	**Healthy (N=26) No (%)**
Sex	Male	133 (60.4%)	19
	Female	87 (39.6%)	7
Age (years) Mean ± SD		21.4 ± 17.5 year	
Age groups (years)	< 5 Years	38 (17.3%)	0
	6 – 15 Years	64 (29.1%)	0
	16 – 30 Years	66 (30.0%)	14
	31 – 45 Years	28 (12.7%)	7
	> 45 Years	24 (10.9%)	5

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Mean of parasite count (parasite/µl) ± SE	59096 ± 924.097	

The Samples were confirmed by detection of *P. falciparum* in stained thick blood film for among all patients (100%) and one sample of healthy participants (3.8%). The nPCR for 18S rRNA gene results were showed positive results in 219 samples (99.5%) among patients and one sample (3.8%) in healthy participants but this positive sample different from sample that detected by microscopy (Table 2).

Table 2. Positive results for *P. falciparum* of both microcopy and 18S rRNA gene by nPCR

Result	**Microscopy**		**18S rRNA gene by nPCR**	
	Patients	Healthy	Patients	Healthy
Positive	220	1	219	1
Negative	0	25	1	25

The validation of nested PCR for 18S rRNA gene was showed the area under the ROC curve was 0.935 with 98.6% sensitivity, 88.6% specificity, 60.1% positive predictive value, and 99.7% negative predictive value. Furthermore, the agreement between nPCR and microscopy by Weighted Kappa was 0.871 (95% CI: 0.770 – 0.972) (Table 3; Figure 2).

Table 3. AUC, sensitivity, specificity, positive and negative predictive value of nPCR for 18S rRNA gene

Diagnostic performance	**18S rRNA gene by nPCR**
Area under the curve (AUC)	0.935
Sensitivity	98.6%
Specificity	88.5%
Positive predictive value	60.1%
Negative predictive value	99.7
Weighted Kappa	0.871

[a] Linear weights, 95% CI:0.770 – 0.972.

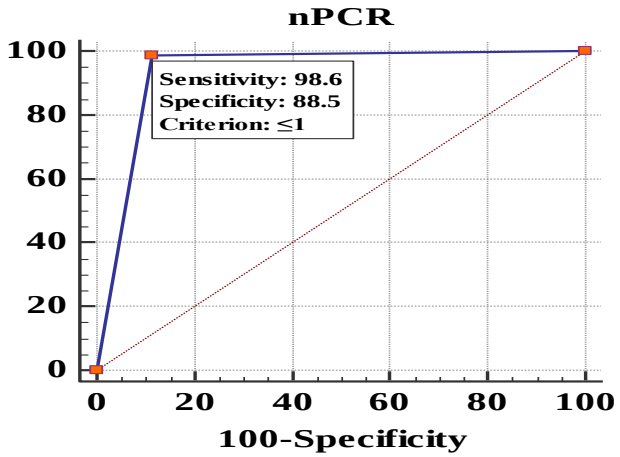

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Figure 1. Receiving operating characteristics (ROC) curve for 18S rRNA by nPCR compared with Microscopy.

III. DISCUSSION:

Falciparum malaria is one of the most widespread parasitic infections and is a major cause of mortality, particularly in endemic area (Mohamedahmed *et al*., 2019). Microscopy remains the gold standard method for laboratory diagnosis of malaria in Sudan due to its simplicity, reducing in time around time and low cost but need high experience examiner. Nested PCR of *P. falciparum* is most sensitive and specific compared with other malaria diagnostic techniques (WHO, 2015). This is study evaluated the performance of 18S rRNA gene by nested PCR for detection of *P. falciparum* against microscopy. The study conducted among referred patients to Wad Medani Teaching Hospitals, Sudan, a known endemic area for *falciparum* malaria. The sensitivity and specificity of nested PCR for 18S rRNA were (98.6% and 88.5% respectively) compared to microscopy, with positive and negative predictive value (60.1% and 99.7% respectively) and agreement by kappa was 0.871 (95% CI:0.770 – 0.972). The overall negative samples were two samples (0.8%) and one sample (0.4%) appears positive but diagnosed as negative by microscopy among both case and healthy. These could be considered as a true negative, as artifacts resulting from slide staining may gave a false positive results. According to recommendation of the WHO to ensure that nPCR is most sensitive and reliable technique for diagnosis of malaria parasites. This finding agrees with studies done in United state which found that the sensitivity was 100% (Johnston *et al*., 2006); other studies were done in Turkey, sensitivity, specificity, and positive predictive values were 100%, 97.2% and 73.3%, respectively (Doni *et al*., 2016); in Malaysia, the sensitivity was 100% (Khool *et al*., 1996); another study done in India showed sensitivity and specificity 90% and 95% respectively (Saha *et al*., 2017). Also, agreed with other studies conducted by Fuehrer *et al*., 2011; Adedoja *et al*., 2019 and Jayasingh *et al*., 2019.

IV. CONCLUSION:

Nested PCR for 18S rRNA is sensitive, specific, reliable technique for diagnosis of *Plasmodium falciparum* in study area and should be introduce as routine confirmatory test, survey and researches in endemic area in Sudan.

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Competing interests:

The authors declare that they have no competing interests

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