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EVALUATION OF USEFULNESS OF POLYMERASE CHAIN REACTION IN THE DIAGNOSIS OF MALARIA IN NIGERIA

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Abstract

Microscopy has been the most common technique for the diagnosis of malaria in south western Nigeria. This study was undertaken to determine the efficiency of PCR for malaria diagnosis in south western Nigeria. A total of 450 samples submitted for malaria diagnosis at Obafemi Awolowo University Teaching Hospital Complex (OAUTHC), Ile-Ife between the months of January and December, 2009 were used. Methods used included Giemsa staining procedure for estimation of parasite densities and polymerase chain reaction (PCR) to detect the presence of malaria parasite in the whole blood. Using microscopy as reference gold standard, patients comprising 120 males and 330 females with age ranging between less than 1 and 60 samples were used. In all, about 255 (56.7 %) of the samples were positive for microscopy, while 75 (16.7 %) with high parasitaemia on microscopy were positive for PCR analysis. The study concluded that PCR for diagnosis of malaria has sensitivity of 29.4% and specificity of 100% using crude method of DNA extraction while the use of DNA extraction kit has sensitivity of 90.2% and specificity of 100%, hence effort should be geared towards increasing the sensitivity and reduce the cost of doing the test in low resource country like Nigeria.

Running Title: Polymerase Chain Reaction in the Diagnosis of Malaria

Key Words: Plasmodium; Polymerase chain reaction; Microscopy; parasitaemia

INTRODUCTION

Malaria is an infection caused by the malaria parasite entering the blood stream (1). The parasite may gain entry through the bite of an infected mosquito or infected blood transferred by blood transfusion or a contaminated injection needle. Malaria is the most important tropical disease, remaining widespread throughout the tropics, but also occurring in many temperate regions (2). It exacts a heavy toll of illness and death especially among children and pregnant women. It also poses a risk to travellers and immigrants with imported cases increasing in non-endemic areas (2). There was an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5 years. About 109 countries were endemic for malaria in 2008, 45 within the WHO African Region (3, 4).

Malaria presents a diagnostic challenge to laboratories in most countries. Microscopic examination of Giemsa-stained thick and thin blood smears has been identified as the most common technique to diagnose malaria since

last 100 years (5, 6). Microscopy continues to be the gold standard for identification of

Plasmodium spp. in the laboratory setting (7, 8). Although easy to apply and cost-effective, this technique assumes that laboratories have certain infrastructure in place with highly skilled professionals and in detecting low level parasitaemia - 10 to 50 parasites/ μ l, so the sensitivity may fluctuate depending upon the skill of technician (9).

WHO has recognized the urgent need for simple and cost-effective diagnostic tests for malaria to overcome the deficiencies of both light microscopy and clinical diagnosis. To overcome such limitations, several methods have been in use including the staining of parasite DNA and RNA with acridine orange: the quantitative buffy coat method (QBC); methods based on the detection of the enzyme lactate dehydrogenase (pLDH), rapid antigen capture assay that detects circulating *P. falciparum* histidine rich protein-2 (PfHRP-II) (10), used mainly in diagnosing malaria in non-

immune individuals or in epidemiologic studies.

Polymerase chain reaction (PCR) based assays have been used mainly for the assessment of the sensitivity and specificity of microscopy and rapid diagnostic tests

(RDTs) and may be of clinical value in some selected situations (11, 12). PCR based tests have shown remarkable capacity to detect malarial parasites in mixed infections and low parasite count and are also sensitive when compared to microscopic examination (13, 14). It has been estimated that PCR can detect malaria infections with parasitemia as low as 5 parasites/ μ l (0.0001% parasitaemia) (15).

The value of PCR as a means of malaria parasite diagnostic technique lies in its sensitivity, its capability of identifying malaria parasites to the species level, as well as its ability to detect five parasites or less/ μ l of blood (16). The aim of this study was to apply the technique of polymerase chain reaction (PCR) in the diagnosis of malaria parasite in a community with limited resources and infrastructural facilities, and compare its sensitivity and specificity with microscopy - a gold standard. This will enable us to appraise the use of molecular technique in the diagnosis of malaria in order to improve the laboratory and clinical diagnosis of this disease.

MATERIALS AND METHODS

Sample collection

A total number of 450 blood samples submitted for blood film in diagnostic laboratory at Obafemi Awolowo University Teaching Hospital Complex (OAUTHC), Ile-Ife between the months of January and December, 2009 were used. These blood samples were collected in EDTA bottles and transported in an ice pack to the Molecular Biology Laboratory of Biomedical Sciences Department, Ladole Akintola University of Technology, Osogbo Campus.

Thick and thin blood films

Thick and thin blood films were prepared according to the methods of (17). Air-dried thick and absolute methanol-fixed thin films were stained with 10% Giemsa solution for 10 minute. Malaria parasites were examined using oil immersion lens objective (\times 100) of a high quality microscope (Olympus) with an incandescent light source.

Parasite density

Thick and thin films were examined microscopically. Each species of *Plasmodium* as well as parasite densities in 100% was identified. The parasite density was counted against 500 leukocytes and was then expressed as the number of trophozoite per microlitre by assuming leukocyte count of 7000 per microlitre (18).

DNA extraction

Kit method: Two hundred microlitres (200 μ l) each from all the samples (450) was subjected to DNA extraction from whole blood collected in EDTA anticoagulant bottle using Ultra Blood Spin kit (Carlsbad, Germany), according to manufacturer's instructions.

Crude method: A modified method of extraction was carried out using digestion buffer. Two hundred microlitres (200 μ l) portion of the blood sample was dispensed in 1.5 ml centrifuge tubes, and the tubes centrifuged at 2,000 g for 5 minutes at room temperature in order to remove the plasma containing proteins. The packed red cell was washed twice in 1 ml distilled water; after which 100 μ l of digestion buffer was added by pipetting up and down with a micropipette. The mixture was incubated at 60°C for 1 hour with occasional mixing, the digest was boiled at boiling temperature (100°C) for 10 minutes, and was snap cooled on ice. The mixture was centrifuged at 12,000 g for 1 minute. The supernatant (DNA) was separated into a new tube for storage in order to prevent inhibition by the sediment which can still contain haemoglobin (19).

Optimization of PCR

Optimization was done in order to know the desired amplification condition for a small subunit ribosomal RNA (ssrRNA) of size 1.7 kb. PCR master mix was prepared to allow for 20 μ l working volume. For a typical 5 reactions, the master mix contained 55 μ l of PCR water, 10 μ l of the forward primer (GGA TAA GTA CGG AAA AGC TGT AGC), 10 μ l reverse primer (CGA CTT CTC CTT CCT TTA AAA GAT), and 20 μ l of the Taq mix (contains the dNTPs and polymerase). Nineteen microlitres (19 μ l) of the master mix was aliquoted into each of the PCR tubes and 1 μ l of the extracted DNA sample was added with positive and negative controls inclusive. The amplification condition was at 1 min denaturation at 94 °C, 1 min reannealing at 56 °C, and 3 min extension at 72°C, for a total of 35 cycles. Maximum care was taken to avoid contamination by ensuring strict physical barrier and working in a well sectorized laboratory of high hygiene.

PCR for the detection of Plasmodium species

PCR master mix was prepared to allow for 30 μ l working volume. For a typical 30 reactions, the master mix contained 512 μ l of PCR water, 96 μ l of the forward primer (GGA TAA GTA CGG AAA AGC TGT AGC), 96 μ l of reverse primer (CGA CTT CTC CTT CCT TTA AAA GAT), and 192 μ l of Taq mix (0.2 mM dNTPs; 1.5 mM MgCl₂; 50 mM KCl; 25 units/ml *Taq* DNA polymerase), and amplification carried out in a thermal cycler, the master mix contained a final concentration of 1 μ M each for forward primer and backward primer, 28 μ l of the master mix was aliquoted into PCR tubes and 2 μ l of the extracted DNA sample was added and mixed. The amplification condition was at 1 min denaturation at 94°C, 1 min annealing at 56°C, and 3 min extension at 72°C, for a total of 35 cycles (Mathiopoulos et al., 1993) using GeneAmp PCR System 9700.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using 1 % agarose gel in 0.5 X Tris borate EDTA buffer (44.5 mM Tris borate and 1 mM EDTA, pH 8.3), agarose was weighed into a clean conical flask containing 95 ml of distilled water, which was made up to 100 ml by addition of 5 ml of Tris borate EDTA buffer. Dissolved agarose was heated briefly for effective dissolution in microwave oven, it was allowed to cool to about 50 °C, ethidium bromide was added. The edge of a clean dry Perspex plate (7 x 7) was tapered with paper tape, molten agarose was poured into a dry Perspex plate sealed with paper tape to form a mould and with inserted combs to create wells for loading the PCR products and allowed to set (20-30 min at room temperature). Combs and paper tapes were removed and gel in the tray was carefully placed in the electrophoretic tank containing electrophoresis buffer. The amplified product was loaded into the agarose

in the tank. To load, the DNA (10 μ l) was mixed with 1 μ l of loading dye (bromophenol blue, xylene cyanole, sucrose) on a Para film "M" paper and was loaded into the agarose well using micropipette. DNA ladder was also loaded to one end of the gel using a micropipette. Electrophoresis was done at 70 Volts for 90 min. The DNA bands were visualised with short wave ultraviolet transilluminator and photograph taken using a Syngene gel documentation system (Syngene, UK). The sizes of the DNA bands were estimated from plots of the relative mobilities against the DNA ladder reference.

Data Analysis.

Data were analysed using statistical package within the Microsoft Excel and Epi-info software for Disease control and prevention, USA. Chi square was used to determine the effect of DNA extraction methods on the data obtained. The p value less than 0.05 was considered to be significant.

RESULTS

On optimisation, amplification was performed in a thermal cycler with a three-step cycling programme: 1 minute denaturation at 94°C followed by 1 min annealing at 56°C and a 3 min extension at 72°C for a total of 35 cycles was successful in respect to both positive and negative control, and amplification of the 1.7 kb product was visible at this condition, and this dictated the condition for overall amplification. Representatives of the PCR products sent for sequencing confirmed the product to be DNA template for *ssrRNA*. The result of PCR showed specific bands of 1.7 kb (Figure 1), 230 of the 450 samples (51.0%) were positive by PCR. Of this, 92 were males and 138 were females. A low proportion, 25 (9.8%) of all microscopy positive samples were negative by PCR and 230 (100%) of PCR positive samples were positive for microscopy (Figure 2).

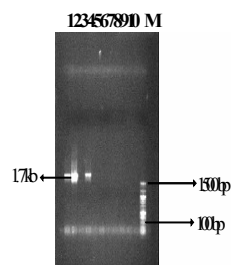


FIG. 1

Figure 1 . PCR detection of *Plasmodium* species.

Lane 1: positive control; Lanes 2-9: samples;

Lane 10: Negative control; Lane M: 100 bp

DNA ladder size marker. Arrow indicating

the expected 1.7 kb DNA fragments

On the basis of repeatedly negative malaria smears and PCR negative results, 180 patients did not have malaria. The overall sensitivity, specificity, PPV, and NPV of the PCR test were found to be 90.2%, 100%, 100%, and 87.8%, respectively. A total prevalence of 56.7 % was obtained using microscopy while prevalence of 51.1% using PCR where DNA extraction kit was used (Figure 2). We also noticed significant difference ($X^2 = 117.62$; $p < 0.01$) in the performance of PCR with respect to DNA extraction method adopted - kit versus crude

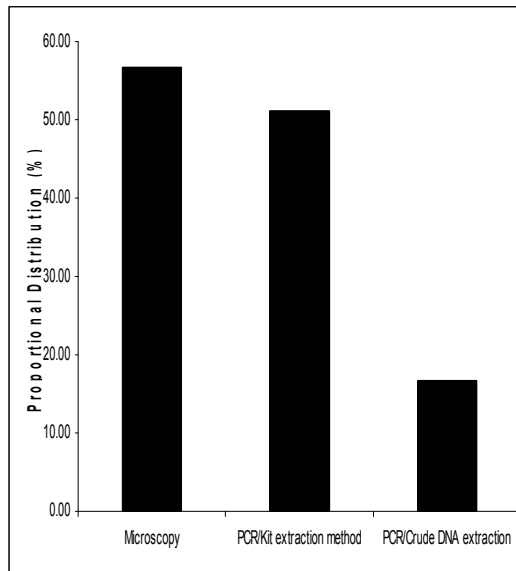


FIG. 2

Figure 2: Distribution of malaria

parasite according to technique employed

method; kit DNA extraction method produced results very close to microscopy (51.1%) while crude method efficiency of detecting malaria parasite was very low (16.7% prevalence, Fig. 2).

A total of 450 samples were used in this study. The age was between 1 and 60 years (mean = 33.4; median = 29.5). Blood film revealed that 255 (56.7 %) had malaria parasite. Seventy five (62.5%) males out of 120 had malaria parasites by blood film while 180 (54.6%) females were blood film positive (Fig. 3).

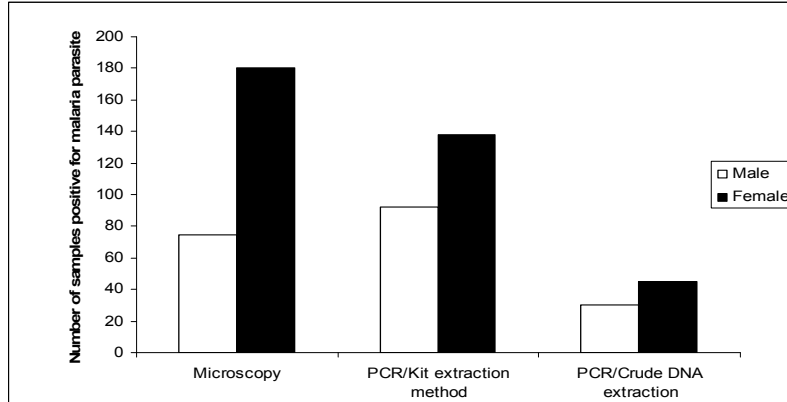


FIG. 3

Figure 3. Sex prevalence of malaria parasite according to the technique

A panel of 144 samples that had non-concordant results were retested and the performance of each assay calculated by comparing the results from the second tests with those of the initial test (i.e. initial test was used as the reference standard for statistical purposes). The data from this test clearly demonstrated that PCR was more repeatable, less subjective test than microscopy with parasite densities of more than 50 parasites/ μ l. Furthermore, the samples that were mistaken to be negative in PCR were found to have low parasite counts. The study also noticed that the crude method provided DNA sample that were PCR inhibited as a result of presence of haemoglobin, while the use of DNA extraction kit ameliorated this condition.

DISCUSSION

In this study, microscopic examination using Giemsa stain showed specific and defined malaria parasites in blood smears. Microscopic examination of Giemsa-stained thick and thin blood smears has been the diagnostic method of choice for species identification in epidemiologic studies and medical diagnosis (20). The method is simple, does not require highly equipped facilities, and in most cases enables differentiation among the four species causing malaria in humans when performed by experienced personnel. However, microscopy is often time-consuming and laborious, and it is estimated that even a skilled person can evaluate only 60 to 80 specimens per day under

field conditions (21). The recommended method and current gold standard used for the routine laboratory diagnosis of malaria is the microscopic examination of stained thick and thin blood films, particularly with the additional sensitivity offered by examination of thick blood films (22).

PCR based methods have been consistently shown to be powerful tool for malaria diagnosis (23, 24). There was extremely poor concordance between microscopy and PCR at relatively low parasite densities. When PCR was considered the reference standard, the performance of microscopy was just as poor as that observed for PCR when microscopy was used as the reference standard. Because it was difficult to determine whether microscopy or PCR was the more accurate assay, all non-concordant samples were retested in order to determine which method was the more repeatable test (assuming that the more repeatable assay was the more accurate). The data from this test clearly demonstrated that PCR was more repeatable. PCR presented with a good performance criteria in terms of reproducibility when compared to microscopy, coupled with 100 % specificity, 90.9% % sensitivity, 100 % positive predictive value (PPV), and 87.8 % negative predictive value (NPV) PCR values, the specificity and PPV can be said to be in agreement with a study carried out by Barker et al. (25), and Speers et al. (26) with 100% specificity. A study from an endemic area of Brazil reported a sensitivity of

PCR to be 73% using blood stained in filter paper strips in condition of low level parasitaemia (27). However, another study has reported an overall sensitivity of 97.4% for PCR compared to microscopy (28), result of 90.9% sensitivity (low), and 87.8 % negative predictive value (high) obtained from this study is not in agreement with the cited studies and this could be as a result of degradation of the target DNA after extraction with repeated freezing and thawing in the presence of *Plasmodium* species sequences that lack sequence recognised by the used primers or technical faults while DNA storage may give false negative results (25) especially when amplifying a large DNA fragment such as 1.7 kb. The repeated freezing and thawing of the samples due to electricity cuts/load shedding might have also contributed to the degradation of the DNA in the samples (29).

In improving sensitivity, it can be recommended that method of extraction should be well monitored in order to prevent PCR internal inhibitors to reduce false negative results. Example of which is haemoglobin in blood which had been shown by this study not to be well cleaned off from the extracted DNA by the crude method of extraction, this was confirmed due to internal inhibition of some samples which were negative for PCR, but were positive with DNA extracted by kit, thereby given kit method of extraction a higher recommendation. Also, investigating smaller amplicon (206 bp, 240 bp, 620 bp) by targeting small subunit ribosomal RNA (ssrRNA) gene can aid more sensitivity as seen in most studies (30, 31) rather than bigger fragment as done for this study.

In spite of having a good Microscopist, this study highlights the difficulty in conducting an active surveillance programme in areas where infection rates and parasite densities are low. Although, sensitivity of PCR can be related to parasite density (30, 31), data here indicates that PCR can be a viable method for conducting active malaria surveillance in south western Nigeria, if sensitivity is increased.

In conclusion, most studies have found that PCR-based tests have shown remarkable capacity to detect malaria parasites in low parasite count, and are also more sensitive when compared to microscopic examination (13, 27). The study concluded that PCR has sensitivity of 90.9 % and specificity of 100 % in malarial infection diagnosis. With this, PCR could be a good tool in confirming the clinically strongly suspected but microscopically negative cases of malaria infection, but effort should be made to increase the sensitivity of PCR to 100% in Nigeria.

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