Comparison of PCR and SD Bioline malaria Antigen test for the detection of malaria in Hadramout Governorate

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Abstract: This investigation was conducted to compare the performance of nested PCR and *SD Bioline malaria Antigen test* with light microscopy test as a mean of detecting *Plasmodium* parasites during active malaria surveillance in Almukalla Hadramout in Yemen. The study was performed during period from years 2009-2010 in the Almukalla malaria centre. *Plasmodium vivax* (PV) and *Plasmodium falciparum* (PF) are the predominant parasite species in this village, followed by *Plasmodium malariae* (PM) and *Plasmodium ovale* (PO), venously blood samples were taken from each participating individual and used to prepare microscopic slides, *SD Bioline malaria Antigen* test and for PCR analysis. Obtained results revealed that PCR was sensitive (100%) and specific (92%) for malaria at low parasite densities; 54% (27/50) of *P. falciparum*- and 0% (0/50) of *P. vivax*. All positive cases detected by PCR were detected positive by microscopic examination except two cases which found to be negative microscopically. However, *SD Bioline malaria Antigen test* was sensitive (56%) and specific (96%), only 10% (5/50) for *P. falciparum,* 10% (5/50) for *P. vivax,* and also (10%) (5/50) for mixed infection at low parasitic densities. Conclusion: PCR appears to be a useful method for detecting *Plasmodium* parasites during active malaria surveillance in Yemen. SD Bioline malaria Antigen test appear as a poor test for detecting malaria parasites at low parasitic densities in spite of having the ability to differentiate between the species of malaria.
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1. Introduction

Malaria is the most important human parasitic disease affecting the tropical and subtropical regions of the world due to its high prevalence and mortality rate. Malaria still continues to be a devastating global public health problem in more than 100 countries with 3.2 billion people being at risk ***(WHO, 2005)***. Of this number, 300-500 million people contract the disease each year, resulting in 2-3 million deaths ***(Snow et al., 2005)***. This includes 1 million children of less than five years of age ***(Sachs and Malaney, 2002).***

Malaria is an endemic disease in Yemen and is responsible for 4.9 deaths per 100,000 populations per year and 43,000 disability adjusted life years lost. Although malaria in Yemen is caused mainly by ***Plasmodium falciparum*** and ***Plasmodium vivax***.

In this study, samples were collected from Almukalla Malaria Centre. The traditional malaria diagnosis is based on the examination of stained blood smears under light microscope. The method remains the gold standard for malaria diagnosis as it is inexpensive and sensitive (5-10) parasites/µl blood ***(Moody, 2002).*** However, it is labor-intensive, time-consuming, and more importantly, requires skill and experienced microscopists.

Recently, alternative methods, such as immunochromatographic assay, molecular amplification method, fluorescence microscopy, mass spectrometry, and flow cytometry have been developed for malaria diagnosis ***(Saito-Ito et al., 2001)***. These methods have some advantages and also some limitations. PCR is considered the most sensitive and specific method, but is expensive, requiring PCR machine, relatively sophisticated and time-consuming procedure, which may not be applicable for malaria diagnosis in remote areas ***(Saito-Ito et al., 2001)***.

A prompt and accurate diagnosis is critical to the effective management and control of malaria. Microscopic examination of stained blood films has been the most widely used technique for detecting malarial parasites, but the correct interpretation of blood films requires considerable expertise, which may not be available at the periphery of the health care system ***(Moody, 2002)***. Recently, rapid antigen detection methods for diagnosing malaria based on the detection of *Plasmodium* lactate dehydrogenase (pLDH) have been developed. However, the accuracies of these tests in diagnosing ***P. vivax*** malaria in areas of endemicity have not been clearly established ***(Iqpal et al., 2003, Ochola et al., 2006)***.

2. Material and Methods:

Studied area:

The present study was conducted in Hadromout governorate with a total population of (871.202) people A total of 50 blood samples were collected from patients clinically suspected of malaria who presented to the malaria centre at Almukalla hospital Hadramout Governorate. The peak time of malaria transmission in the coastal areas occurs in winter (October-April), while in the western mountains, the peak occurs in the summer (May-September). ***Anopheles arabiensis*** is the main vector in the country but ***Anopheles culicifacies*** plays an important role in the transmission of malaria in the coastal area. ***Anopheles sergenti*** has also been reported in the mountainous hinterland and highland areas **(*NMCP, 2002)***.

Subjects and blood sample collection:

A total of 50 patients aged from 5 to over 50 years, who sought treatment at the Malaria Health Center in Almukalla malaria centre Hadramout Governorate in a period from 2009-2010, were invited to participate in the study.

The patients were selected randomly from different groups (symptomatic and asymptomatic individuals of both genders, etc.). Finger-prick blood samples were collected and thick and thin blood smears were prepared for microscopical observation. Samples to be used for PCR were stored frozen with EDTA at -20 C° until used. These samples were also used for the ***SD Bioline malaria Antigen test***.

Two milliliters blood samples for PCR assay were obtained by informed consent before treatment from patients with either slide-confirmed ***P. falciparum*** or ***P. vivax*** infection or from slide-negative patients who had been referred to the clinic because of fever and headache. A history of fever, symptoms, and drugs taken, if any, was recorded for each subject.

Examination of parasitaemia

Giemsa-stained thick blood films were routinely used for the detection of parasites without any quantitative estimation of parasitaemia. In this study, both thin and thick blood films were prepared and sent with blood samples to the Parasitology Department Assiut University, in order to determine the parasitaemia and to confirm the parasite species by PCR analysis. Thick and thin films were interpreted as negative only after examination with an oil immersion lens at × 1,000 magnification for at least 100 oil immersion fields. All the samples were air-dried, fixed in methanol and then stained for 15–30 minutes in Giemsa (BDH Ltd); a 1:10 diluted Giemsa (pH 7.2) was used. The stain was washed off with tap water and the smears were examined by × 1,000 magnification. The percentage of the parasitaemia was calculated from a total count of 1,000 red blood cells (RBCs)counted in a Giemsa stained thin blood film.% Parasitaemia = Total no. of infected RBCs × 100/Totalno. RBCs

Polymerase chain reaction (PCR):

The PCR analysis for malaria was done for all the samples. DNA extraction and purification from EDTA blood samples was done using the QIAamp DNA Mini spin columns kit from (Millipore Corporation). The extracted DNA was thereafter amplified by the PCR using a set of primers as;

1st- round: (sense); 5- T T A A A A T T G T T G C A G T T A A A A C G – 3\

(Anti sense): 5l – C C T G T T G T T G C C T T A A A C T T C-3l

2-nd round:

***P. falciparm* primers:**

(sense); 5l T T A A A C T G G T T T G G G A A A A C C A A A T A T A T T -3l

(Antisense): 5l- A C A C A A T G A A C T C A A T C A T G A C T A C C C G T C -3l

***P VIVAX*, primers**:

(sense): 5 l - C G C T T C T A G C T T A A T C C A C A T A A C T G A T A C-3l

(Antisense); 5l- A C T T C C A A G C C G A A G C A A A G A A A G T C C T T A-3l

DNA extraction:

The DNA extraction from blood samples was carried out using QIAamp Mini spin columns, according to Snounou (1996).

Set up a PCR (as provided by manufacturer):

-Add 3ml extracted DNA
-Add 2ml of forward and reveres primer mix.
-Add 6.5ml of DNAse free water
-Add 12.5ml of 2x green PCR Master Mix.
-Mix the reaction.
-Put the tube in the thermo-cycler.

The thermo-cycler was programmed as follows. Hot start- 95 °C for 10 min; initial denaturation-95 °C for 45 sec; annealing -58°C for1 min; extension-72 °C for 2 min; final extension 72 for 10 min. A total of 40 cycles were used and final annealing and extension was for 5 min. after which the reaction was stopped and the amplified product was detected by electrophoresis on 1% agarose gel containing ethidium bromide and visualized on an ultraviolet transilluminator. Gel photographs were taken and specific amplified bands at 100 bp were looked.

SD Bioline Malaria Antigen Test (home Test, one-step *P.f/ P.v*, rapid test):

One step malaria ***P.f / P.v*** rapid test: (cat. No.

05FK40,SD Standard DIAGNOSTICS,Yongin,Republic of Korea). It is a recently introduced malaria diagnostic test contains a membrane strip, which is pre-coated with polyclonal antibodies directed against isoforms of the enzyme (pLDH), an enzyme produced by sexual and asexual forms of the parasite. Differentiation of malaria parasites is based on antigenic differences between the pLDH isoforms. If the target antigen is present in the blood, a labeled antigen antibody complex is formed and it migrates up the test strip to be captured by the pre-deposited capture antibodies specific against the antigens and against the labeled antibody (as a procedural control). This test device has three line letters "Test Line 1" specific for *Plasmodium falciparum*, "Test Line 2" specific for non *Plasmodium falciparum* (*P. v*) and test line C, "Control Line" on the surface of the case.

1) 10µ of anticoagulated blood was added to the sample well (S) of the test device, then 3 - 4 drops of assay diluents was added and start the timer.
2) As the test began to work, a purple color was seen moving across the result window in the center of the test device.
3) Test results was Interpreted at 10 minutes. A positive result will not change once it has been established.

3. Results

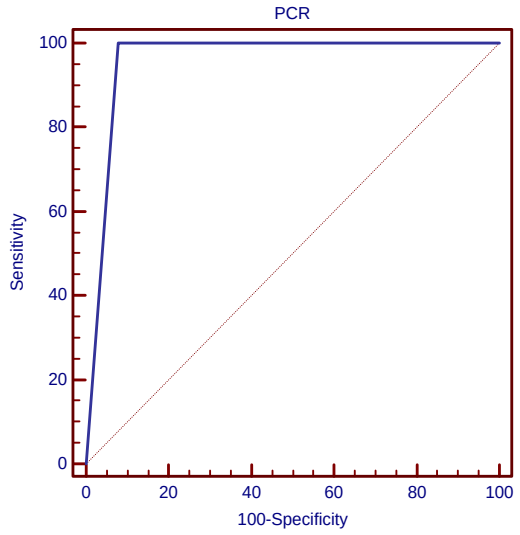
As shown in table (1), 22 (44%) were males, and 28 (56%) were females, participated in this study, aged between 5 to over 50 years old. 50 suspected malaria patients were examined by thick blood film analysis by light microscopy. 7 (14%) were identified as having only ***P. vivax*** and 18 (36%) as only ***P. falciparum*** infections. 25 (50%) were slide-negative. The thin films showed that parasitaemia ranged from 0.001% to 5%. PCR analysis for detection of the ***Plasmodium*** genus and species determination is also shown in Table (2) and Figs (1, 2). The percentages of ***P. vivax*** mono infection, ***P. falciparum*** mono infection, and mixed infections were 0 (0%), 27 (54%) and 0 (0%), respectively. A typical gel is shown in Figure (2). Two out of 50 specimens microscopically diagnosed as negative were positive as ***P. falciparum*** with nested-PCR. In order to ensure that the results are true and not due to technical errors such as cross contamination, assays were all carried out with concurrent human negative controls. However ***SD Bioline malaria Antigen test*** *was* sensitive (56%) and specific (96%), only 10% for ***P. falciparum*** and 10% for ***P. vivax*** and 10% for mixed infection at low parasitic densities. result shown in Table (2) and Figs (3,4).

Table (1): Personal characteristics of 50 suspected cases of malaria.

	No. (n= 50)	%
Age: (years)		
< 30	17	34.0
≥ 30	33	66.0
Mean ± SD (Range)	33.8 ± 8.2 (20 – 48)	
Sex:		
Male	22	44.0
Female	28	56.0

Table (2): Comparisons between Micoscopic examination, PCR and SD Bioline antigen test

Results	PCR (n= 50)		One-step anti-malaria (n= 50)		Microscopic examination (n= 50)	
	No.	%	No.	%	No.	%
Negative	23	46.0	35	70.0	25	50.0
Falciparum	27	54.0	5	10.0	18	36.0
Vivax	0	0.0	5	10.0	7	14.0
Mixed	0	0.0	5	10.0	0	0.0



Sensitivity: 100.0% Specificity: 92.0%
Area under the ROC curve (AUC): 0.960

Fig. (1): Sensitivity AND Specificity OF PCR

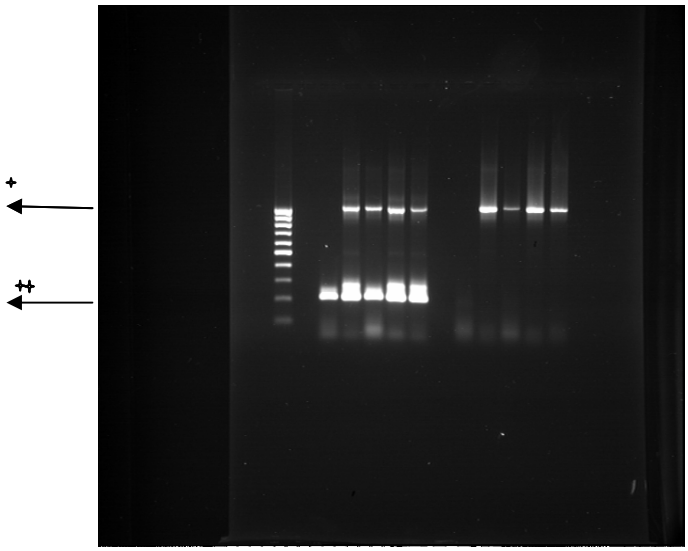
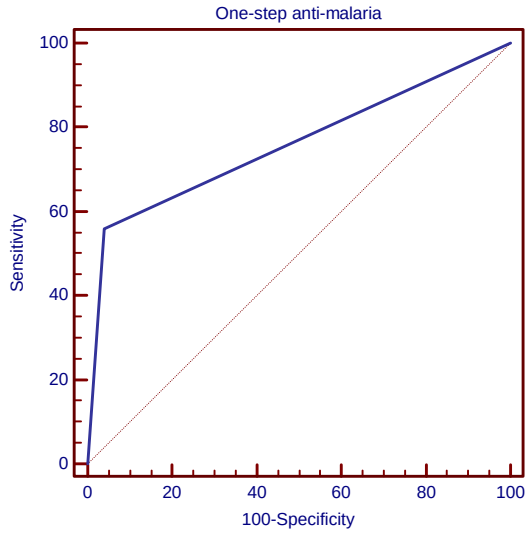


Fig. (2): Schematic representation of agarose gel electrophoresis of nested PCR product using specific primer for ***Plasmodium vivax*** and ***Plasmodium falciparum***. marker is 100 bp ladder. PCR amplification product of 1100 bp were seen in all. The representative ***Plasmodium vivax*** were negative by using ***Plasmodium vivax*** specific primer (right panel) and the representative ***Plasmodium falciparum*** were positive at 205bp by using ***Plasmodium falciparum*** specific primer (left panel).
+1100bp ++1100bp



Sensitivity: 56.0% Specificity: 96.0%
Area under the ROC curve (AUC) 0.760

Fig. (3): Sensitivity and specificity of SD Bioline Antigen Test.

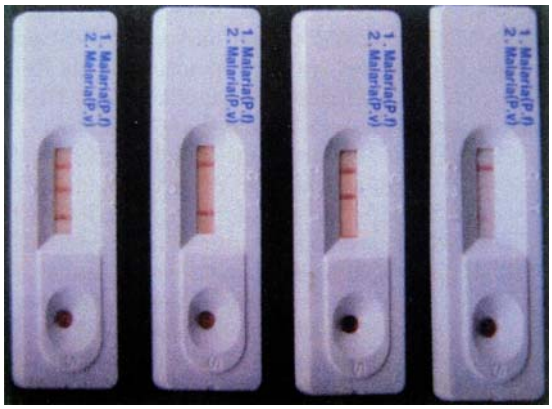


Fig. (4): Results of SD Bioline Antigen Test.

4. Discussion:

Microscopy has historically been the mainstay of the diagnosis of malaria. A clinical diagnosis of malaria currently depends on the visualization of parasites by light microscopy of Giemsa-stained thick and thin blood smears. This procedure is cheap and simple, but it is a labour intensive procedure and requires well-trained personnel ***(Kimura et al. 1995)***. Many studies have demonstrated the greater sensitivity and specificity of PCR compared to thick blood films. The detection of low ***P. vivax*** and ***P. falciparum*** parasitaemia by PCR, at levels undetectable by microscopy, has been reported. ***(Brown et al., 1992***; ***Sethabutr et al., 1992;*** **Snounou** ***et al., 1993; Wataya et al., 1993; Black et al., 1994; Khoo et al., 1996; Roper et al., 1996 and Singh et al., 1996)***. The present study between microscopy and nested PCR assay showed that the results obtained by PCR were equivalent or superior to those obtained by microscopy, in that all microscopy-positive samples were positive by PCR.

With the spread of parasite resistance to anti-malarial drugs in Yemen and the increasing difficulty in controlling malaria in these areas, it is important to diagnose malaria accurately and to treat it correctly. Microscopic observation of parasites stained with Giemsa in thick smears is an inexpensive a method that is still used in these areas with malaria transmission. And where the diagnosis of malaria is part of primary Health care. Several malaria infections from endemic Countries are sub-patent, with very low parasitaemia, and our results also showed this has occurred in our study area. In these cases, an accurate malaria diagnosis is very important so that a possible recrudescence after an incorrect treatment of infected individuals can be avoided.

Mixed infections with asexual blood forms of ***P. falciparum*** and ***P. vivax*** are well- described but relatively uncommon compared to single species infections. Shute (1951) described the frequency of mixed infections as less than 1% among the hundreds of British troops he examined in southern Italy during 1943 ***(Brown et al., 1992)***.Traditionally, ***P. falciparum*** has been thought to inhibit the parasitaemia of ***P. vivax (Garnham, 1966)***. In contrast, there are several lines of evidence suggesting that ***P. vivax*** may have a suppressive effect on ***P. falciparum***. ***(James, 1931)***, in his classic review of studies of induced malaria was impressed that ***P. vivax*** was the predominant species. In their studies of induced malaria, ***(Boyd & Kitchen, 1937)*** often used small doses of quinine which has long been known to have a greater suppressive effect on ***P. vivax*** than on ***P. falciparum (Covll and Nicol, 1951)***. In three instances where ***P. vivax*** parasitaemia rose and no drug was administered, asexual ***P. falciparum*** parasitaemia fell to submicroscopic levels ***(Boyd & Kitchen, 1937)***. Drug selection for the treatment of malaria depends on species of malaria present.

Patients with mixed species infections were four-times less likely to present with patent ***P. falciparum*** gametocytemia when compared with pure ***P. falciparum*** infections. Furthermore, the density of these gametocytes tended to be lower in the mixed infection group of patients. Furthermore, when ***P. vivax*** was also found mixed with ***P. falciparum*** in the admission, the likelihood of observing ***P. falciparum*** gametocytes during treatment follow-up (4–9 weeks) was decreased threefold as compared with that in patients with a pure ***P. falciparum*** infection on admission (***Georges and Nicolas,2004***).

Here we have reported on the development and application of a PCR-based test for the diagnosis of malaria and the differentiation between ***P. falciparum*** and ***P. vivax*** infections in our study. This method permits the detection of four parasites per microliter, which is equivalent to a 0.0015% parasitemia. One of the major advantages of the technique is the minimal need for sample preparation. It was possible to amplify old blood spot samples that had been stored at room temperature up to 3 years by using the L1 and L2 primers as well as the multiplex Pf1-Pf2 and Pv1-Pv2 primers ***(Tham et al. , 1999).***

Our strategy for the PCR amplification was to use one step from the extraction DNA of *Plasmodium*. Detection of a malarial infection was done with genus-specific primers made from the conserved 18 s small sub unit tribosome rRNA gene, and detection of the two main human *Plasmodium* species, ***P. falciparum*** and ***P. vivax,.*** These primers were then used in a nested PCR system. The ability to perform nested PCR to differentiate the species decreases the number of PCR assays required to be performed with each blood sample.

Our comparative study of microscopy, the home test kits, and the PCR test showed that the results obtained by PCR were nearly equivalent or superior to those obtained by microscopy, in that all microscopy-positive samples were positive by PCR. In addition, the PCR test was not able to detect mixed infections. This could probably be due to the tendency for one species to be dominant over another species ***(Kimura et al., 1995)*** also this result not agree with ***Jill et al., (1999)*** who mentioned that PCR could diagnose mixed infection more than microscope.

The ability to detect the presence of the parasite during the course of treatment could be demonstrated by PCR amplification. Similar results have been reported when PCR was used to assess the response to antibiotic treatment for ***Borrelia burgdorferi*** infection ***(Nocton et al., 1994)*** and acyclovir treatment of herpes simplex encephalitis ***(Aurelius et al., 1991)***. In both cases, PCR results were negative following the course of successful drug therapy ***(Aurelius et al., 1991 and Nocton et al., 1994)***. This was also demonstrated when ***P. vivax***-infected patients undergoing chloroquine treatment were monitored by PCR amplification of the circumsporozoite protein gene, whereby no amplicons were observed after the 4th day of treatment ***(Kain et al., 1993)***, and ***Jill et al., (1999)*** agree with this result.

The amplification of the targeted regions could be applied to detect the persistence of very low-grade parasitemia. PCR assays have been used to study patients whose ***P. falciparum*** parasite densities were below the microscopic threshold. ***(Sethabutr et al. 1992)*** demonstrated by the PCR technique that ***P. falciparum*** DNA in the blood of infected patients could be detected transiently at a time when the parasite could no longer be detected microscopically. The recurrence of ***P. vivax*** infections cannot be theoretically predicted by PCR assays because PCR cannot detect relapses of the ***P. vivax*** hypnozoite liver stage ***(Kimura, 1995).***

In the present study, we evaluated the accuracy of rapid antigen detection in diagnosing ***vivax, falciparum*** malaria using the recently introduced SD Bioline Malaria Antigen test. This test showed sensitivity (56%) and high specificity (96%) in the diagnosis of ***vivax, falciparum*** malaria. The home test of Malaria Pf-P-v kit produced only 10 false-negative result and no false-positive results. The home test of Malaria Pf-P-v kit it detect five ***P.***

falciparum infections and five P-v and five mixed infection. Recent studies have verified the usefulness of rapid antigen diagnosis in ***falciparum*** malaria, while relatively inconsistent data have thrown doubt on its role in ***vivax*** malaria. The optimal test, which detects pLDH, is the most widely evaluated rapid diagnostic test for ***vivax*** malaria. The test has shown a wide range of sensitivities for detecting clinically suspected cases of ***P. vivax*** in areas where ***vivax*** malaria is endemic ***(Palmer et al., 1998; Iqpal et al., 1999; Ferro et al., 2002; Iqpal et al., 2003 and Palmer et al., 2003)***.

The ICT Malaria ***Pf/ Pv*** test is another rapid diagnostic test that detects malarial histidine-rich protein 2. However, data on the ICT Malaria ***Pf/ Pv*** test are scarce, and this test has shown unsatisfactory results for ***vivax*** malaria ***(Iqpal et al., 1999)***. The SD Bioline Malaria Antigen test showed a lower sensitivity for ***falciparum*** malaria than other rapid antigen tests ***(Ratsimbasoa et al., 2007)***,

In this study, the SD Bioline Malaria Antigen test showed superior or at least comparable sensitivity and specificity to those of previously used methods in the rapid diagnosis of ***vivax*** malaria.

In conclusion, we have described a nested PCR test system for the diagnosis of malaria. The test detects plasmodial infection and can detect ***P. falciparum***. via a nested reaction. No preparation or treatment of the blood samples is required prior to the amplification. The test can be completed within 3 h with a high degree of sensitivity and specificity. Although our PCR test for the detection and differentiation of malaria parasites requires more time than the home test assays (approximately 15 min), it has been shown to be a more sensitive assay and the home test have the ability to differentiate plasmodial species and detect mixed infections. The production cost of our PCR assay for the diagnosis of malaria is more expensive to that of the commercially available home test assays also it need PCR machine that not available in remote country. Further development and evaluation of the dipstick detection system are under way. This PCR diagnostic assay can easily be developed for mass screenings through automation and could thus be an effective diagnostic tool that is sensitive, specific and less labor intensive than currently used methods. We would like to present this system as a simple and reliable test for the diagnosis of malaria.

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