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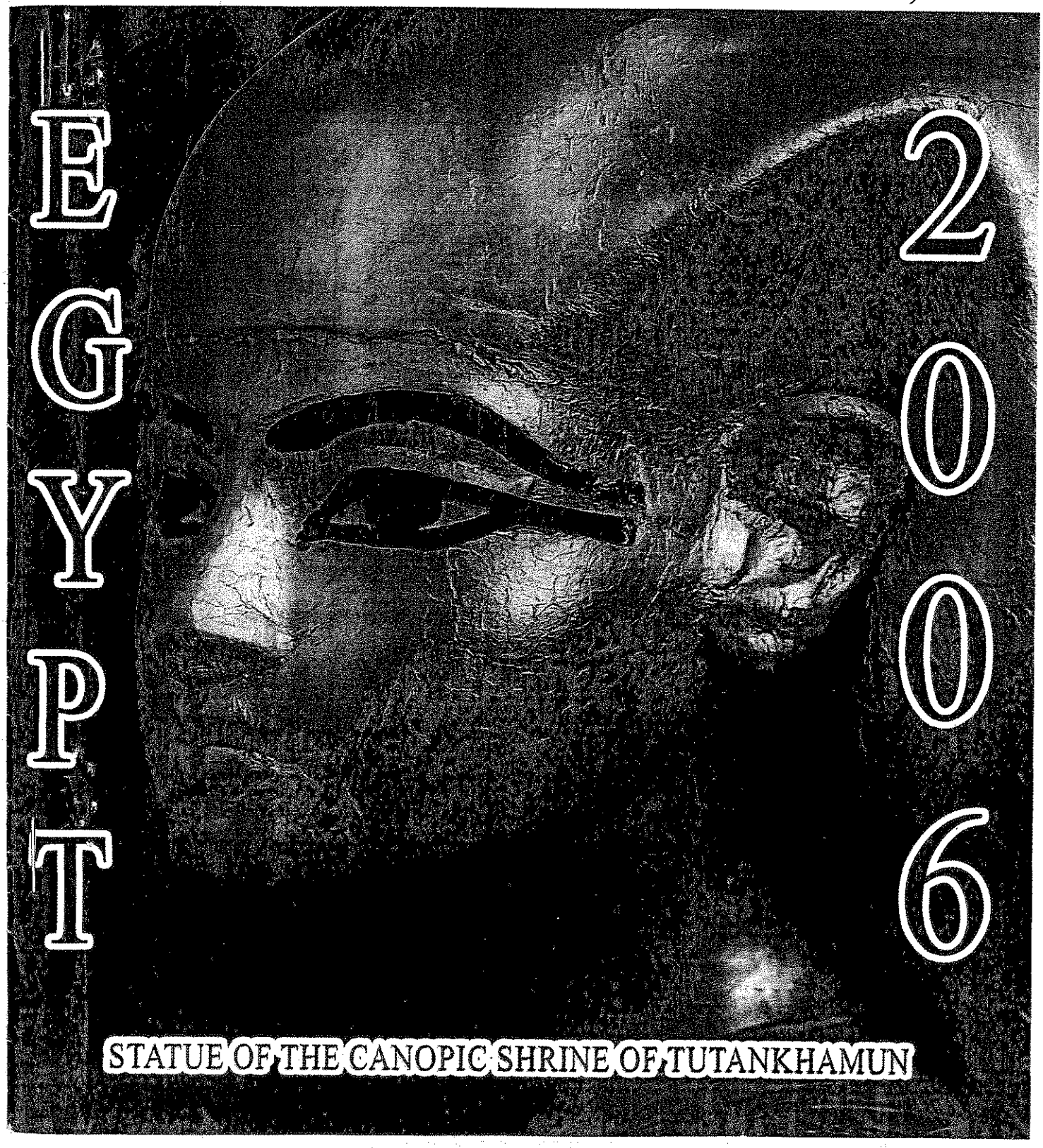

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# DETECTION OF MALARIA IN SAUDI ARABIA BY REAL-TIME PCR

By

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## Abstract

Malaria transmission occurs in Saudi Arabia and mainly endemic in the lowlands of Asir region, the Southwester Province. Imported cases have been reported. Sensitive routine laboratory techniques for rapid and accurate malaria diagnosis are therefore desirable to facilitate the identification of individuals infected with the malarial parasites and to follow up the progress of 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 method of diagnosis used for malaria diagnosis in Saudi Arabia. Molecular diagnostic techniques based on the detection of nucleic acids (as PCR; Real-time PCR) are now highly considered. Real time-PCR a new methodology has been recently applied to detect human malaria. In this study a total of forty four samples, using whole-blood, dried blood and thick smears were examined by PCR and Real-time PCR. Both techniques showed a higher sensitivity than the microscopy. Parasites were detected in twenty nine samples out of forty four, compared to twenty six of thirty nine were positive with thin blood film. The real-time PCR

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assay offers a practical and positive alternative for rapid and accurate diagnosis for malaria infection. The application of such technique will be significantly valuable especially for screening for malaria infection in endemic areas.

## Introduction

In Saudi Arabia malaria transmission still occurs in the north, western and especially endemic in the lowlands of Asir Region in the southwestern province. Its existence is perpetuated there by continuous importation from Yemen (El-Refaie *et al.*, 1984; El-Sebai and Makled, 1987; Vassallo *et al.*, 1985; Warrell, 1993; Bashwari *et al.*, 2001; Alkhalife, 2003). Besides, there was a high threat of introducing malaria by pilgrims during Hajj and Umrah (Al-Hassan and Roberts, 2002a; Khan *et al.*, 2002). Studies also indicated a risk of malaria transmission by blood transfusion. Nevertheless, there were no approved laboratory tests available to screen blood for malaria (Pomper *et al.*, 2003). Typically, malaria produces fever, head-ache, back-ache, malaise, gastrointestinal symptoms (vomiting, abdominal pain and diarrhea) and other flu-like symptoms. If miss diagnosed or drugs are not available for treatment or else the parasites are resistant to them, the infection can progress rapidly to become life-threatening as in the case of *Plasmodium falciparum* (Al-Hassan and Roberts, 2002b). Malaria is mainly diagnosed by the clinical symptoms and detection of the parasites in peripheral blood by thick and thin blood smears stained by Leishman's or Giemsa stain (WHO, 1991; Avila and Ferreira, 1996). Many research laboratories have invested their efforts on the progress 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 & immunofluorescence). Other methods were based on detecting anti-plasmodial antibodies in serum such as in-direct immunofluorescence, enzyme immunoassay, western blotting (Avila and Ferreira, 1996). DNA-based techniques (such as Polymerase Chain Reaction (PCR), nested PCR, and Real-time PCR) increasingly used to improve sensitivity, specificity and high throughout analysis. PCR-based techniques

have significantly effective for the diagnostic and epidemiological malarial investigation (Makler *et al.*, 1998; Hanscheid, 1999; Roshanravan *et al.*, 2003; Farcas *et al.*, 2004). DNA-based technique was used to study genetic variation in malaria parasites and have practical significance for developing strategies to control the disease (Menegon *et al.*, 2000). Bruna-Romero *et al.* (2001) described highly sensitive real-time PCR to detect and measure the development of the liver-stage of malaria parasites in mice. Witney *et al.* (2001) determined the liver stage parasite burden using real-time PCR as a method for evaluating pre-erythrocytic malaria vaccine efficacy. Hermsen *et al.* (2001) detected *P. falciparum* parasites in vivo by real-time PCR method. Polanco *et al.* (2002) determined the parasitaemia in *P. vivax* infection in monkeys using real-time PCR. Cheesman *et al.* (2003) validated a real-time PCR technique for analysis of genetically mixed infection of malaria parasites. Monbrison *et al.* (2003) assessed the chloroquine sensitivity for *P. falciparum* single nucleotide using real-time PCR. Bell and Ranford-Cartwright (2004) made a real-time PCR assay to detect *P. falciparum* infections in the mosquito vector. Wilson *et al.* (2005) monitored the anti-malarial drug resistance using real-time PCR. The multicopy 18S (small subunit) rRNA genes of *Plasmodium* sp. that infect humans have been demonstrated to be highly stable and conserved and assays to detect them have displayed no cross reactions to human DNA or other human pathogen DNA or RNA, including non-human *Plasmodium* sp. (Snounou *et al.*, 1993). In Saudi Arabia microscopic diagnosis of thick and thin blood film smears remained to be the main diagnostic method used for malaria (Bashwari *et al.*, 2001). Accurate and rapid diagnosis of malaria is essential for proper treatment of malaria patients. Studies have showed there is a need to improve accuracy of routine laboratory diagnosis of malaria. Technical faults that may significant effect on the accuracy of diagnosis were reported (Milne *et al.*, 1998). PCR and Real time-PCR was developed and used for malaria diagnosis (Ann-Lee *et al.*, 2002; Perandin *et al.*, 2004; Mangold *et al.* 2005, Swan *et al.*, 2005; Elsayed 2006; Espy *et al.*, 2006). This technique approved valuable for high-throughout rapid screening of hundreds of samples with a high sensitivity and specificity.

The present objective was to evaluate PCR and Real time-PCR in the detection of *Plasmodium* malaria species in febrile patients from different samples including, whole blood, dried blood spots and thick blood smears.

## Material and Methods

Specimens included 24 blood samples from patients living in the attending local hospitals and malaria clinics; five dried blood samples collected on filter papers and 15 thick smear slides. Samples collected between March and September, 2003. All sam-ples were taken randomly from patients presented with fever and admitting physicians suspected malaria infection.

For microscopy, blood samples, 5 ml of whole blood were collected into and EDTA vial for each patient, these were the routine sample provided by the clinical staff for microscopy. Thin smears were made, then stained with stained with 1% Giemsa stain in phosphate-buffered saline (pH 7.0) and examined under oil lenses at x100 for malaria parasite.

DNA extractions from whole blood specimen and dried blood samples were carried out from 200 $\mu$l using QIAamp DNA Blood Mini Kit (Cat. no.51104), according to Manufactures Instruction. DNA recovered from thick smear slides was preformed as follow: the smear was detached using a razor; the detached smear debris was collected into 0.5ml micro-centrifuge tube. 100 µl of absolute methanol in micropipette and used to wash debris to tube bottom. Tubes were incubated 15 minutes; then micro-centrifuged at 13,000 for 2 minutes. Methanol was removed. The wash maybe repeated if the supernatant was densely colored with stain. The residual methanol in the tube was allowed to evaporate at room temperature for 15 minutes. The dried pellet was gently resuspended in 50 µl water and incubated in a heat block at 65°C for 10 minutes with gentle agitation. 5 µl of resuspended extract was used directly in 25 µl primary PCR, and the reminder was stored at -20°C until use.

The primers used were described by Ann Lee *et al.* (2002). Briefly, this PCR procedure was based upon amplification in microtubes performed in a DNA thermal cycler Gene Amp PCR system 9700 (AB Applied Bio-systems). The cycling conditions

were as follows: an initial activation at 95°C for 15 minutes, followed by 45 cycles of 94°C for 30 seconds; 60°C annealing temperature for 30 seconds and 72°C 30 seconds, extension time cycle was 72°C for 4 minutes and hold at 4°C. The PCR product was analyzed on 1% agarose gel stained with ethidium bromide.

Real time PCR assay was used on smart cycler II (Cepheid) platform. It is specific amplification and detection of a species-conserved 85-bp region of the *Plasmodium* 18S rRNA gene of all four human malaria species. The primers and probe used have been previously described by Ann Lee *et al.* (2002). A total reaction volume of 25 µl: 5 µl aliquot of the DNA extract, 900 nM Mach 60, 300 nM Mach 61, 200 nM Mach 62, and 1x TaqMan universal PCR master mix (Applied Biosystems). The following setting were used for PCR: an initial denaturation at 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds and 60°C for 1 minutes.

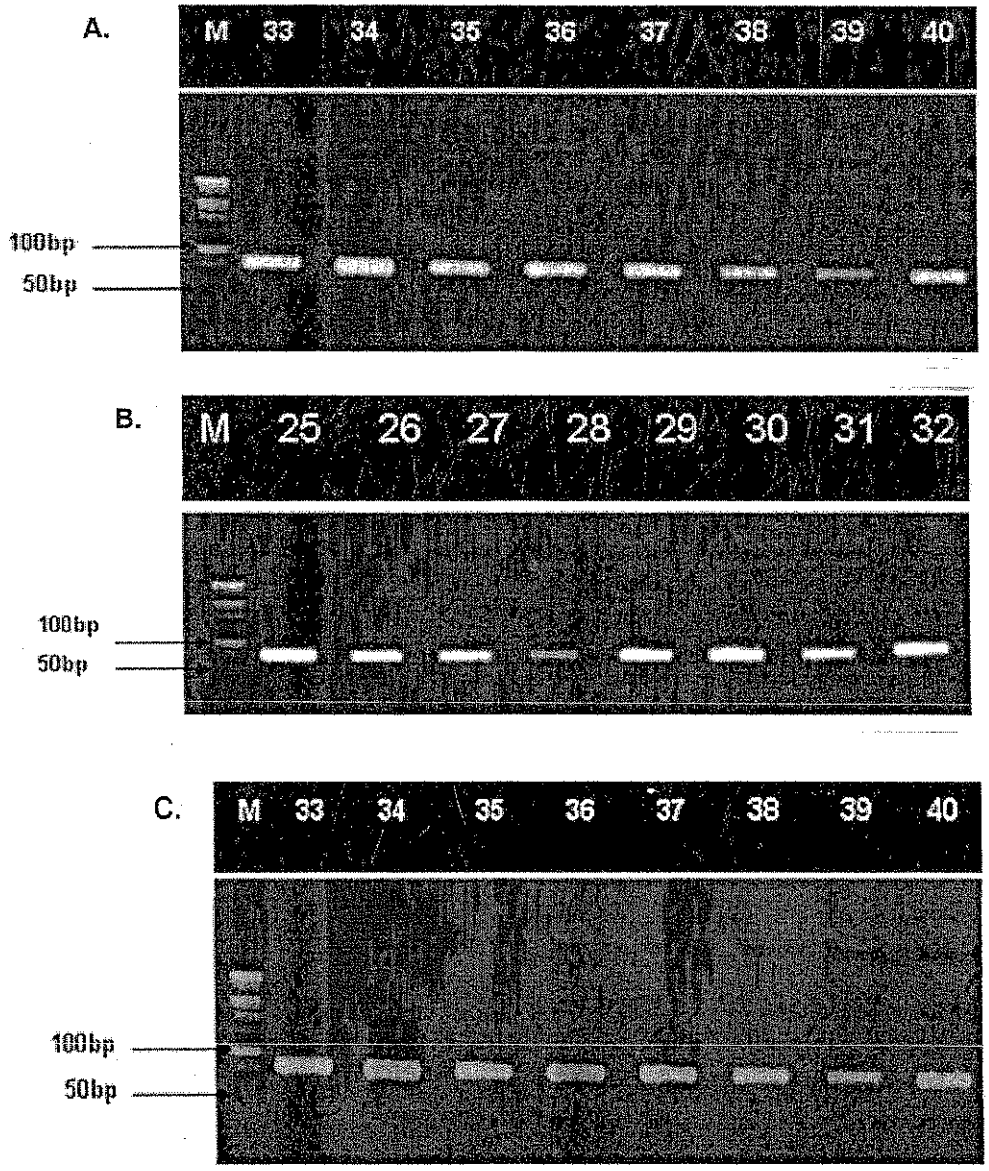
## Results

The total numbers of samples analyzed were 44 samples using PCR and Real-time PCR. Both PCR and Real-time PCR detected 29 (67%) positive samples out of the total 44. Thin smears were not provided for 5 samples (dried blood samples), which were not included in the results. Twenty six (59%) of 39 samples were positive by microscopy. The remaining 15 samples were negative. All 29 positive samples generated a single product of about 85 base pair as expected using PCR. The blood film failed to detect the parasites in three samples while both PCR & Real-PCR were positive (Fig.1A, B & C).

PCR products for the set of primer pairs used have the same melting temperature. The samples reactions showed a series of curves in order of DNA concentration and dilution (Fig.1A, B, C & Fig. 2 A, B). The figures represent the agarose gel electrophoresis of PCR products obtained of studied samples.

## Discussion

Malaria is a major tropical infection causing more than 300 million acute illnesses, and at least one million deaths annually




PCR, Real-time PCR and thin blood film for malaria parasites detection. Total samples were 39 samples. Both PCR and Real-time PCR detected 24 positive samples out of 39 samples while thin blood film 20 positive samples. C). Three techniques used were PCR, Real-time PCR and thin blood film for malaria parasites detection. Total samples used for comparison are 39 samples. Five samples were omitted from the analysis because thin smear results were not provided. Both PCR and Real-time PCR detected 24 positive samples out of 39 samples while thin blood film 20 positive samples.

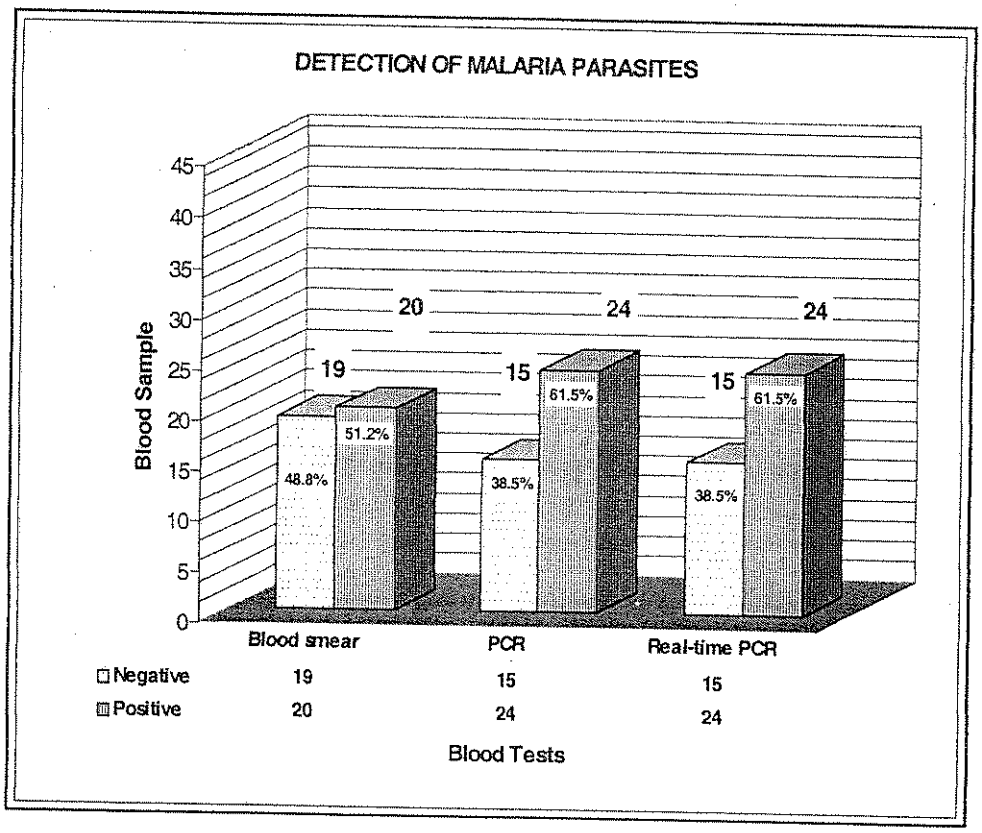



Figure 3: PCR & Real-time PCR showed a higher sensitivity than microscopy.

in more than 100 countries (WHO, 2004). In Saudi Arabia, malaria transmission occurs especially in the endemic South-western region. Also, there was always high risk of introducing infection to non-endemic areas by expatriate or pilgrims during Omara or Hajj (El-Refaie *et al.*, 1984; El-Sebai and Makled, 1987; Vassallo *et al.*, 1985; Warrell, 1993; Bashwari *et al.*, 2001; Al-Hassan and Roberts, 2002; Khan *et al.*, 2002; Alkhalife, 2003). The transmission is seasonal and occurs mainly during the winter months where the percentage of rain falls is higher (September to February). This seasonal variation played a major part in the number of blood sample collected in the study. For proper treatment of malaria patients, accurate and rapid diagnosis of malaria is essential. Traditional diagnosis, based on the microscopic examination of Giemsa-stained thick and thin films remains the main standard for diagnosis.

In the present work, dried blood samples used gave very promising results, which will be highly considered in future sample collection. PCR and Real-time PCR detected 24 out of

the 39 samples (61.5%). Malaria parasites were detected in 20 out of 39 samples with thin blood smear (51.2%). Both PCR and Real-time PCR showed a higher sensitivity than the microscopy. Similar findings have been reported which support that PCR-based methods have the advantages over traditional microscopic methods, especially in cases of low parasitemia and mixed infections (Makler *et al.*, 1998; Hanscheid, 1999; Roshanravan *et al.*, 2003; Farcas *et al.*, 2004; Perandin *et al.*, 2004; Mangold *et al.*, 2005; Swan *et al.*, 2005; Elsayed 2006; Espy *et al.*, 2006).

Samples detected parasites by microscopy were also positive with PCR and Real-time PCR. Three infections were brought to light by both PCR techniques, Thus, compared to the gold standard nested-PCR assay, real-time PCR is highly sensitive (100%) for the simultaneous detection of *Plasmodium* species.

The main advantages of real-time PCR over PCR assays included is far less labour-intensive (only one PCR step); it is performance in a closed system where post-PCR handling is not required (i.e., transfer of amplified template from the primary to secondary amplification reaction and agarose gel electrophoresis for the detection of PCR products. This constitutes a major advantage since the risks of contamination are minimal); and finally (iii) the result of assay can be obtained in only 2 hr, versus a minimum of 3 hr with PCR. The real-time PCR assay offers a practical and clinically acceptable alternative to rapid and accurate diagnosis of malaria infections in patients presenting with symptoms indicative of this disease.

The Real-time PCR is useful in laboratories lacking well trained and experienced microscopist in malaria. The assay is suitable for routine screening of *Plasmodium* sp. Infections in clinical laboratories as an adjunct to other diagnostic methods such as blood film examination. The assay might be useful for *in vivo* drug efficacy studies, to detect resistant parasites before they reached the high levels and result in a clinical recrudescent episode. Menegon *et al.* (2000) stated that using Real Time-PCR provides a good tool in strategy of malaria control.

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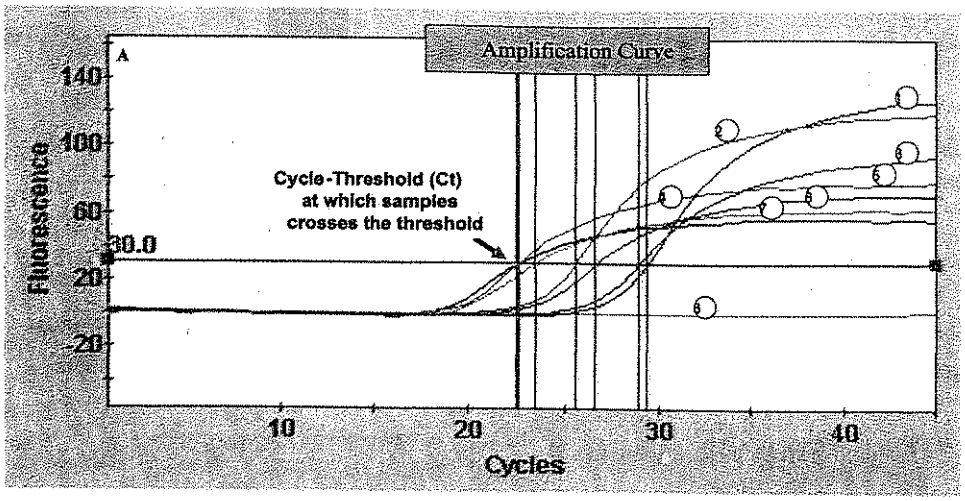
## Explanation of plate

Figure 2: A, B & C represented peaks flu of super-Green I in amplified PCR products after completion of run in a manner similar to ethidium bromide used in conventional PCR but more sensitive. Cycle number shown along the X-axis and arbitrary fluorescence units (actually fold increased over background fluorescence) as on Y-axis. Plots represented 24 replicates (tab. 2). Replicates showed different starting quantity & represented clearly starting in samples (A: 7, 5 & 6; B: 15, 29 & 30; C: 34, 31, 35). Sensitivity of detection allowed acquisition of data when PCR amplification in exponential phase, determined by identifying cycle number at which reporter dye emission intensities raised above background noise; cycle number called threshold cycle ($C_t$). $C_t$ determined at most exponential phase of reaction & more reliable than end-point measurements of accumulated PCR products used by traditional PCR methods. As PCR reaction progresses, samples began to amplify. Amplification occurred exponentially, that a doubling of product (amplicon) occurred every cycle. As reaction progressed, some reagents consumed as a result of amplification, reactions started to slow down at each cycle. The linear amplification seen in linear phase of reaction. Real-Time PCR detected accumulation of amplicon during reaction using SYBR Green - dye. When, SYBR Green dye bonded to double stranded DNA, intensity of fluorescent emissions increased. As more double stranded amplicons produced super-Green dye signal increased.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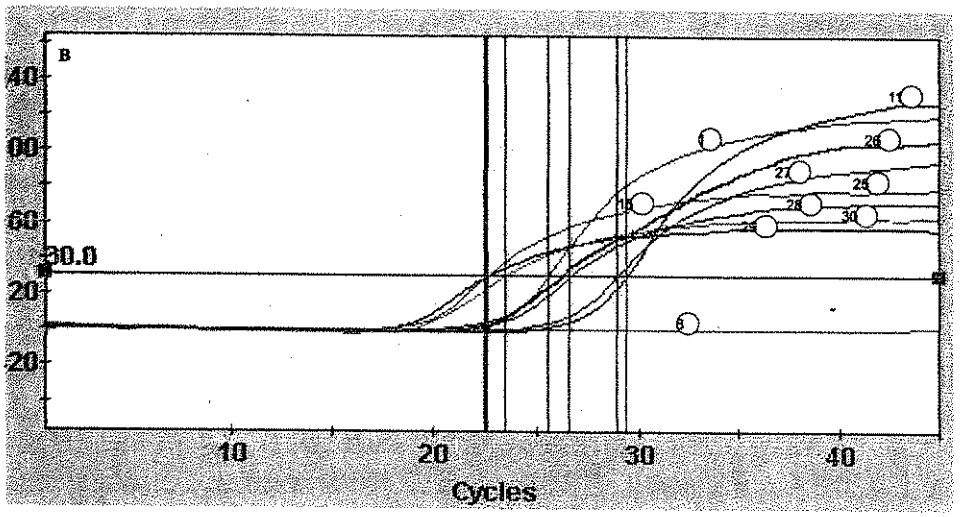
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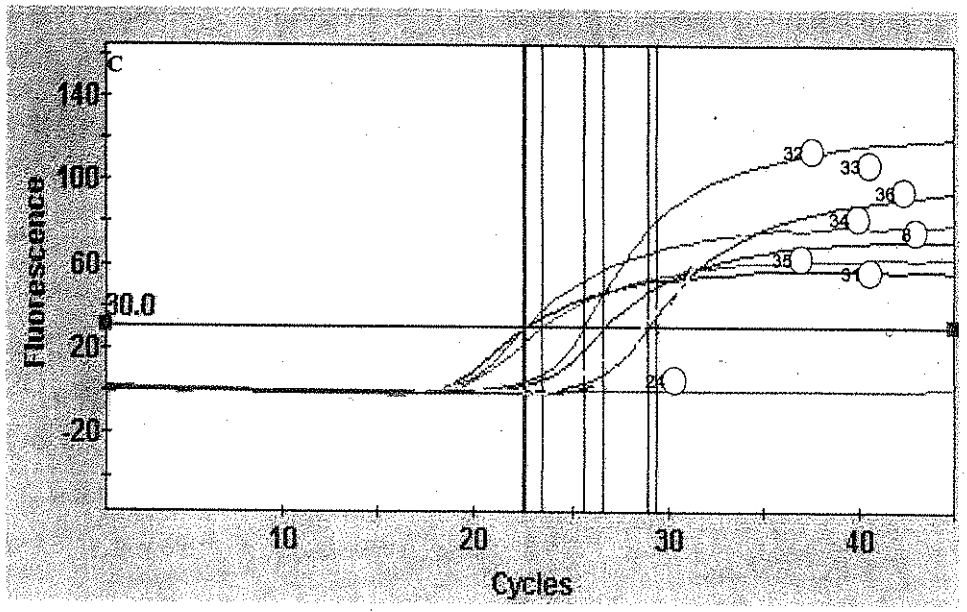
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