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RESEARCH ARTICLE PUJ

Laboratory Diagnosis of Malaria Infection in Clinically Suspected Cases Using Microscopic Examination, OptiMAL Rapid Antigen Test and PCR

Maha M. Abdel-Wahab[1], Khadiga A. Ismail[2], Nagwa M. El-Sayed[1]

Parasitology Departments of Research Institute of Ophthalmology, Giza[1], and Faculty of Medicine, Ain-Shams University, Abbassia, Cairo[2], Egypt

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ABSTRACT

Background: Malaria diagnosis depending on clinical conditions is often unreliable due to the inconsistent signs and symptoms of malaria, leading to over-diagnosis and over-treatment. Correct diagnosis is important for effective management of malaria cases and to reduce wastage of costly drugs.

Objective: This study was conducted to detect malaria infection in patients complaining of fever of unknown origin, highly suspected clinically to be due to malaria. OptiMAL rapid antigen test and polymerase chain reaction (PCR) were used in comparison with microscopy.

Subjects, Material and Methods: A total of 120 expatriate patients attending King Faisal specialized hospital, Taif, KSA, complaining of fever of unknown origin were screened for malaria parasites by microscopy of Giemsa-stained blood smears, OptiMAL rapid antigen test and genus specific PCR. The diagnostic performance of these methods was statistically compared.

Results: Out of 120 clinically suspected cases, 54 (45%) were positive for *Plasmodium* infection by using microscopy, and of these 45 (83.3%) were infected by *P. vivax*, 6 (11.1%) by *P. falciparum*, 1 (1.9%) by *P. malariae* and 2 (3.7%) were mixed infections (*P. vivax* and *P. falciparum*). Correspondingly, OptiMAL test and PCR detected malaria infection in 51(42.5%), and 56(46.7%) patients respectively. The differences in detection rates of these diagnostic tests were not statistically significant ($P>0.05$). Using direct microscopy as gold standard, OptiMAL test showed 5 false-positive samples that were negative by microscopy and 8 false-negative samples that were positive by microscopy. At the same time, PCR showed 3 false-positive and one false-negative results. PCR showed a higher sensitivity (98.1%), specificity (95.5%), positive predictive value (94.6%), negative predictive value (98.4%) and diagnostic accuracy (96.6%) than OptiMAL test (85.1%, 92.4%, 90.1%, 88.4%, 89.1%, respectively).

Conclusion: Consideration of fever alone as a presumptive prompt diagnosis for anti-malarial treatment would result in huge over-treatment. The use of OptiMAL test and/or PCR assay is a valuable complement to microscopy because these methods help expand the coverage of parasite-based diagnosis and minimize exclusive clinical diagnosis.

Keywords: Malaria, Diagnosis, Microscopic Examination, PCR, OptiMAL test.

Corresponding author: Khadiga A. Ismail, khadigaahmed68@yahoo.com

INTRODUCTION

Rapid and accurate diagnosis is the key to effective management of malaria cases in order to reduce morbidity and mortality caused by delayed or poor management of patients[(1)]. In many instances, a presumptive diagnosis of malaria is based upon the presence of fever alone which leads to the overuse of antimalarial drugs. Fever paroxysms, the hallmark of malaria, occur when infected RBCs rupture and release parasite-derived molecules that stimulate the production of pro-inflammatory cytokines by the host[(2)]. Under ideal circumstances, the clinical suspicion of malaria would be confirmed by a laboratory test that is simple to perform, rapid, sensitive, specific, and inexpensive. Hence traditional malaria diagnosis based on the examination of stained blood smears under light microscope remains the gold standard for malaria diagnosis. In addition it can routinely detect parasitaemia levels as low as 40 parasites/µl, and experienced

microscopists can detect as low as 5-10 parasites/µl of blood[3]. However, it is labor-intensive, time-consuming, requires technical expertise and the availability of a good quality microscope. Therefore, several alternative methods have been developed for malaria diagnosis including immunochromatographic (ICT) assays and molecular amplification methods[4-6]. Each of these methods has strengths and weaknesses in terms of test parameters, cost and technical complexity.

Malaria rapid antigen tests are commercially available; most of them are ICT dipstick assays, based on the detection of malaria antigen in blood flowing along a membrane containing specific anti-malaria antibodies. Most tests which detect *P. falciparum* are based on the histidine rich protein 2 (HRP-2), which is specific to that species. Other tests detect the parasite enzyme lactate dehydrogenase (LDH), using either monoclonal antibodies which react with LDH of all species including *P. falciparum* (so called PAN or pLDH), or antibodies specific for *P. falciparum* LDH[1]. Of these tests, OptiMAL test has been used for the specific detection of pLDH, an enzyme produced by metabolizing malaria parasites[7]. This assay was found to be accurate, rapid and easy to perform and interpret. Moreover, it can be a useful tool for the detection of malaria in countries where both plasmodial species are co-endemic and where laboratory support is limited[5]. Its sensitivity has proved similar to the sensitivity of microscopy in both developing and developed countries[4,8].

Molecular amplification methods such as conventional PCR, nested PCR and Real- time PCR, have significantly affected the diagnostic and epidemiological malaria investigations. These methods allow species differentiation and can be used to identify mutation, which can be correlated to drug resistance acquired by the parasite. They were also used to study genetic variation in malaria parasites and have practical significance in developing strategies to control the disease[6,9-11]. The amplification principle allows picking up a negligible amount of parasite DNA sequence and multiplying it million times for easy detection. It has been estimated that PCR was able to detect as few as four malaria parasites per microliter[12]. These tests are relatively sophisticated, expensive and require a PCR setup. Their use for routine clinical diagnosis is therefore limited because the analysis is time consuming due to the need of multiple reactions per sample, and may not be applicable for malaria diagnosis in remote areas[13].

The objective of this study was to statistically evaluate the diagnostic performance of direct microscopy, OptiMAL rapid antigen test and PCR in detection of malaria infection in clinically suspected cases.

SUBJECTS, METERIAL AND METHODS

Study Type: Descriptive analytical study.

Subjects: A total of 120 expatriate patients enrolled in this study, presented with fever > 37.5°C, associated in some cases with shivering and body aches, were clinically diagnosed as malaria infection. The patients were 90 males and 30 females with an average age of 18-55 years, attending the internal medicine department at King Faisal specialized hospital, Taif, KSA, in the period from January 2007 to November, 2010. All patients were immigrant workers from Southeast Asia (India, Pakistan, Sri Lanka and Philippines) where malaria is endemic and some of them had been treated at least once with chloroquine in their countries. Finger-prick blood samples were collected and tested for the presence of *Plasmodium* parasites using microscopic examination of thick and thin blood films, OptiMAL test and PCR amplification. 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, labeled and stored in a plastic bag with silica gel. Further processing was done at the molecular laboratory of Pediatric Genetic Unit, Ain Shams University, Egypt.

Microscopic examination of blood smears: Thick and thin blood films were prepared, stained with fresh 10% Giemsa's solution and examined using X l000 oil immersion magnification. The slides were reported negative only when no parasites were detected in 200 fields of each thick film. Stained thin film preparations of positive thick films were examined to determine the species: *P. falciparum*, *P. vivax*, *P. malariae* or mixed infection[14]. Parasitaemia was evaluated in 100 fields of thin films against the leucocytes counts taken from records of the patients, based on the equation: number of parasites/µl = total parasite count/WBC count X the total leucocyte count/µl[15]. Samples with high level of parasitaemia were used as positive control for PCR.

OptiMAL test: The OptiMAL rapid malaria test (Diamed, Flow Inc. Portland, Oreg.) was performed according to the manufacturer' instructions. Briefly, a drop of blood was added to a well in a microtiter plate and mixed with two drops of lysis buffer A, which disrupts the red blood cells and releases the pLDH. The specimens were then allowed to migrate to the top of the pLDH strip. After eight minutes, the strips were placed in washing buffer B, which clears the hemoglobin from the strip. Positive and negative control samples were included with each batch tested. The entire process took approximately 15 min, and results were visually interpreted immediately. A positive

control line should always 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, indicates the presence of a non-*P. falciparum* malaria parasite (*P. vivax*, *P. ovalae*, or *P. malariae*). When a third line is also present, this indicates a positive response for *P. falciparum* infection (Figure 1).

PCR amplification: DNA was isolated from dried blood samples on filter paper using the genomic DNA purification Kit #K0512 (Fermentas, EU) which has been validated for extraction from human tissue samples. The PCR procedure included two genus specific primers**:** L1 (biotin-5-GAC CTG CAT GAA AGA TG-3) and L2 (5-GTA TCG CTT TAA TAG GCG-3)[13]. To 10 µl of DNA extract, 50 µl of Go Tag® Green master mix (Promega, USA), and 0.8 µg/µl of each primer were added. Amplification involved 40 cycles. Each cycle consisted of 1 min of denaturation at 90°C, 2 min of annealing at 56°C, and 1 min of primer extension at 72°C with an additional extension at 72°C for 5 min after the last cycle. Positive and negative controls were used for each run. Positive control was DNA extracted from pooled known positive blood samples, by blood film, with high parasitaemia > 200 parasite/µl; while negative control was a blank containing all PCR reagents but no DNA. For detection of PCR products**,** 10 µl of each PCR mixture and DNA molecular size marker were electrophoresed in a 1.0% agarose gel for 1.5 hour and was stained with a 0.5 µg/ml ethidium bromide for 5.0 min, visualized in a UV transilluminator then photographed. The positive control lane showed a specific band at 600 bp, negative control lane was free from any band and samples showing a band opposite to the positive control band were considered as positive (Figure 2).

Statistical analysis: Statistical analysis was performed using the computerized software program SPSS version 10. The variables measured were the numbers of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN). Sensitivity was then calculated as TP/(TP+FN)×100, specificity as TN/(TN+FP)×100, the positive predictive value (PPV) as TP/(TP+FP)×100, and the negative predictive value (NPV) as TN/(FN+TN)×100, diagnostic accuracy (DA) as TP+TN/Total No. of patients×100. Differences in detection rates of the diagnostic tests were also tested for significance using Chi-square test. A probability value of less than 0.05 was considered statistically significant.

Ethical considerations: All patients included in the study were informed of the study objectives and a written signed consent was taken from each one of them.

RESULTS

Results are shown in tables (1-2) and figures (1-2).

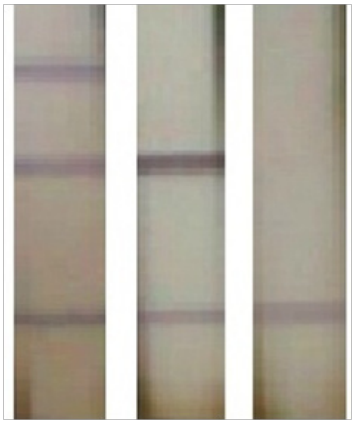
Table (1): Validation of OptiMAL test and PCR results for diagnosis of malaria using direct microscopy as gold standard

Applied tests	Direct microscopy			Sensitivity %	Specificity %	PPV %	NPV %	DA %
	+ve (%)	-ve (%)	Total (%)					
OptiMAL								
Positive	46 (38.3%)	5 (4.2%)	51 (42.5%)					
Negative	8 (6.7%)	61 (50.8%)	69 (57.5%)					
Total	54 (45%)	66 (55%)	120 (100%)	**85.1**	**92.4**	**90.1**	**88.4**	**89.1**
PCR								
Positive	53 (44.2%)	3 (2.5%)	56 (46.7%)					
Negative	1 (0.8%)	63 (52.5%)	64 (53.3%)					
Total	54 (45%)	66 (55%)	120 (100%)	**98.1**	**95.5**	**94.6**	**98.4**	**96.6**

OptiMAL vs direct microscopy, $P > 0.05$ = no significant difference.
PCR vs direct microscopy, $P > 0.05$ = no significant difference.

Table (2): Results of microscopy for detection of malaria species

Direct microscopy	Malaria species				Total
	P. vivax	*P. falciparum*	*P. malariae*	Mixed	
Positive	45 (83.3%)	6 (11.1%)	1 (1.9%)	2 (3.7%)	54 (45%)
Negative					66 (55%)
Total					120 (100%)



(3) (2) (1)

Figure (1): Result reaction on the OptiMAL test strip.
(1) Negative,
(2) Positive for non-*P. falciparum*,
(3) Positive for *P. falciparum*

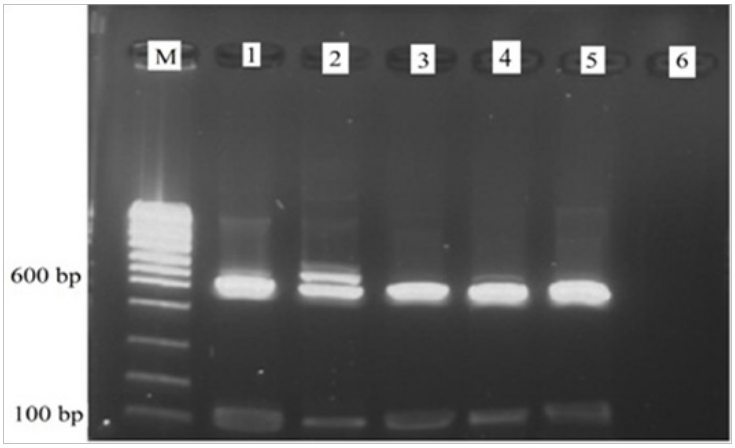


Figure (2): Agarose gel electrophoresis of PCR products based on *Plasmodium* parasite (Ls-rRNA gene).
M: Molecular size marker, **Lane 1:** Positive control,
Lanes 2-5: Positive cases (600 bp band) and **Lane 6:** Negative control

DISCUSSION

Malaria diagnosis has for a long time, and particularly at community level, depended on clinical diagnosis. However, this is unreliable due to the non-specific nature of signs and symptoms of malaria leading to over-diagnosis and over-treatment[16]. Dependable diagnostic services for malaria are critical in order to reduce wastage of costly drugs, and reduce drug selection pressure. In developed countries, rapid diagnostic tests (RDTs) used in screening febrile returnees from endemic areas[4,17] and for self-use by travelers, however, produced variable outcomes[18,19]. In developing countries, RDTs render the sole dependence on clinical diagnosis for malaria unfavorable, especially in remote areas where good microscopy has failed or is unavailable. RDTs are also recommended in situations exceeding microscopy capability, such as in an outbreak or in occupationally exposed groups[20].

Our results showed that the differences in detection rates of microscopy, OptiMAL test and PCR (45%, 42.5%, and 46.7% respectively) in 120 clinically suspected cases, were not statistically significant (P >0.05). Our findings are in agreement with another observational study[21] in which malaria was diagnosed in 46.9% among patients with undifferentiated fever; and are more or less similar to those obtained by other reported studies from different countries of South Asia: Sri Lanka[22], Pakistan[23] and Thailand[24] that demonstrated 38%, 42%, 53% malaria positive among studied groups using microscopy and RDTs. Our results also agree with other reports indicating that RDTs have shown a comparable level of accuracy to microscopy in clinical settings[15,25].

Species differentiation in the positive samples by blood film examination showed higher prevalence of *P. vivax* (83.3%), than *P. falciparum* (11.1%), and *P. malariae* (1.9%). Mixed infections by *P. vivax* and *P. falciparum* presented 3.7% with preponderance of *P. vivax*. This coincides with reports on the high levels of *P. vivax* disease activity in South Asia[26]. In addition, Fernando *et al*.[22] reported that *P. vivax* malaria accounts for up to 70% of infections in Sri Lanka. 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[27]. 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 is vital to obtain follow-up specimens from malaria-positive patients to monitor therapy outcome and detect drug failure[7].

Misdiagnosis can lead to inappropriate or delayed treatment that has been implicated in malaria-associated deaths in developed countries[28]. In the current study, OptiMAL test showed 5 false-positive samples that were negative by microscopy and 8 false-negative samples that were positive by microscopy, which is compatible with results obtained from previous studies[23,29]. In explanation of false positivity researchers hypothesized that RDT positive cases missed by microscopy might be individuals who had been treated but in whom antigenemia persists[15,20]. Other reasons include persistence of antigens due to sequestration of malaria parasites from peripheral blood[30], incomplete treatment,

delayed clearance of circulating antigen (free or in antigen-antibody complexes), and cross reaction with non-*falciparum* malaria, rheumatoid factor[31] or heterophile antibodies[32]. False negative results of RDTs have been 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[31]; and also inability of OptiMAL test to detect parasitaemia levels blow 100 parasites/µl of blood[3,25].

With the PCR protocol used in our study, *Plasmodium* parasites were missed in one blood sample that was positive by direct microscopy. It has been shown that PCR false-negative results are obtained when the DNA isolation protocol is not appropriate[33]; for example, not removing the inhibitors from the sample and not preventing the action of enzymes that degrade DNA. On the other hand, PCR-positive results are obtained only when the DNA is extracted from samples containing live parasites, while dead parasites or parasites cleared by drug treatment or immune system pressure do not register as positive by PCR amplification[12]. Our recorded false positive PCR results (2.5%) as compared to microscopy may be attributed to the possibility of low levels of parasitaemia below the detection level of the microscope. Using microscopic examination of Giemsa stained blood smears as gold standard method, PCR showed a higher sensitivity (98.1 %), specificity (95.5%), PPV (94.6%), NPV (98.4%) and DA (96.6%), than OptiMAL test (85.1%, 92.4%, 90.1%, 88.4%, 89.1%, respectively). The sensitivity and specificity of OptiMAL test when used by others ranged from low 25% to high 100%[7]. Factors that may contribute to these diverse findings include test kit storage conditions in the field (manufacturers usually recommend 4-30°C as the optimal temperature range and practically, exposure of RDTs to > 70% humidity and/or > 30°C frequently occurs in the tropics); in addition to inadequate adherence to the test protocol, or levels of parasitaemia below the detection limit of the OptiMAL test[7]. Moreover, markedly variable sensitivity and specificity for this commercially available RDT may be influenced by the use of different gold-standards, as well as possible geographic variation in malaria antigens[31]. Iqbal *et al*.[29] showed that the sensitivity of the OptiMAL test is 97% at a high level of parasitaemia (>100 parasites/µl), but drops to 59% when the level is <100 parasites/µl and to 39% when it is <50 parasites/µl. Although RDTs require minimal skills and are easy to read, which allow them to be used by moderately trained health workers, their accuracy (sensitivity and specificity), storage under field condition and application for treatment of malaria remain a challenge[15,34,35]. Even though microscopy is considered the "gold standard," it is not 100% sensitive and specific, even when practiced by skilled and experienced technologists in countries where malaria is endemic. This is due to low-level parasitaemia and the occurrence of frequent errors in species identification in mixed infections; in addition interpretation of results is often ambiguous, and procedures for preparation of slides and enumeration of parasites are inconsistent[36,37].

The multicopy Ls-rRNA genes of *Plasmodium* have been demonstrated to be highly stable and conserved, and assays to detect them have displayed no cross reactions with human DNA or other human pathogen DNA or RNA including non human *Plasmodium sp*.[38]. Our results obtained by genus specific PCR proved it to be superior to microscopy based on the report that PCR assay could detect as few as three to four parasites /µl of blood with either the genus- or the species-specific primers[12]. However, the use of this promising technique is limited today to research labs, as it involves a high cost as well as trained personnel; and the possibility of contamination (false positive) of the blood product remains another drawback of PCR. Moreover, the technique is not quantitative and 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[39].

In the present study, pools from blood samples (irrespective of species) directly spotted on filter paper were used for PCR amplification. The dried blood spot technique is far more practical, inexpensive, technically simple, and once dried, the nucleic acids are stable over a wide range of temperatures and over time[40]. It was possible to amplify old blood spot samples that had been stored at room temperature up to 3 years[12]. This technique avoided the use of heparin because of its high inhibitory effect on *Taq* polymerase[28] and also EDTA which is known as a PCR inhibitor by depletion of free Mg^{2+}[41]. Although the use of dried whole blood spots on filter paper may result in a minor loss of sensitivity[10] however, the advantages in collection, transport, and storage outweigh any slight loss in sensitivity. So, dried blood samples used gave very promising results, which will be highly considered in future sample collection.

Based on the results of this study, the use of fever alone as a presumptive prompt for anti-malarial treatment would result in a huge over-treatment burden. OptiMAL test and PCR assay are valuable complements to microscopy because they help expand the coverage of parasite-based diagnosis and minimize exclusive clinical diagnosis.

Author contribution: MM Abdel-Wahab proposed the research idea and the study design, collected the study samples and shared in laboratory performance of the work. KA Ismail shared in study design, assisted in performing the laboratory work and revised the manuscript. NM El-Sayed assisted in performing the laboratory work, interpreted the results, wrote the manuscript and helped in the study design. Each author examined carefully the prepared slides separately and all agreed on the positivity of samples by microscopy.

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التشخيص المعملي للاصابة بالملاريا في الحالات المشتبه فيها اكلينيكيا باستخدام الفحص المجهري واختبار المستضد الأمثل السريع وتفاعل البلمرة المتسلسل

مها محمد عبد الوهاب[1]، خديجة أحمد اسماعيل[2]، نجوى مصطفي السيد[1]
قسمي علم الطفيليات بمعهد بحوث أمراض العيون، الجيزة[1] و بكلية الطب، جامعة عين شمس[2]

المقدمة: تشخيص الملاريا اعتمادا على التشخيص الاكلينيكي لا يمكن الاعتماد عليه نظرا للطبيعة الغير المحددة من علامات وأعراض الملاريا، مما يؤدي إلى عدم دقة في التشخيص. لذا فإن التشخيص الصحيح يعتبر مهما للحد من هدر الأدوية المكلفة.

الهدف من البحث: أجريت هذه الدراسة للكشف عن الإصابة بالملاريا لدى المرضى الذين يشكون من الحمى مجهولة المنشأ والمشتبه فيهم الإصابة بالملاريا اكلينيكيا باختبار مستضد الأمثل السريع وتفاعل البلمرة المتسلسل، ومقارنة هذه الاختبارات باستخدام الفحص المجهري لدعم التشخيص الاكلينيكي.

طرق البحث: تم فحص ١٢٠ مريض من خلال الفحص المجهري باستخدام صبغة الجيمسا، اختبار المستضد الأمثل السريع الذي يكشف انزيم اللاكتات التي تنتجها طفيليات الملاريا وتفاعل البلمرة المتسلسل المحدد للجنس، ثم مقارنة فاعلية هذه الاختبارات بالتحليل الاحصائى.

نتائج البحث: ولقد أظهرت النتائج الاصابة بالملاريا في 54 حالة من بين ١٢٠ حالة بنسبة ٤٥٪ باستخدام الفحص المجهري منهم ٤٥ حالة (٣و٨٣٪) كانت الملاريا نتيجة الاصابة بطفيل الفيفاكس و٦ حالات (١١,١٪) نتيجة الاصابة بطفيل الفالسبارم وحالة واحدة نتيجة الاصابة بطفيل المالاري، اما الباقي (٣,٧٪) نتيجة الاصابة بخليط من طفيل الفيفاكس والفالسبارم. وباستخدام اختبار المستضد الأمثل السريع وتفاعل البلمرة المتسلسل كانت الاصابة بالملاريا في ٥١ (٤٢,٥٪)، و٥٦ (٦٤,٧٪) حالة علي التوالي. وكانت الاختلافات في معدلات الكشف عن الاصابة بالملاريا بهذه الاختبارات التشخيصية لا يعتد به إحصائيا ($P > 0.05$)، ومقارنة باستخدام الفحص المجهري المباشر، أظهر اختبار المستضد الأمثل ٥ عينات ايجابية كانت سالبة باستخدام الفحص المجهري المباشر، كما أظهر ٨ عينات سالبة كانت موجبة باستخدام الفحص المجهري المباشر. أما باستخدام تفاعل البلمرة المتسلسل وجدت ٣ عينات ايجابية كانت سالبة باستخدام الفحص المجهري المباشر وعينة واحدة سالبة كانت موجبة باستخدام الفحص المجهري. ووجد أن تفاعل البلمرة المتسلسل كان الأعلي حساسية (٩٨,١٪)، والنوعية (٩٥,٥٪)، والقيمة التنبؤية الإيجابية (٩٤,٦٪)، القيمة التنبؤية السلبية (٩٨,٤٪)، ودقة التشخيص ٩٦,٦٪ من تلك التي باختبار المستضد الأمثل السريع (٨٥,١٪، ٩٢,٤٪، ٩٠,١٪ و٨٨,٤٪، ٨٩,١٪، على التوالي).

الخلاصة: استخدام الحمى وحدها في تشخيص المالاريا من شأنه أن يؤدي للاسراف في العلاج وأن استخدام المستضد الأمثل السريع وتفاعل البلمرة المتسلسل بالاضافة للفحص المجهري يساعد علي توسيع نطاق التشخيص ويحد من الزيادة في التشخيص الاكلينيكي.