Molecular and Microscopic Studies of Malaria Parasites (Plasmodium)

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Abstract: Malaria causes significant morbidity and mortality worldwide. Improvements in malaria diagnostics should facilitate the identification of individuals infected with the malarial parasites and the treatment of such cases with appropriate drugs. Traditional diagnosis, based on the microscopic examination of Giemsa-stained thick and thin films remains the main standard for diagnosis. Although it has good sensitivity and allows species identification and parasite counts, it is time consuming, requires microscopically expertise and maintenance of equipment. Advanced diagnostic techniques based on the detection of nucleic acid (including PCR) are now available. A total of eighty six (86) blood specimens were collected from all patients associated with symptoms that admitting physicians on duty considered indicative of a malaria infection (fever, abdominal pain, jaundice and black urine). Among the 86 suspected cases revealed 46(56%) were positive while 40(44%) were slide negative by microscopic examination compared to 61(70%) examined by PCR diagnosed positive for Plasmodium genus given 153 base pair PCR product. The thin films showed parasitaemia ranged from 1+ to 4+.
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1.Introduction

Malaria is the most common victor-borne disease and causes significant morbidity and mortality worldwide, there were an estimated 247 million malaria cases among 3.3 billion people at risk in 2008 causing nearly a million deaths mostly of children under age of 5 years and pregnant woman. 109 countries were endemic for malaria up to 2008, most of them (45 countries) within the African region (WHO, 2008). Malaria has been a well-known disease in Egypt since ancient times (Hassan, 2006). It has been endemic in parts of the country but in recent years Fayoum Governorate has represented the only focus. Historical data on malaria parasite prevalence were provided by the Department of malaria, Filariasis and Leishmaniasi, MOHP, covering the period 2006- 2008 indicated that Fayoum has a significantly higher percentage of identified hydrogeological unite (45.3%) within its boundary compared to the low risk governorates (3.3%) (Kenawy, 2008). Improvements in malaria diagnostics should facilitate the identification of individuals infected with the malarial parasites and the treatment of such cases with appropriate drugs. Morphological characteristics of malaria parasites can a determine parasite species however, microscope occasionally fail to differentiate between species in cases where morphological characters overlap especially (*Plasmodium virax and P. ovale*) as well as in cases where parasite morphology has been altered by improper storage of the sample. In such cases, the plasmodium species can be determined by application on molecular diagnostic tests. In addition, molecular tests such as PCR can detect parasites in specimens however parasitemia may be below the detectable level of blood film examination additionally malaria diagnosis based on the microscopic examination of Giemsa-stained thick and thin films remains the main standard for diagnosis. Although it has good sensitivity and allows species identification and parasite counts, it is time consuming, requires microscopically expertise and maintenance of equipment (Brown *et al.,* 2002). Advanced diagnostic techniques based on the detection of Nucleic acid including PCR are now available (Whiley *et al.,* 2006).

The present study aimed to diagnosed and detect *plasmodium* parasites by microscopic examination and to evaluate the PCR as advanced molecular technique compared to conventional microscopic method.

2.Material and Methods

Twenty four cases were hospitalized in Abassia fever and tropical Department, Al-Hussien Hospital during a period from August 2008 till November 2009 designed medical sheet were filled out on each case including personal history, clinical examination, laboratory results and treatment blood specimens.

A total of eighty six (86) blood specimens were collected from all patients with fever associated with symptoms that admitting physicians on duty considered indicative of a malaria infection (fever,

abdominal pain, jaundice and black urine). A data sheet for each sample with patient consent was filled. Two milliters of blood was drawn into sterile tubes with EDTA and thin and thick blood films stained with Giemsa 1% stain which prepared at the time of sample collection and examined under the light microscope followed by DNA extraction from whole blood. The DNA was recovered from 200µl of whole blood according to manufactures instruction transferred into a 1.5ml micro centrifuge tube. It was then centrifuged in a micro centrifuged at (13.000 g) for 10 seconds to pellet the cells and remove the PBS 600µl of nucleic lysis solution was add to lyse the cells and pipette until no visible cell clumps remained. 3µl of RNase solution was added to the nuclear lysate. Inverting the tube 25 times mixed the sample. The mixture was incubated for 15-30 minutes at 37°C in a water bath, and then allowed to cool to room temperature for 5 minutes before proceeding, 200µl of protein precipitation solution was added and vortexes vigorously at high speed 20 seconds. The sample was chilled on ice for 5 minute and centrifuge 4 minutes (13.000 g). The precipitated protein formed a tight white pellet. Supernatant containing the DNA was carefully removed, and transferred into to a clean 1.5ml micro centrifuge tube containing 600µl of room temperature isopropanol. The solution was gently mixed by inversion until white thread-like stands of DNA formed a visible mass. The isopropanol was carefully aspirated and the tube was inverted on a clean absorbent paper and air dried for 15 minutes. 100ml of DNA rehydration solution was added and the DNA was incubated over night at 4°C. Periodically the solution was mixed gently by tapping the tube. A rapid and simple DNA extraction procedure was performed with the QIA amp mini kit (Westburg, lensdes, the Netherlands), according to the manufactures instructions except for the elution, which was performed in 100µl of elution buffer instead of 200µl. Samples were immediately used for PCR or stored at-20°C. A set of primers were designed to amplify a region of 153 base pair, the small submit ribosomal gene of plasmodium

5'AGTTAAGGGAGTGAAGACGAT-3'
5'CCAAAGACTTTGATTTCAT-3'Small-submit rRNA gene (ssurRNA) diagnostic PCR. This method is used for detection of the four species of Plasmodium parasites responsible for human malaria (Ciceron *et al.,* 1999). The protocol was modified from the original publication of Ciceron *et al.*(1999). A 25µl PCR reaction was used. Primers final concentration was 0.25 mM. Many attempts were made to optimize the PCR reaction. 12.5µl of 2xPCR Master-mix were used. Different concentration of template and $MgCl_2$ were used. Accordingly, DNA template and primers were used then completed by dH_2O to complete the volume to total of 25µl .The 200µl PCR tubes were then run into thermo-cycler PCR machine. Amplification reaction was carried out using a PCR thermal cycler (P x E 0.2, USA). The PCR reaction program start with an initial denaturation step at 95°C for 5 min, followed by 40 cycles of a denaturaion step at 95°C for 30 sec, an oligonucleotides annealing step at 55°C for 30 sec and an extension step at 72°C for 1 min. A final extension step at 72°C for 10 min was applied ones the 40 cycles were concluded. The PCR products were separated on basis of their sizes by electrophoresis on agarose gels containing ethidium bromide (EtBr) at a final concentration of 1 ug/ml. Once completely run, the gels were visualized using UV transilluminator. The molecular size of visualized DNA bands in positive PCR reactions were determinate by comparison with the known bands size migrating at the same distance on the agarose gel of a 100 pb scale DNA ladder loaded alongside. Species was used as positive control specimen. Negative control blood samples from healthy individuals were also used as negative controls.

3.Results

Details of patients profile are given in Table (1). Among the 24 patients, 16(67%) were males and 8(33%) were females. There are 18 Egyptian patients (75%) and 6 foreign patients (25%). The age group range from 19 years to over 50 years old most of them suffering fever 21(87.5%), jaundice 18 (75%) and spleenomegaly 13(54%) in additional to pallor 9 cases (37.5%), rigor 7 cases (29.16.5%) and black urine 6 cases(25%).

Table (1): Detailed of patients enrolled in the study

Preliminary data		Clinical data	Microscopic data		Molecular data	
			+ve	-ve	+ve	– ve
Age	19-50	Fever 21(87.5)	46(56%)	40(44%)	61(70%)	25(29.9%)
Sex male	16(67)	Pallor 9(37.5)				
Female	8(33)	Diarrhea 3(12.5)				
Nationality		Rigor 7(29.16)				
Egyptian	18(75)	Jaundice 18(75)				
Nigeria.	1(4.1)	Black urine 6(25)				
Sudanese	5(20.19)	spleenomegaly 3(12)				

The microscopic examination was performed in the local hospital as well as in the research lab. Among the 86 suspected cases revealed 46 (56%) were positive while 40 (44%) were slide negative. The thin films showed parasitaemia ranged from 1+ to 4+. Among the 86 sample whole blood samples examined by PCR 61 (70%) were diagnosed positive for Plasmodium genus given 153 base pair PCR product. The remaining 25 (29.9%) samples were negative visible.

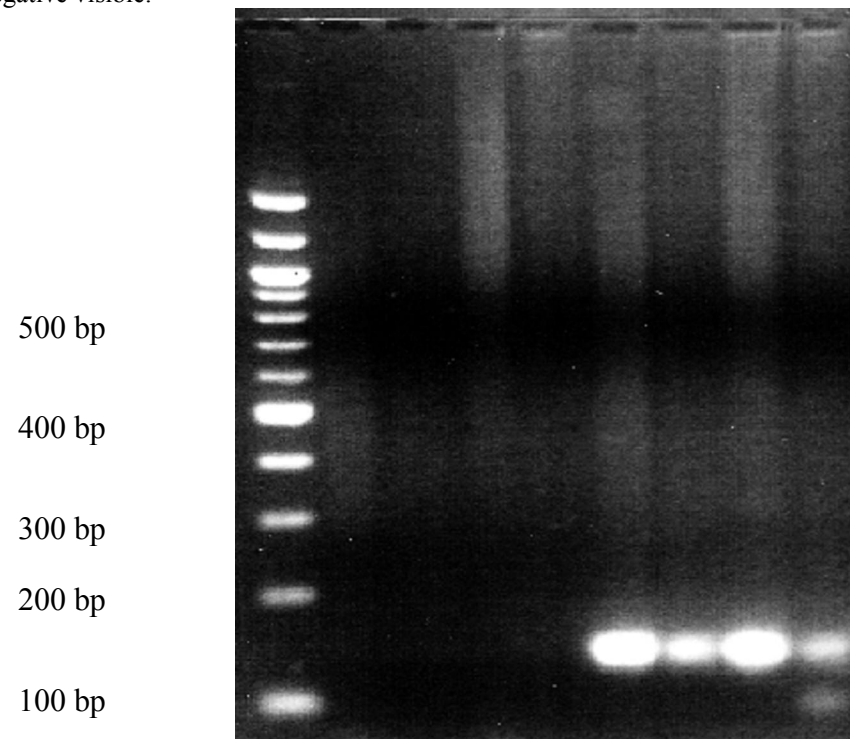

Fig (1): Performance of optimized Plasmodium rRNA 25µl PCR reaction. Gradual extracted DNA templates volumes: 2 µ l (lane 5), 5 µ l (lane 6) and 10 µl (lane 7) from a confirmed positive sample (2 µl in lane 8 and 5 µl in lane 9). Two volumes, 2 µl (lane 3) and 5 µl (lane 4) were analyzed for the negative blood sample. dH_2O was used as a reaction control (lane 2), 15 µl of the reaction products (153bp) were separated onto 2% agarose gel with EtBr, at 75 volts. Amplicons sizes are determined comparatively to a 100bp ladder (lane 1).

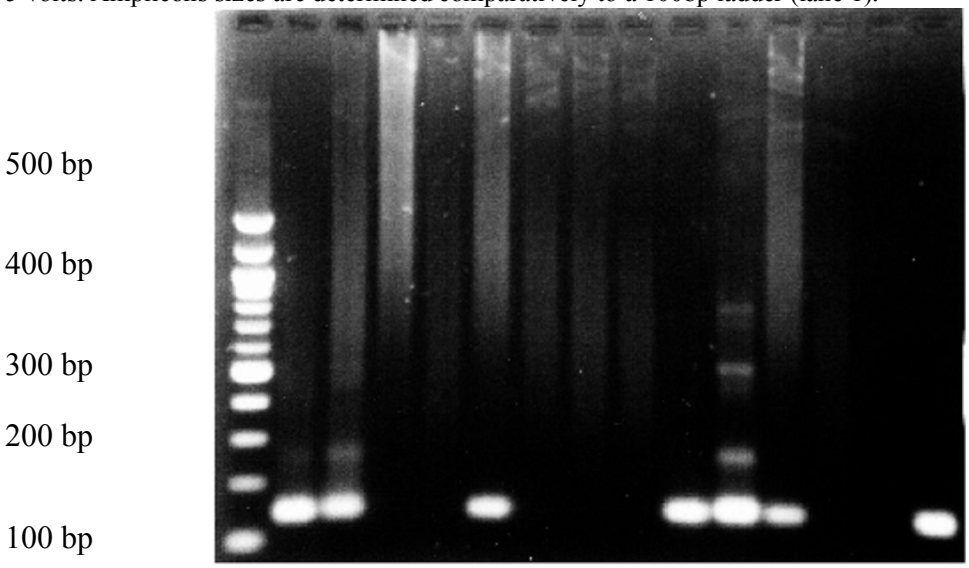

Fig (2): Schematic representation of agarose gel electrophoresis of PCR products from clinical specimens.

4.Discussion

Microscopic diagnosis of blood film, however, has many drawbacks such as how the blood film is prepared, the level of training of the observer, the adequacy of material and equipment and mainly its fair sensitivity. Therefore, many research laboratories have invested their efforts on the development of alternative methods for malaria diagnosis. Such methods include detection of plasmodia within erythrocytes (fluorescent microscopy, Quantitative Buffy coat (QBC), dark field microscopy, nucleic acid probes and immunofluorescence. Other methods based on detecting antiplasmodial antibodies in serum such as indirect immunofluorescence, enzyme immunoassay, western blotting (Avila and Ferreira, 1996). Recently, DNA-based techniques (such as Polymerase Chain Reaction "PCR", nested PCR, and real-time PCR) have been increasingly used to improve sensitivity, specific and high throughout analysis. PCR based techniques have been significantly effective for diagnostic and epidemiological investigation of malaria (Makler *et al.,* 1998; Hanscheid, 1999; Roshanravan *et al.,* 2003). DNA based studies also used to study genetic variation in malaria parasites and have practical significance for developing strategies to control the disease (Menegon *et al.,* 2000). Several PCR assays have been developed for the detection of malaria DNA from blood samples. The small-subunit 18S rRNA gene have been used as target for the differentiation of Plasmodium spp. Nowadays, developed methods using conventional multiplex, nested, hemi-nested or real-time PCR enable all four species to be identified (Whileya *et al.,* 2006). The predominant Plasmodium species are *P. falciparum* and *P. vivax*. Although, that microscopic examination remains the method of choice for the diagnosis of human malaria, in recent years, considerable attention has been given to molecular methods, including the PCR technique. The use of PCR as a diagnostic tool for malaria is well documented in the literature (Barker *et al.,* 1992; Brown *et al.,* 1992; Snounou *et al.,* 1993; Scopel *et al.,* 2004). It is also widely used in malaria research for analysis of field samples. Moreover, many studies have demonstrated the greater sensitivity and specificity of PCR compared to thick blood films. The detection of low parasitaemia by PCR, at levels undetectable by microscopy, has been reported by 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; Singh *et al.,* 1996; 1999; Zakeri *et al.,* 2002. It has also been recognized that the success of the technique depend on the quality of DNA used, DNA amount, blood collecting and amplification factors can inhabit the PCR assay (Brown *et al.,* 1992). Farnert *et al.*(1999) investigated the loss of PCR sensitivity in relation to blood sampling (anticoagulants, culture medium, filter paper), storage (temperature, time and immediate lysis) and handling (repeated thawing and freezing). The study clearly established that the mode of collection and storage of blood samples may influence the sensitivity of detection of malaria parasites by PCR. This may be critical in studies including individuals with low parasitaemia, mixed infections and comparison of data from different settings. This study has evaluated the sensitivity of PCR to detect malaria parasites according to the blood sample collection. The PCR applied is targeting regions of the 18S rRNA gene, allowing for the simultaneous diagnosis of malaria species in human samples. The study showed that the sensitivity of the PCR were 75% for whole blood samples which is relatively much higher than that obtained by microscopy (56%). The PCR assay showed that the results obtained by PCR were superior to those obtained by microscopy, in that all microscopy positive samples were positive by PCR. In addition, the PCR test was able to detect 15 infections that were missed by microscopy.

In conclusion the PCR using the 18S ssrRNA gene of *plasmodium* is useful as an optional to conventional microscopy in for the diagnosis of low level parasitaemia. It showed high sensitivity and it could be performed relatively quickly when many samples must be tested. Also, the PCR is not affected by the subjectivity of the observer. Therefore, it is an excellent tool for obtaining accurate epidemiological data. This method also useful to guide the anti malarial drug when required were parasitaemia level is usually low and can be easily missed by microscopy. Further studies should be carried out to improve the quality of DNA extraction from dried blood spots to reduce the cost of diagnosis by PCR for epidemiological purposes.

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دراسة الإصابة بطفيلى الملاريا باستخدام الفحص المجهري وتقنية البلمرة التسلسلية

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مرض الملاريا من الأمراض الوبائية المهمة والتي تتسبب في عدد كبير من الوفيات حول العالم. وتدل الإحصاءات على وجود مرض الملاريا في مناطق متعددة في العالم وخاصة في أفريقيا لذا فإن تشخيص المرض بتقنيات حديثة وفعالة لها دور مهم في الحد من انتشار المرض.

ويعتبر الفحص المجهري للدم باستخدام المسحات الرقيقة والسميكة هي الطريقة المثلى التقليدية والتي تعتمد في المقام الأول على خبرة الفاحص (PCR) وقد أشارت دراسات حديثة من عدة دول أن تقنية البلمرة التسلسلية طريقة دقيقة لاكتشاف وتشخيص مرض الملاريا إذا ما قورن بالتقنيات المستخدمة حالياً.

الهدف الرئيسي من هذه الدراسة هو تطوير طريقة للكشف عن الطفيلي باستخدام تقنية البلمرة التسلسلية عن طريق جمع عينات دم. ومن ثم استخلاص الحمض النووي للطفيلي منها وهذه الطريقة في التشخيص تعتبر من الطرق الحديثة للكشف عن الأمراض الوبائية التي تصيب خلايا الدم ويمكن استخدامها في المسح الميداني للسكان في المناطق الموبوئة بديلاً للفحص المجهري.

تم جمع 86 عينة دم من حالات ظهرت عليهم أعراض الإصابة بطفيلي الملاريا من مستشفى الحميات بالعباسية ومرضى قسم الأمراض المتوطنة بمستشفى الحسين الجامعي بالقاهرة. جمعت عينات الدم لكل حالة بطريقتين مختلفتين : شريحة سميكة وأخرى رقيقة للفحص المجهري وأخذ عينة دم 2مل في أنبوب مانع التجلط.

أظهرت النتائج عن وجود طفيلي الملاريا في (46 حالة) 56٪ باستخدام الفحص المجهري مقارنة بـ (61 حالة) 70٪ باستخدام تفاعل البلمرة وذلك عند استخلاص الحمض النووي من من العينات المحفوظة في أنابيب مانعات التجلط.

تبين من هذه الدراسة أن تفاعل البلمرة يتمتع بحساسية عالية تفوق الفحص المجهري في الكشف عن طفيلي الملاريا عند استخلاص الحمض النووي من 200 مايكرو ليتر دم وتعتبر الطريقة المثلى والحديثة في التشخيص