

**A COMPARATIVE STUDY OF BLOOD FILM,
IMMUNOCHROMATOGRAPHIC TEST AND POLYMERASE CHAIN
REACTION IN THE DIAGNOSIS PLASMODIUM VIVAX INFECTED
PATIENTS IN KHARTOUM STATE SUDAN**

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ABSTRACT

Background: Malaria is a major public health problem in Sudan. It is the most important human parasitic disease throughout the tropical and subtropical regions due to its high prevalence and mortality rate, blood film, ICT and PCR are laboratory methods used for diagnosis of malaria parasite. **Objectives:** The aim of this study was to compare between three methods (blood film, immunochromatographic test and PCR) used for detection of *Plasmodium vivax* among Sudanese patients with malaria. **Methods:** Cross-sectional study was conducted during the period from March to April 2018, fifty samples were collected from known patients with malaria, blood film, ICT and PCR was used to detect *Plasmodium* species from each sample; Data

were collected using structural questionnaire, analysis was carried out by means of statistical package for social science (SPSS version 21). **Results:** From fifty malaria samples caused by *Plasmodium* species, there are fifty samples 48(96%) and 43 (%) were positive by blood film and ICT respectively, but by using PCR technique there were fifteen 15(30%) of patients samples were positive for *Plasmodium vivax* and *P. falciparum*. **Conclusion:** The high number of false negatives in microscopy, it is necessary to reinforce training of laboratory staff on malaria microscopy, the “Gold Standard” in endemic areas.

KEYWORDS: Malaria, P-vivax, BFFM, ICT, PCR and Sudan.

INTRODUCTION

Malaria is the most important human parasitic disease throughout the tropical and subtropical regions of the world due to its high prevalence and mortality rate, *Plasmodium falciparum* and *vivax* causes the most severe form of the disease and is responsible for most malaria morbidity and almost all malaria mortality.^[1,2]

Malaria in Sudan is the major public Health Problem. It leads to an estimated 7.5- 10 million cases and 35000 deaths every year. The burden of the disease on the health system is a reality. Out of total outpatients' attendance, admission and deaths malaria represents 20-40%, 30-50% and 15-20% respectively. These figures bring Sudan on the top of WHO / EMRO countries, as Sudan shouldered 50% of cases and 70% of deaths in the region (WHO/EMRO). Malaria is endemic throughout the Sudan. The endemicity level varies from hypo-endemic in the north – mesoendemic in the central part and hyper-and holo-endemic in the south (Map 1). Considering other factors serve as a background for malaria in Sudan; meteorological, Malaria in Sudan is the major public Health Problem. It leads to an estimated 7.5- 10 million cases and 35000 deaths every year. The burden of the disease on the health system is a reality. Out of total outpatients' attendance, admission and deaths malaria represents 20- 40%, 30-50% and 15-20% respectively. These figures bring Sudan on the top of WHO / EMRO countries, as Sudan shouldered 50% of cases and 70% of deaths in the region (WHO/EMRO). Malaria is endemic throughout the Sudan. The endemicity level varies from hypo-endemic in the north – mesoendemic in the central part and hyper-and holo-endemic in the south (Map 1). Considering other factors serve as a background for malaria in Sudan; meteorological, Malaria in Sudan is the major public Health Problem. It leads to an estimated 7.5- 10 million cases and 35000 deaths every year. The burden of the disease on the health system is a reality. Out of total outpatients' attendance, admission and deaths malaria represents 20- 40%, 30-50% and 15-20% respectively. These figures bring Sudan on the top of WHO / EMRO countries, as Sudan shouldered 50% of cases and 70% of deaths in the region (WHO/EMRO). Malaria is endemic throughout the Sudan. The endemicity level varies from hypo-endemic in the north – mesoendemic in the central part and hyper-and holo-endemic in the south (Map 1). Considering other factors serve as a background for malaria in Sudan; meteorological, Malaria in Sudan is the major public Health Problem. It leads to an estimated 7.5- 10 million cases and 35000 deaths every year. The

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min are needed to examine each slide, which is often the case in endemic areas for malaria or when drugs are taken inappropriately.^[4]

On the other hand, the immunodiagnostic tests (ICT) used for the detection of Plasmodium are easy to use and can produce fast results, but they usually have lower sensitivity and specificity than conventional microscopic examination. Many of them have been designed to specifically detect *P. falciparum* and *vivax*, some of them can detect the other malaria species non-specifically.^[5,6]

Finally, the use of molecular biology for the diagnosis of malaria has proved to be highly sensitive for the detection of the disease, but the equipment required is not widely available in many of the endemic areas and the protocol is more complex and needs better trained technicians. The use of the polymerase chain reaction (PCR), however, is extremely useful for decision making in disease control and treatment, for example, for the detection of mixed infections which play a modulatory role in the severity of the symptoms.^[6,9,10] The greatest advantage, however, is the ability of PCR to detect infections with parasitemia as low as 5 parasites/ μ L blood.^[11,12] However, there are some problems with false-negative results when the DNA isolation protocol is not appropriate^[13], for example, not removing the inhibitors from the sample and not preventing the action of enzymes that degrade DNA^[14]; however this study was designed to compare between three methods (blood film, ICT and PCR) used for detection of plasmodium *vivax*, among Sudanese patients in Khartoum state.

MATERIALS AND METHODS

Study design and Study area: Cross-sectional study was conducted at Khartoum teaching hospital during the period from March to April 2018. The survey was carried out in Khartoum state Sudan. The State has a tropical climate with one dry seasons (October to April) alternating with rainy seasons (May to September).

Study population

A Fifty samples from known patients who attending Khartoum teaching hospital with malaria caused by *P-vivax*, aged from 10-70 years; Permission of this study was obtained from to local authorities in the area of the study. An informed consent was obtained from each participant in the study after explaining objectives of the study. Interview and questionnaire was used to collect data.

Samples collection and sampling technique

Sampling was carried out using non probability sampling technique (namely convenient sampling method, we collect 50 blood samples from participants. A total of 5 ml of venous blood was collected from each participant; 3 ml of blood samples were taken for blood film (Microscopy), and ICT, the remainder 2 ml was stored in -80 for PCR.

Microscopy: Thin and thick slides were made in the Slides of the peripheral blood specimens were made immediately after collection on a clean, grease-free microscope slide and allowed to air dry. The films were stained with 10% Giemsa solution (Applichem, Panreac ITW Companies) for 10 min and Then were allowed to air dry and subsequently examined by light microscopy using an oil immersion objective lens. All slides were examined by microscopists and checked by senior lab specialist from Al-Neelain University faculty of Medical Laboratory Science, Department of Medical Parasitology. A slide was declared negative only after observing 100 microscopic fields without finding parasites. For ensuring a quality control; Giemsa stain was checked, and the microscopists observed the slides without knowing the previous diagnosis obtained with the RDTs.

Rapid diagnostic test

® Malaria 4 species test (Test cassette) (Nalvon Minden, Moers, Germany) was used as the RDT in situ. The test enables differential diagnosis between *P. falciparum*, *Plasmodium malariae*, *P. vivax*, and *P. ovale* in human whole blood samples. The test is based on the detection of HRP2 specific for *P. falciparum* and pLDH specific for *Plasmodium* sp. The test detects the HRP2 and pLDH proteins; the cut-off level was 1–50 parasites/μl of blood for HRP2 and 51–100 parasites/μl of blood for pLDH. To perform the malaria test, 5 μl of whole blood was collected with the provided capillary pipette and transferred to the sample well. Four drops of the assay diluent were added to the diluent well according to the manufacturer's protocol. The results were read after 15 min.^[15]

DNA extraction and molecular analysis

DNA was extracted from blood samples using salting out technique. SnM-PCR was performed as described previously.^[16,17] The method is based on features of the small subunit nuclear ribosomal RNA gene (ssrDNA), a multicopy gene possessing both highly conserved domains and domains characteristic of each of the four human malaria parasites. The first reaction in SnM-PCR includes a universal reverse primer with two forward primers specific for *Plasmodium* and mammals, respectively. The mammalian-specific primer was included as

a positive control to distinguish uninfected cases from simple PCR failures. The second PCR reaction includes a *Plasmodium*-specific forward primer plus species specific reverse primers for *P. falciparum*, *P. vivax*, (common malaria species in Sudan). The technique is more sensitive and specific than the standard microscopic examination.^[18] Diagnostic PCR was performed for all samples positive for malaria by the other methods.

Statistical analysis

Statistics was performed using statistical package for windows (SPSS v21). Frequencies with 95% confidence intervals (CIs) were used for categorical variables. Associations were assessed by the Chi square test. The level of significance was set at $P \leq 0.05$. Sensitivity and specificity calculations for microscopy and RDT were performed using MedCalc statistical software and were calculated using SnM-PCR as the reference technique, “Gold Standard”.

Ethics

This study was approved by institutional Review Board at Al Neelain University Faculty of Medical laboratory Sciences. Written informed consent was obtained from all participants.

RESULTS

Fifty samples from patients with febrile episodes were enrolled in the study. Males (60%) recruited in the study were more frequent than females (40%) figure 1. Statistical analysis showed a significant variation (0.00) in detection of plasmodium species in patients samples used blood film, ICT and PCR when compared to other (Table 1). According to -PCR results, they were false positives in RDT and microscopy (43 positive samples by RDTs and 48 by microscopy). Compared to RDTs microscopy had higher sensitivity (88.24; 95% CI 63.56% - 98.54%) (Table 2). RDTs specificity was higher than microscopy specificity (44.87; 95% CI 33.59% - 56.56%). (Table 3) summarized the frequency of plasmodium species detected by microscopy; among the study participants, 96% (48) patients were malaria-positive by microscopy. Of those, 29% (14/48) and 71% (34/48) were diagnosed as *P. falciparum* and *P. vivax*, respectively.

Table. 1: Diagnostic results with each method.

| Technique | Positive | Negative | P. value |
|-----------|----------|----------|----------|
| PCR | 15 (30%) | 35 (70%) | 0.00* |
| RDTs | 43 (86%) | 7 (14%) | |
| BFFM | 48 (96%) | 2 (4%) | |

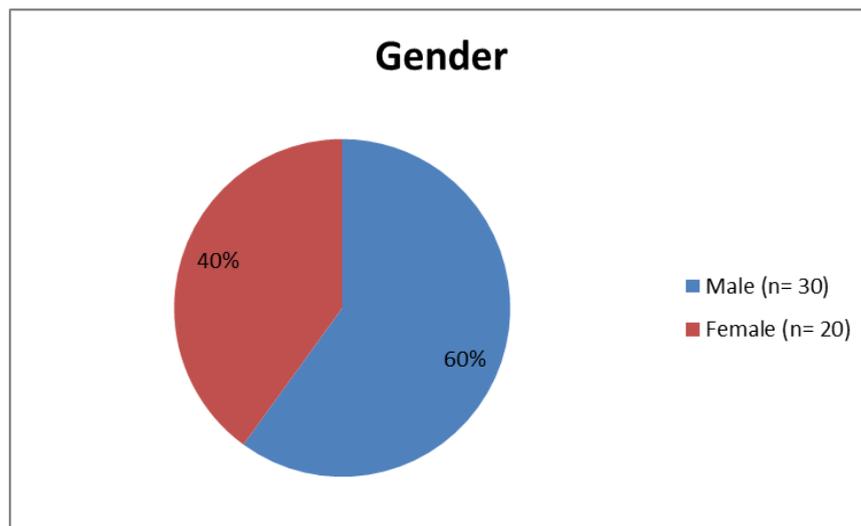
*P-value less than 0.05 consider as significant

Table. 2: Sensitivity and specificity of microscopy and RDTs.

| | PCR/Microscopy Value | 95% CI | Value | PCR/RDTs 95% CI |
|--------------------|----------------------|-----------------|---------|-----------------|
| Sensitivity | 88.24% | 63.56% - 98.54% | 68.18% | 45.13% - 86.14% |
| Specificity | 42.17 % | 31.40% - 53.51% | 44.87 % | 33.59% - 56.56% |

Table. 3: The frequency of plasmodium species detected by microscopy.

| Technique | Species | |
|------------------------|----------------------|-----------------|
| | <i>P. falciparum</i> | <i>P. vivax</i> |
| Microscopy n=48 | 29% (14) | 71% (34) |
| RDTs n=43 | 33%(14) | 67%(29) |

**Figure. 1: Gender distribution in case group.**

DISCUSSION

Accurate diagnosis of *Plasmodium* species is important not only for establishing the correct treatment regimen, but also for applying effective malaria control strategies in endemic regions like Sudan. Misidentification of the *Plasmodium* species could result in severe public health concerns due to inappropriate treatments, leading to recrudescence and even drug resistance.^[19] Malaria control requires a high quality diagnostic method to detect the parasite before prescribing anti-malarial treatment following the WHO's guidelines. Malaria parasitological diagnosis targets treatment, supports characterization of the treatment response, and enables early identification of the parasite.^[20]

A total of 65 febrile patients with malaria based on clinical presentation were enrolled in the study and screened for Plasmodium parasites. Among the study participants, 96% (48) patients were malaria-positive by microscopy. Of those, 29% (14/48) and 71% (34/48), by Giemsa microscopy about 15 samples it was not possible to determine if were positive or

negative because the staining was not good and there was stain deposit. Nested PCR was used as reference method and 30% (15/50) participants tested positive by nested PCR. Significant differences were found among the positive and negative samples and in the different species detected when comparing the three diagnostic methods (Table 1).

Microscopy yielded a prevalence of 100%. This finding is disagree with result of nested PCR observed by Alemu *et al.*^[21] Microscopy is subjective and the result obtained is dependent on the quality of training of the observer. In this study, senior Laboratory technicians of malaria were recorded the result of Giemsa stained smear as being positive when all of microscopists recorded a positive result. The difference found by this study and that of Alemu *et al.*^[21] may be due to the subjective nature of results obtained by malaria microscopy and may be due to quality of Stain used.

Even though conventional microscopy is the reference method and the one most used for the diagnosis of Plasmodium spp, its sensitivity and specificity are limited to the number of tests that can be analyzed per microscopic and his/her training, especially for low-parasite densities, when more time is needed for an accurate diagnosis (2,13). These limitations could explain the false results obtained in the microscopic diagnosis of the endemic populations like Sudan. The study concerned about possible deficiencies of diagnosis by microscopy and RDT, in Sudan. SnM-PCR was used as the gold standard to compare the results obtained by Giemsa microscopy and a RDT. A false negative rate of 40% and 10% was found for microscopy and the RDT, respectively. Alemu *et al.*^[21] also used nested PCR as a reference technique in their study in the north of Gondar (Ethiopia), detecting a false negative rate of 13.1%. False negatives are a big public health problem because there is a part of the population that returns home without a correct diagnosis and treatment, not complying with the rule “fast and correct diagnosis, and treatment with confirmed presence of the parasite”., The use of rapid diagnostic tests (RDTs) based on histidine-rich protein 2 (HRP2) synthesized by Plasmodium falciparum has been widely advocated to save costs and to minimize inappropriate treatment of non-malarial febrile illnesses. HRP2-based RDTs are highly sensitive and stable; however, their specificity is a cause for concern, particularly in areas of intense malaria transmission due to persistence of HRP2 antigens from previous infections. The result obtained was come in agreement with Tarekegn A Abeku *et al.*^[22] who conclude that (RDTs may be effective when used in low endemicity situations, but high false positive error rates may occur in areas with moderately high transmission). Although RDTs

are used as diagnostic methods, diagnosis by microscopy should never be abandoned because it is the gold standard in endemic areas. In addition, microscopy allows the calculation of parasitic densities and identification of all species and is cheaper than the other methods. Although it is the best diagnostic method with high sensitivity and specificity, PCR is still costly and not very useful for routine diagnosis especially in developing and endemic countries.

CONCLUSIONS

The high number of false negatives in microscopy, it is necessary to reinforce training of laboratory staff on malaria microscopy, the “Gold Standard” in endemic areas. Taking into consideration the results obtained with the RDTs, an exhaustive study of the deletion of the *hrp2* gene must be done in Sudan to help choose the correct RDT for this area.

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