

1 **Original Research Article**
2 **Detection and species identification of malaria**
3 **parasites by nested-PCR: comparison with light**
4 **microscopy and with SD BIOLINE Malaria Ag**
5 **test in Luanda, Angola.**

6

7 **ABSTRACT**

Background: Malaria is the infectious disease causing the highest morbidity and mortality in Angola. Existing tools for the diagnosis of malaria include microscopy, rapid diagnosis tests (RDTs) and molecular tools. Nested-PCR is commonly used as a reference technique in the diagnosis of malaria due to its high sensitivity and specificity. The present study aims to evaluate the accuracy of light microscopy and SD BIOLINE Malaria Ag in the detection of *Plasmodium* spp. infection, using the nested-PCR as a reference method, and to determine the *Plasmodium* species in the study populations (Luanda, Angola) using [this](#) molecular tool.

Methods: Blood samples were obtained from patients with clinical suspicion of malaria. Malaria was diagnosed by light microscopy, SD BIOLINE Malaria Ag and nested-PCR, used as a reference method, with *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* being detected when possible. The sensitivity, specificity, positive, and negative predictive values (PPV and NPV) of microscopy and SD BIOLINE Malaria Ag were compared using the McNemar's test and the weighted generalized score Chi-squared test for paired data.

Results: A total of 225 subjects were studied. SD BIOLINE Malaria Ag ~~were~~was significantly

more sensitive than microcopy (87.65 % versus 71.60 %), ~~showed and was~~ substantially correlated ($\kappa = 0.64$) with the reference method. Nested-PCR detected 36.0 % (81/225) cases, 80 cases (98.8 %) infected with *P. falciparum* and 1 case as *P. malariae* (1.2 %), with no mixed infections.

Conclusion: The findings of this study support the need of using to use RDT in the diagnosis of *Plasmodium*. PCR could appear to be a useful method for detecting *Plasmodium* parasites during active malaria surveillance in Angola. This study contributes to wide knowledge about the presence of *Plasmodium* species in Angola.

Keywords: nested-PCR, RDT, Microscopy, Malaria, *Plasmodium*

1. INTRODUCTION

Malaria is an ancient protozoan parasitic disease that is a major health problem in tropical and subtropical countries throughout the world [1]. Largely based on Polymerase Chain Reaction (PCR) results, the number of species of human malaria parasites detected has increased from four to six within only 10 years [2]. The known causative agents of human malaria include *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and the recently included *Plasmodium knowlesi*, which is still recognized as a zoonotic species. Molecular methods also highlighted both the existence of two distinct non-recombining species of *Plasmodium ovale* (classic type *Plasmodium ovale curtisi* and variant type *Plasmodium ovale wallikeri*) [3,4].

In 2013, malaria produced 198 million cases and caused an estimated of 584 000 deaths, mostly among African children. In Angola, malaria is a major public health problem, with 3.7 million cases per year reported by National Malaria Control Program, two-thirds of which occur in children under 5 years [5]. According to Ministry of Health estimates, *P. falciparum* accounts for 92% of infections, followed by *P. vivax* in about 7% of cases and *P. malariae* (3%) [5,6]. Fançonny et al. [6] reported the existence of single and mixed infections with the two *P. ovale* species (*P. ovale curtisi* and *P. ovale wallikeri*) in Angola patients. Malaria misdiagnosis is an important cause of additional morbidity and mortality [7]. Appropriate diagnosis is not only important for ensuring prudent use of anti-malarial medications, and facilitating correct prognosis based on appropriate differential diagnosis [8], but also for tracking malaria elimination

efforts [9-11]. However in general the information regarding malaria species in Angola is limited [6-7]; for that reason it is important to contribute to more completely determine the species in different study population and regions of Angola, including the detection of the five human species previously described.

Existing tools for the diagnosis of malaria include light microscopy, rapid diagnostic tests (RDTs) and nucleic acid detection test [12-13]. Microscopic detection of parasites on Giemsa-stained blood smears has been the mainstay for malaria diagnosis in laboratories for more than a century. This method is relatively simple and ~~has a low used~~required minimal and training of the microscopist, with an average sensitivity of about 50–100 parasites per microliter, ~~which make~~ it is an imperfect gold standard [14-15]. Consequently, the quality of the results varies considerably from one lab to another, mainly due to the level of expertise of microscopists, quality of reagent and equipment, procedures, workload capacity and inefficient quality control procedures [7, 16].

RDTs are immunochromatographic tests designed to detect parasite products in human blood. These methods are increasingly being used for malaria diagnosis because they are rapid and easier to use especially in resource limited settings and do not require trained personnel or special equipment [9]. However, their use is limited due to lack of sensitivity for *P. vivax*, *P. malariae* and *P. ovale* [17]. To improve the accuracy of malaria diagnosis and avoid unnecessary treatment, the World Health Organization (WHO) recommends parasitological confirmation of malaria whenever possible, by microscopy or by RDT [16,18].

Molecular detection methods of *Plasmodium* have an important role to play in the efforts to control, **eliminate** and eventually eradicate malaria [19]. There are two basic approaches for species detection, single PCR and nested-PCR. In general nested-PCR is more sensitive than single PCR [19]. The nuclear small subunit (SSU) rRNA genes are targets extensively used for the molecular detection of human malaria parasites. ~~This gene is~~These genes are known to have highly conserved regions and their copy numbers range from 4 to 8. For these characteristics ~~is a~~these are suitable genes for phylogenetic studies and molecular detection of *Plasmodium* spp. parasites [2]. Molecular detection methods for malaria parasites detect low-level malaria parasitemias that are missed by microscopy and rapid diagnostic tests (RDTs) [20]. The PCR is highly sensitive and has been widely used for diagnosis, confirmation of diagnosis, epidemiological studies, drug efficacy assessment and to measure the accuracy of microscopy

and RDTs [21-26]. The PCR has been used in surveillance as a performance measurer of microscopy and RDTs [7,22,27,28].

The goals of this study were to determine the accuracy of light microscopy and the RDT SD BIOLINE Malaria Antigen (Ag) (05FK40, Standard Diagnostic, Korea) in the detection of *Plasmodium* spp. infection in Luanda, using the nested-PCR as a reference method, and to identify the *Plasmodium* species in the studied populations.

2. METHODOLOGY

2.1. Patients and study design

A total of 225 patients with clinically suspected malaria admitted in 10 health centers of Luanda (Kassequel, Samba, Rangel, Sapú II, Zango I, Boa Vista, Funda, Zango II, Km 12 and Kilamba), Angola, during December 2013 to March 2014 were included in the study. Freshly finger-prick blood samples for light microscopy and SD BIOLINE Malaria Ag were collected from each patient.

In addition, 3-5 drops of blood were spotted onto filter paper (Whatman® 3MM Chr, Whatman International Ltd., Maidstone, England). Each filter paper sample was dried at room temperature and stored at 4°C in separate plastic packets to avoid cross contamination for nested-PCR analysis. The whole blood sample and dried filter paper were collected simultaneously. The dried filter paper samples were transported to Molecular Biology Laboratory, Parasitology Department, Institute of Tropical Medicine "Pedro Kourí" (IPK), Havana, Cuba in iceboxes (0-5 °C). The DNA extraction and nested-PCR ~~was~~ were performed at IPK.

2.2. Microscopy

Thick and thin blood films were prepared. The slides were stained with 10% of Giemsa (Merck, Darmstadt, Germany) for 15 min and screened under an oil immersion (Olympus microscope CX21, Tokyo, Japan) for *Plasmodium* spp. parasites by four independent technicians. Slides were considered as positive only after being confirmed by two of more technicians. Parasite density was determined as the number of parasites

per 200 leukocytes on the assumption of an average leukocyte count of 8000/ μ l of blood. A result was considered negative if no parasites were detected per 200 leukocytes.

The four technicians¹ for microscopy detection were well trained, weeks before by theoretical and practical sections on: Malaria diagnosis, blood collection, thick and thin blood smear preparation, Giemsa staining and slide reader [29].

2.3. Rapid diagnostic tests (RDTs)

SD BIOLINE Malaria Ag test kit (05FK40, Standard Diagnostic, Korea) is a three-band lateral-flow immunochromatographic antigen detection test in a cassette format. Testing was carried out according to the manufacturer's instructions. Finger-prick blood was used for the rapid assay. The kit is a one step, rapid, qualitative and differential test for the detection of *Plasmodium* lactate dehydrogenase (pLDH) specific to *P. falciparum* and pan specific to other *Plasmodium* species (*P. vivax*, *P. malariae* or *P. ovale*). A unique positive PfpLDH line represents a *P. falciparum* infection whereas a unique pan-pLDH line indicates an infection with one or more of the non-*falciparum* species. The presence of both test lines indicates either an infection with *P. falciparum* or a mixed infection with *P. falciparum* and one or more of the non-*falciparum* species. In cases where the control line did not appear, the results were interpreted as invalid and the test repeated with a new device.

2.4. DNA extraction

Parasites² DNA was extracted from the blood spotted on Whatman® 3MM filter paper samples. A 3-mm² square piece of blood-impregnated filter paper (~15 μ l) was excised using a puncher and transferred into a 1.5 ml tube and stored at -20°C until use. DNA was extracted using a QIAamp DNA mini kit (Qiagen, Germany), according to the manufacturer's instructions, and then eluted into 100 μ l of sterile double-distilled water. DNA was stored at -20°C, and 2.0 μ l aliquots were used for each PCR.

2.5. Nested-PCRs

For the detection and identification of *Plasmodium* species we performed and optimized the nested-PCR method described as NP-2013 protocol, an update for the widely used NP-1993 to NP-2005 (SSU rRNA) protocols for all human malaria parasites molecular detection [2]. For the *Plasmodium* genus detection a first PCR was done with specific primers ~~specific~~ (rPLU1 and rPLU5) followed by a second reaction using the primers rPLU3 and rPLU4. All genus specific nested PCR-positive results were analyzed to species level using the second nested-PCR in 5 separate reactions as reported previously: rFAL1/rFAL2 (*P. falciparum*); rVIV1/rVIV2 (*P. vivax*); rMAL1/rMAL2 (*P. malariae*); PkF1140/PkR1550 (*P. knowlesi*) and rOVA1WC/rOVA2WC (*P. ovale*) (Table 1), using the PCR amplification of the first reaction (rPLU1 and rPLU5) as a DNA template.

Table 1. PCR primers and conditions

Species/ genus	Primer name	PCR primers(5'-3')	Size (base pair, bp)	Reagent Concentrations ^a	Amplification conditions	Refer ence
Genus— nested 1	rPLU1 rPLU5	TCAAAGATTAAGC CATGCAAGTGA CCTGTTGTTGCCT TAAACTTC	~1,670	Primer: 0.25µM MgCl ₂ : 2mM	Initial denaturation, 95°C for 5 min; 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; final extension, 72°C for 5 min	[30]
Genus— nested 2	rPLU3 rPLU4	TTTTTATAAGGATA ACTACGGAAAAGC TACCCGTCATAGC CATGTTAGGCCAA TACC	240	Primer: 0.25µM MgCl ₂ : 2mM	Initial denaturation, 95°C for 5 min; 30 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 2 min; final	[30]

					extension, 72°C for 5 min	
<i>P. falciparum</i>	rFAL1	TTAAACTGGTTTG GGAAAACCAAATA TATT	206	Primer: 0.25µM MgCl ₂ : 2mM	Initial denaturation, 95°C for 5 min; 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; final	[30]
	rFAL2	ACACAATGAACTC AATCATGACTACC CGTC				
<i>P. malariae</i>	rMAL1	ATAACATAGTTGT ACGTTAAGAATAA CCGC	145	Primer: 0.25µM MgCl ₂ : 2mM	extension, 72°C for 5 min	[30]
	rMAL2	AAAATTCCCATGC ATAAAAAATTATAC AAA				
<i>P. vivax</i>	rVIV1	CGTTCTAGCTT AATCCACATAAC TGATAC	121	Primer: 0.25µM MgCl ₂ : 2mM		[30]
	rVIV2	ACTTCCAAGCCG AAGCAAAGAAAG TCCTTA				
<i>P. ovale</i> spp.	rOVA1WC	TGTAGTATTCAA ACGCAGT	659– 662	Primer: 0.25µM MgCl ₂ : 2mM		[31]
	rOVA2WC	TATGTACTTGTTAA GCCTTT				
<i>P. knowlesi</i>	PkF1140	GATTCATCTATT AAAAATTTGCTTC	410	Primer: 1-µM MgCl ₂ : 2mM	Initial denaturation, 95°C for 5 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; final	[32]
	PkR1550	TCTTTTCTCTCCG GA GATTAGAACTC				

^aPrimer concentrations are listed per primer; the total MgCl₂ concentration in the reaction mix is given (1.5 mM of which is included with the PCR buffer, as explained in Material and Methods).

All PCR was performed in 25 µl total volume, each containing 1X Q solution and 1X CoralLoad PCR buffer including 1.5 mM MgCl₂, 125 µM of each deoxynucleoside triphosphate and 0.5 U HotStar Taq Plus DNA Polymerase (Qiagen, Hilden, Germany). The additionally added MgCl₂ and primers varied as detailed in Table 1. The first PCR round used 5 µl of extracted DNA from the blood spotted on filter paper samples. The second PCR reactions used 2 µl of the PCR product obtained for the first PCR. The amplification conditions for all ~~PCRs~~ PCR reactions are shown in Table 1.

Thermal cycling was performed in a MyCycler™ (Bio-Rad, Foster City, CA, USA). Each amplification run included two negative controls (ultrapure water and a negative control of DNA extraction) and one positive control (DNA extracted from positive clinical samples of each *Plasmodium* spp). For nested-PCR reactions, an additional negative control was added, consisting of 2 µl of the negative control reaction of the first run of PCR.

To visualize the products of the nested-PCR, analysis on a 2% agarose gel in 0.5 X tris-borate-EDTA buffer and stained with ethidium bromide (0.5 µg/ml) was used to verify the amplified product size and to check for non-specific amplification. Electrophoresis visualization was carried out using a compact imaging system U:Genius (Syngene, UK). PCRs were considered positive if an specific amplicon of the expected base pair (bp) size was observed (Table 1).

2.6. Statistical analysis

Predictive values and diagnostic sensitivity and specificity of microscopy and RDT, with nested-PCR as the “reference method” were determined using 2 x 2 contingency tables. Exact 95 % confidence intervals were calculated. All the results were calculated using the EPIDAT version 3.1 program (Dirección Xeral de Saude Pública, Organización Panamericana de la Salud, Galicia, Spain). In order to assess whether the various methods rendered significantly different results in terms of parasite detection sensitivity and specificity and predictive values, *P*-values were calculated with the McNemar test (sensitivity and

specificity) [33] or the weighted generalized score Chi-squared test (PPV and NPV) for paired data [34]. Each **2×2** comparison was analyzed with the online module of Graphpad (<http://graphpad.com/quickcalcs/McNemar1.cfm>). Statistical significance was considered at the probability level of $P < 0.05$. The kappa coefficient (Cohen's kappa coefficient as a measure of agreement for qualitative items) was determined to confirm the consistency of the results among the diagnostic tools. The kappa values were used to categorize the strength of agreement between the microscopic examination and RDT with the nested-PCR. Values were interpreted with the Landis and Koch classification [35]: poorly correlated (<0), slightly correlated (0-0.20), fairly correlated (0.21-0.40), moderately correlated (0.41-0.60), substantially correlated (0.61-0.80), and perfectly correlated (0.81-1.0).

2.7. Ethical approval

Ethical approval for the study was obtained from de Angola Ministry of Health Ethics Committee. ~~Writing~~ Written informed consent was obtained from all adults willing to participate in the study with a parents or guardian respond giving consent from children. Blood samples were collected only after written consent was given. Patients who tested positive by microscopy or RDTs were provided with antimalarial treatment by a nurse or physician according to national guidelines [36].

3. RESULTS

A total of 225 patients with suspected malaria based on clinical presentation were enrolled and screened for *Plasmodium* parasite in the course of the study. Among the study participants, 34.7 % (78/225) ~~of the~~ patients were malaria positive by microscopy, all diagnosed as *P. falciparum* infection. No other *Plasmodium* species, either in single or mixed infections, were detected by the microscopists. However, 44.4 % (100/225) cases were positive by RDT, all with specific diagnosis of *P. falciparum*. Nested-PCR was used as a reference method and 36.0 % (81/225) cases were positive to *Plasmodium* spp.: 80 cases (98.8 %) infected with *P. falciparum* and 1 case with *P. malariae* (1.2 %). No mixed infections were detected (Table 2). Fig. 1 ~~showed~~ shows the ~~PCRs~~ PCR results of 19 clinical samples.

Table 2. Results of *Plasmodium* spp. detection using light microscopy, RDT and nested-PCR in patients from Luanda Health Centers.

	Microscopy	RDT	Nested-PCR
Positive	78 (34.7 %)	100 (44.4 %)	81 (36.0 %)
Negative	147 (65.3 %)	125 (55.6 %)	144 (64.0 %)
Total	225	225	225

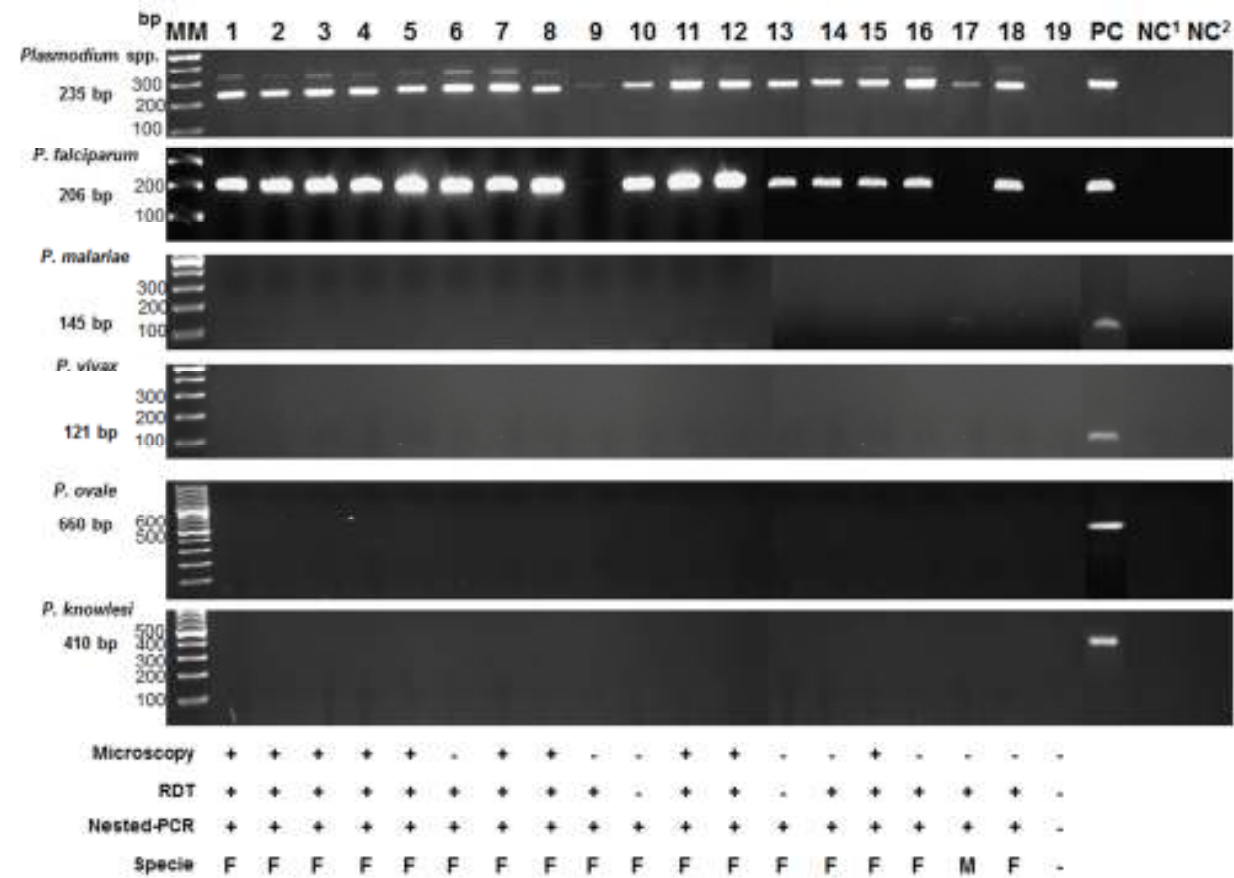


Fig. 1. Agarose (2 %) gel electrophoresis (2 %) analysis of *Plasmodium* nested-PCRs from 19 clinical samples of patients with clinical suspicion of Malaria attended in Luanda Health Centers.

MM: Molecular weight marker GeneRuler™ 100 bp DNA ladder (MBI Fermentas), with band sizes indicated on the left in base pairs. Size indication of the PCR fragments are depicted on the left of the gels in base pairs. On top the code of clinical samples. PC: positive control. NC¹: negative control. NC²: negative control of the first PCR runs (rPLU1-rPLU5). The results (positive or negative) of microscopy examination, RDT and nested-PCR (rPLU3-rPLU4) including *Plasmodium* specie detected (F: *P. falciparum*; M: *P. malariae*) are shown at bottom.

202

203 Microscopy showed a ~~moderately~~ moderate measure of agreement ($\kappa = 0.58$) with the reference method,
 204 nested-PCR for *Plasmodium* detection. Microscopy correctly identified 58 out of 81 PCR-positive
 205 *Plasmodium* spp. infections (71.6 % sensitivity; CI₉₅ 61.2-82.0) and 124 out of 144 PCR-negative samples
 206 (86.1 % specificity; CI₉₅ 80.1-92.1), with PPV of 74.4 % and NPV of 84.4 %.

207

208 SD BIOLINE Malaria Ag correctly identified 71 out of 81 PCR-positive *Plasmodium* spp. infections (87.7 %
 209 sensitivity; CI₉₅ 79.9-95.4) and 115 out of 144 PCR-negative samples (79.9 % specificity; CI₉₅ 72.9-86.8),
 210 with PPV of 71.0 % and NPV of 92.0 % (Table 3). RDT showed a substantially correlated ($\kappa = 0.64$) with
 211 the reference method. In direct comparison to microscopy, the RDT had significantly higher sensitivity and
 212 negative predictive value. However the specificity and positive predictive value had significant higher for
 213 microscopy in comparison to RDT (Table 3).

214

215 **Table 3. Sensitivity, specificity, predictive values of microscopy and RDT with nested-PCR as**
 216 **reference method.**

217

		PCR Positive (n=81)	PCR Negative (n=144)	Sensitivity (%) (CI ₉₅)	Specificity (%) (CI ₉₅)	PPV (%) (CI ₉₅)	NPV (%) (CI ₉₅)
Microscopy	Positive (n=78)	58	20	71.6 (61.2-82.0)	86.1 (80.1-92.1)	74.4 (64.0-84.7)	84.4 (78.1-90.5)
	Negative (n=147)	23	124				
SD BIOLINE Malaria Ag	Positive (n=100)	71	29	87.7 (79.9-95.4)	79.9 (72.9-86.8)	71.0 (61.6-80.4)	92.0 (86.8-97.2)
	Negative (125)	10	115				
P value				0.0009	0.0077	<0.0001	<0.0001

218

For diagnosis of *P. falciparum* and *P. malariae* the results of microscopy and RDT showed the same results ~~obtaining were obtained for~~ overall for the samples (Table 4). However for diagnosis of *P. malariae* in only one ~~samples-sample~~ was detected by nested-PCR; this sample was positive by RDT but not by light microscopy.

Table 4. Sensitivity, specificity, predictive values of microscopy and RDT with nested-PCR as reference method for *Plasmodium* species detected.

<i>Plasmodium</i> spp.	Diagnostic tool	Sensitivity (%) (CI ₉₅)	Specificity (%) (CI ₉₅)	PPV (%) (CI ₉₅)	NPV (%) (CI ₉₅)
<i>P. falciparum</i>	Microscopy	72.5 (62.1-82.9)	86.2 (80.3-92.2)	74.4 (64.0-84.7)	85.0 (78.9-91.1)
	SD BIOLINE	87.5	79.3	70.0	92
	Malaria Ag	(79.6-95.4)	(72.4-86.3)	(60.5-79.5)	(86.8-97.2)
<i>P. malariae</i>	Microscopy	0	100 (99.8-100)	0	100 (99.8-100)
	SD BIOLINE	100	100	100	100
	Malaria Ag	(50.0-100)	(99.8-100)	(50.0-100)	(99.8-100)

The concordant and discordant results of light microscopy, SD BIOLINE Malaria Ag and nested-PCR are summarized in Table 5.

Table 5. Comparative details of light microscopy, SD BIOLINE Malaria Ag and nested-PCR for *Plasmodium* detection and species identification.

Category according to nested-PCR	Number of samples	Light microscopy	SD BIOLINE Malaria Ag	nested-PCR
CONCORDANT				
True positive	58		<i>P. falciparum</i>	

True negative	115		Negative	
DISCORDANT				
True positive	1	Negative	Pan	<i>P. malariae</i>
True positive	12	Negative	Pf	<i>P. falciparum</i>
True positive	10	Negative	Negative	<i>P. falciparum</i>
False positive	9	Negative	Pf	Negative
False positive	20	Positive	Pf	Negative

Pf: *Plasmodium falciparum*; Pan: specific to other *Plasmodium* species (*P. vivax*, *P. malariae* or *P. ovale*).

Thirteen (56.5 %) of the 23 false negative microscopy (which were negative by microscopy, but positive by nested-PCR as a reference method) were positive by RDT. Ten (43.5 %) of the 23 false negative microscopy samples were negative by RDT. Twenty (100%) of the false positive microscopy (which were positive by microscopy, but negative by nested-PCR as a reference method) were positive by RDT.

The false negative RDT (10 patients) (which were negative by RDT, but positive by nested-PCR) were negative by microscopy. Twenty (69 %) of the 29 false positive RDT samples (which were positive by RDT but negative by nested-PCR) were confirmed to be positive by microscopy. Nine (31 %) of the 29 false positive RDT samples were negative by microscopy.

Among microscopy and RDT negative patients (125), 10 cases tested positive by nested-PCR, all infected with *P. falciparum*; this represents 8 % of the infected subjects that were not detected by microscopy and RDT.

4. DISCUSSION

Malaria is endemic throughout much of the Angolan territory, and is by far the highest cause of morbidity and mortality particularly among children less than five years old and pregnant women. The true burden of malaria in Luanda has been a matter of debate [37].

The *Plasmodium* positive values determined by microscopy, RDTs and nested-PCR in this study varied between 34.7 - 44.4 % according to the selected diagnostic in patients (children's and adults). According to nested-PCR the positive was 36.0 %. Higher prevalence was obtained in asymptomatic children's under

five year (45 %) in a different province of Angola [27]. However, lower prevalence were described also in this country by Mendes et al. [38] (28.9 %) and Fançony et al. [6,28] (15.9 %) when studied in asymptomatic children older than two month and a group of children's up to 15 years and adult mothers, respectively.

It has long been established that a proportion of individuals with malaria have low-density infections that are unlikely to be detected by conventional microscopy. In our study, 23 cases were negative by microscopy but infection was detected by nested-PCR. Microscopy is reported to detect about 75 % of malaria infection in high transmission areas, whereas in low transmission areas, this method has been reported to miss up to 88 % of infections [39].

The use of nested-PCR as a reference method was done when it was noted that it presented the highest sensitivity in comparison to microscopy and RDTs [21-26]. Considering the principle of this molecular assay, in which small fragments of *Plasmodium* DNA can be detected, the results were not surprising [40].

In the present study, the sensitivity of microscopy for the diagnosis of *P. falciparum* was considerably higher (71.60 %) in comparison to other studies developed in Angola by Fortes et al. [27] and Fançony et al. [28], whose studied 1012 asymptomatic children's and 3307 people, respectively. The sensitivity of the microscopy taking into account the nested-PCR as a gold standard was 59 % and 60 %, in these studies respectively. Moura et al. [7] reported 66.7 % of the total sensitivity of microscopy using the real-time quantitative polymerase chain reaction (qPCR) as a gold standard, in one study developed in different hospital laboratories for three provinces of Angola (Luanda, Benguela and Bengo). In spite of the inherent limitations of malaria microscopy, the quality of microscopic diagnosis largely depends on the quality of training. On the one hand, adequate training can increase the yield of accurate malaria diagnosis. This result was possible for the adequate intervention developed prior to this study in order to increase the knowledge and expertise in the microscopy of *Plasmodium* species [29]. Moura et al. [7] assess the impact of the training course ~~ef-on~~ on the knowledge of technicians's, quality of blood smears preparation, and accuracy of microscopy malaria diagnosis using qPCR as a reference method in Angola. However, no significant increase of sensitivity or specificity after the training course was registered, but in some laboratories the sensitivity and specificity increase after completing the training course. For example, the microscopy sensitivity in the Luanda laboratory was 75 % after the course (72.9 % before course)-); this

289 result showed the same sensitivity as that in our study. The high sensitivity is particular important because
290 of the risk of untreated cases of malaria, ~~and~~ a potentially lethal disease [7]. The better microscopic
291 diagnosis helps to reduce illness, potential death, mistreatment and persistently high disease burden while
292 at the same time saving vital resources for malaria control [41].

293
294 The high specificity of microscopy is important for reducing the waste of limited resources, the increase of
295 costs for drugs, unnecessary exposure of patients to the adverse effect of anti-malarial medication, for
296 avoiding drug resistance, and also for prompting clinicians to look for other causes of illness [7,42]. The
297 specificity of microscopy in our study was 86.1 %. Moura et al. [7] showed 83.8 % of total microscopy
298 specificity using qPCR as a gold standard, ~~however~~ higher specificity of 91.1 % and 98.8 % were
299 reported in the Luanda and Benguela laboratories, respectively. Fançonny et al. [28] reported 92.5 % of
300 specificity using PCR as a gold standard. However, lower values (73 %) were showed by Fortes et al. [27].

301
302 One case was identified by RDT as non *P. falciparum* and by nested-PCR as *P. malariae*, but none by
303 microscopy. Parasite density of non-falciparum Plasmodium infection is usually low and therefore easily
304 missed by microscopy. Fançonny et al. [6] also could not identify malaria parasites other than *P. falciparum*
305 by microscopy.

306
307 The non-concordant microscopy positive/nested-PCR negative (20) cases can also be attributed to blood ~~fi~~
308 ~~im~~ if the preparation generates artifacts commonly mistaken for malaria parasites even after the training
309 course, including bacteria, fungi, stain precipitation, and dirt and cell debris or normal blood components
310 such as platelets also confound diagnosis [43]. However, it is possible also that the degradation of parasite
311 DNA or low parasitemia combined with degradation of parasite DNA were responsible for negative nested-
312 PCR results [44].

313
314 RDT ~~were~~ was significantly more sensitive than microcopy. Although SD BIOLINE Malaria Ag test is a
315 sensitive and relatively specific assay; it is not applicable for differentiation between malaria species. The
316 accuracy (sensitivity and specificity) of RDT is mostly dependent on the parasite species, transmission
317 intensity, parasite density, amount of circulating antigens, local polymorphisms of target antigen and
318 persistence of antigens after treatment [45-46].

319

According to nested-PCR as a reference method, 29 patients were false positive by RDT. RDT ~~are~~is known to produce false-positive results by cross-reactivity with the heterophile antibodies [47] and because of residual antigen, which can persist for weeks after treatment [48]. The pLDH enzyme - expressed by viable parasites - declines rapidly after therapy but is also expressed by gametocytes [45]. The false negative RDT (10 patients) were due to low parasitaemia as in the majority of cases or by interpreting the RDT before the test line has fully developed [49]. Recently, Dzakah et al. [50] demonstrated that a combination of aldolase and LDH in RDTs for the rapid diagnosis will enhance the sensitivity of the assay and reduce misdiagnosis. Other RDTs will be evaluated in Angola context in future ~~researches~~research.

Nested-PCR allows for the detection of low density infections and even more importantly of mixed infections, which are routinely missed in microscopy [51], and this makes nested-PCR an ideal confirmatory test for malaria diagnosis [41]. In the present study, the molecular assays revealed 23 additional single infections (22 *P. falciparum* and one *P. malariae*) missed by microscopy, probably due to the very low presence of *Plasmodium* spp. [30,52-53]. Among them 10 (8 %) samples were also considered negative. These results like many others already described in the literature demonstrate the considerably high potential of molecular methods for detecting low parasite densities and subclinical infections, negative ~~for~~by microscopy and/or RDT [26,30,41,52-53], and emphasizes once again the usefulness of molecular methods in the diagnosis of malaria as compared to the traditional microscopic or RDT.

The literature regarding malaria species other than *P. falciparum* in Angola is limited [6]. The dominant specie in this study was *P. falciparum* (98.8%). This result is in concordance with the *Plasmodium* species reported ~~in~~that concluded *P. falciparum* is a responsible for more than 90% of all infections [54]. *P. malaria* was less prevalent (1.2 %) which is the same ~~to as~~ previously reported in Angola [6,27,38]. No *Plasmodium* mixed infections were reported in our study. However, in Angola ~~were reported~~ the mixed infection has been reported by different authors [6,27,38].

5. CONCLUSION

In this study we demonstrated that RDT were significantly more sensitive than microscopy, and that this test is a rapid diagnostic test, are is not labor-intense but are is more costly than microscopy. These findings support the need of using RDT in the diagnosis of *Plasmodium*. However the sensitivity of microscopy was

higher in comparison with other ~~studied-studies~~ reported in Angola ~~and~~ may be ~~for due to~~ the microscopist training course developed before the study. The nested-PCR is time- and labour-consuming and required expensive reagents and infrastructure. However, our results confirmed the considerable sensitivity of nested-PCR for detection of cases that remain undiagnosed by microscopy and RDT. PCR ~~could- appears~~ to be a useful method for detecting *Plasmodium* parasites during active malaria surveillance in Angola. This study contributes to the ~~less-limited~~ literature regarding malaria species in Angola. Only single infections were detected, with the *P. falciparum* infection ~~was-~~ reported in the majority of positive cases, and the other specie ~~less-~~ detected to a limited extent was *P. malariae*.

CONSENT

All authors declare that written informed consent was obtained from the patients.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

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