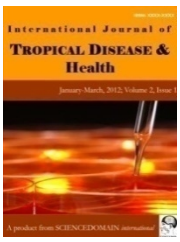

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Low Prevalence of *Plasmodium vivax - Plasmodium falciparum* Mixed - Infection in Patients from Central and Eastern Part of Sudan: Implication for Case Management in Sudan

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Authors' contributions

This research was conducted in collaboration between all authors. All authors involved in the study design, protocol writing, results interpretation and the final manuscript draft, reading and approval. Authors AAT, SZ and BYMN managed the field data collection. Authors AAT, SP and BYMN performed the laboratory techniques and the statistical analysis. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Accurate diagnosis of malaria parasite species is crucial for rational treatment that is a key success for a malaria control and elimination programmes. The main objective of this investigation was to correct species identification and re-assessment of diagnosis method in central and eastern part of Sudan. The blood samples were collected from 71 febrile

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cases infected with *P. vivax* in Eastern and Central Sudan, diagnosed by light microscopy and also by nested-PCR assay, using 18S small sub-unit ribosomal RNA (ssrRNA) gene. The nested-PCR were detect 92.9% (66/71) and 2.8% (2/71) *P. vivax* and *P. falciparum* mono-infection, respectively. Based on microscopy method, the level of mixed - Infection was zero; however, nested-PCR assay detected 4.2% (3/71) mixed infections in collected samples. In detecting *P. vivax* infection, microscopy had high sensitivity (97%) and specificity (50%). In conclusion, the present data point to the need of improving microscopy diagnosis method in malaria endemic region and also suggest that although molecular techniques are not practical for diagnosis of *P. vivax* and *P. falciparum* mixed infections in any areas; these could be used to collect epidemiological facts for control and elimination of the disease in Sudan.

Keywords: Malaria diagnosis; microscopy; nested PCR; mixed infection; Sudan.

1. INTRODUCTION

The Prompt and effective treatment of malaria remains a key intervention in reducing the burden of disease and death from malaria. The aims of this study consistent with the strategies of malaria control in Sudan that introduced by the National Malaria Control Programme (NMCP) to reduce the burden of malaria in Sudan. These strategy include many directions: Prompt and reliable diagnosis and effective treatment, effective prevention measures and detection and control of malaria epidemics. Sudan will ensure the availability of quality artemisinin-based combination antimalarial therapies as well as the effective antimalarial drugs for severe and complicated cases in public health care facilities [1]. The quality diagnostic services remain the major challenges of malaria diagnosis in control programme in Sudan; hence, the rapid and reliable diagnosis and effective treatment could help to interrupt the malaria transmission to reduce malaria burden in Sudan. In this case, we can prevent malaria epidemic due to mis-diagnosis of *P. vivax* and/or *P. falciparum* mainly in low parasitaemia and mixed infections.

Detection of malaria parasites by light microscopy of Giemsa-stained thick and thin films remains the standard laboratory method for the diagnosis of malaria. In Sudan and many other malaria endemic regions, there are problems and limitations associated with reliance on microscopic diagnosis of malaria for both *P. falciparum*, the main prevalence malaria species in Sudan, and *P. vivax* the most recently species that prevalence mainly in Eastern Sudan [2]. These limitations include lack of skilled microscopists, variation in individual training and/or experience, limited supply of microscopes and reagents as well as variation in equipment maintenance, and inadequate quality control. However microscopical diagnosis is still the main tool for the diagnosis of malaria. The nested-PCR has greater sensitivity and specificity than light microscopy [3,4], particularly in situations of low-level of parasitaemia [5]. Furthermore, it is a more powerful technique to detect mixed infections of malarial species [6]. However, it is an expensive technique which requires sophisticated laboratory and skillful personnel. Therefore the aims of this study was to re-assess malaria diagnosis using sensitive nested-PCR technique and also define the prevalence of *P. vivax* and *P. falciparum* mixed infections in the study area. These species cause a major global health problem with the broadest geographic distribution of the flive malaria species known to infect humans [7]. There are about 2.85 billion people at risk of malaria and an estimated 80 to 300 million clinical cases of *P. vivax* annually [7,8]. Although *P. vivax* is mainly endemic in Southeast Asia and Latin America [9] and Brazil Amazon Region [10] but, *P. vivax* was recently increased in Sudan and Ethiopia [11,12]. Malaria is an important health problem in

Sudan, and in 2002, an estimated 9 million disease episodes and 44,000 deaths from the disease occurred [12]. Therefore, in this investigation in view of uncertainty in the real malaria diagnosis and the level of mixed infection in Sudan the malaria diagnosis method (microscopy) in Central and Eastern Sudan was reassessed and compared with highly sensitive PCR-based diagnostic method to ensure early and rational treatment. This would facilitate malaria control and elimination programs for eliminating mortality, minimizing morbidity and reducing prevalence of the disease.

2. STUDY SITES AND BLOOD SAMPLE COLLECTION

This study was conducted in three health facilities in Central and Eastern Sudan, where closed to Ethiopia which *P. vivax* was high distributed [11]. Wad Medani Paediatric teaching hospital, Wad Medani teaching hospital, Elgariya ashara health center and Alkuawaty Kassala hospital,. Wad Medani town is located in Central Sudan in the western bank of the Blue Nile river, in 187Km south of Khartoum. Wad Medami is the main town of the irrigated area of Gezira Agricultural Scheme where malaria is stratified as mesoendemic to hyperendemic with unstable transmission pattern. In this areas, malaria occurs around the year with one main peak transmission, from August to December with *P. falciparum* as the predominant species in Gezira State (97%) and the second species are *P. vivax* estimated to be 3% , in Gedarif state the prevalence of *P. vivax* was 4.8% and *P. malariae* was 2.4% and in Kassala *P. vivax* represent 6.1% as found by malaria indicators survey [13].

All samples were collected from January 2012 to January 2013. In Sudan, the burden of malaria declined gradually over the last few years from 1,465,496 total cases in 2010 to 1,246,833 in 2011 [13]. Recently *P. vivax* infection is increasing in eastern Sudan as in Gezira state from 3% in 2012 to 5% in 2013 (Ministry of Health unpublished data). In this study, All samples were collected from the patients (aged from 4 to 70 years old) with *P. vivax* mono-infection (n.=71) with asexual parasitemia between 1200 and 72,800 parasites/μl. All *P. vivax* clinical isolates were diagnosed by light microscopic examination of Giemsa-stained blood smears.

Prior to the treatment, after cleaning a finger prick blood sample was collected onto the Whatman no. 3 (Whatman International, Ltd., Maidstone, England paper) and detection of Plasmodium species in all samples was performed by both microscopy and nested-PCR amplification. Patients with positive microscopy results were treated with a standard regimen according to Sudan malaria treatment policy [14].

3. MALARIA PARASITE DETECTION BY MICROSCOPY

Thick and thin blood smears were made from finger-prick samples, all the blood slides were air-dried, fixed in methanol and then stained with 1:10 dilution of Giemsa (pH 7.2) for 15–30 minutes. The stain was washed off with tap water. Further, the slides were read by local expert microscopists with the routine methods × 100 oil immersion lens at × 1000 magnification for at least 100 fields at the health centers in the study area. The estimation of the parasiteamia was calculated according to the formula that stated by WHO-basic malaria microscopy guideline.

4. PREPARATION OF DNA FROM FILTER PAPER

Briefly, genomic DNA of Plasmodium parasite was extracted from dried blood spot by using Tris-EDTA buffer-based extraction as described previously [15] and the DNA was kept at -20ºC until use.

5. DETECTION OF PARASITES BY NESTED-PCR ASSAY

Plasmodium species DNA (*P. vivax* and *P. falciparum*) were detected by nested-PCR amplification of the small sub-unit ribosomal ribonucleic acid (ssrRNA) genes using the primers and cycling parameters as described previously [6,16]. The positive controls were genomic DNA prepared from a patient infected with *P. vivax* and was confirmed with microscopy and molecular methods. For *P. falciparum*, DNA from the 3D7 strain of *P. falciparum* was used as positive control. Two negative controls were included in each set of amplification reactions (one was blank without DNA and the second was contain filter paper cut without blood). In order to prevent cross-contamination, different sets of pipettes and different work areas were used for template preparation, preparation of master mix for PCR and addition of template to the first and second nests and PCR assays. The nest 2 PCR for amplification of *P. vivax* gene were done according to [17] programme and the nest 2 PCR for amplification of *P. falciparum* gene were done according to [6,16,18] method.

6. RESULTS

6.1 Demographic Data of Patients

As indicated in (Table 1), 40/71 (56.3%) males and 31/71 (43.7%) females cases of P. vivax malaria, aged between 4 – 70 years attending to Wad Medani Paediatric teaching hospital and Wad Medani teaching hospital – Gezira state (n= 46), Elgaria ashara health center in Gedarif state (n=11), and Alkuawaty Kassala hospital in Kassala state (n=14) were enrolled in this study. All the patients had a fever, 15/71 (21.1%) headache, 7/71 (9.8%) back pain, 6/71 (8.4%) vomiting, 4/71(5.6%) has shivering and 2/71 (2.8%) were in coma. One of the patients with coma was a 5 years old child (with 72,800 parasite/µl) from Kassala state, who was died in hospital. The second patient was 40 years old from Gezira state with 17,710 parasite/µl. The past history of malaria infection among the enrolled cases ranged between 1 month to 5 years (Table 1).

6.2 Microscopy Results

In (Table 1), 71 thin and thick blood film from febrile cases showed positive *P. vivax* mono-infection and the parasitaemia ranged from 1,150 to 72,800 parasites/µl of blood with mean average of 12,990 parasites/µl of blood. Most of cases has different asexual stages of *P. vivax* from young trophozoite to schizont.

6.3 Nested- PCR Results

The nested-PCR were performed for amplification of small subunit of 18s ribosomal ribonucleic acid gene of both *P. vivax* and *P. falciparum* and the results showed that 66/71 (92.9%) mono- infection with *P. vivax,* 3/71 (4.2%) were mixed infection of *P. vivax* and P. falciparum. The three were from Gezira state (25 years old), Gedarif state (27 years old) and Kassala State (4 years old) with parasite count of 8500 parasite/µl, 24100 parasite/µl

and 2400 parasite/ µl respectively. (Fig. 1) while 2/71 (2.8%) were mono-infection with *P. falciparum.*

Table 1. Baseline characteristics of the enrolled cases infected with *P. vivax* malaria

Demographic characteristics	
Gender	40/71 (56.3%) males and 31/71 (43.7%) females
Age	between 4–70 years
Location	Gezira state: 46/71 (64.8%), Kassala State:14/71 (19.7%) Gedarif State: 11/71 (15.5%)
Past history of malaria	Ranged between 1month to 5 years
Temperature(oC)	Ranged from 37.5-39.8
Mean parasitaemia	12,990 parasites/µl of blood (ranging from 1,150 - 72,800)
Parasite stages	Different asexual stages: From young trophozoite to schizont in 71 cases

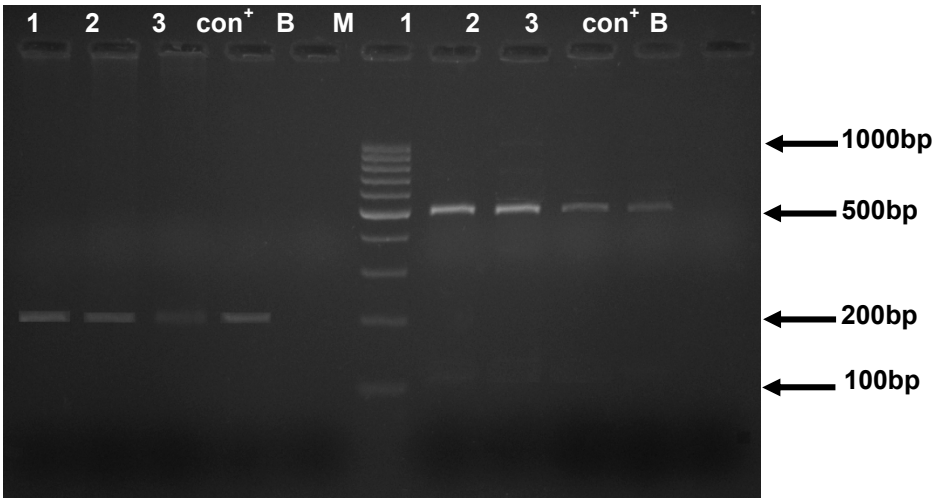

Fig. 1. Schematic representation of nested-PCR products for molecular diagnosis of Plasmodium species in Sudaness samples

Lane 1-3 are the *P. falciparum* positive samples . Lane con+ is for *P. falciparum* positive control, lane B is the negative control of PCR, Lane M is the 100 bp DNA ladder (Fermentas), lane 1-3 are the same samples that had the bands of P. vivax, Lane con+ is *P. vivax* positive control and lane B is for negative control PCR.

7. DISCUSSION

Malaria has been a major public health problem in Sudan and the quality of malaria diagnostic services remains the major challenges in control and elimination programme in this country. Therefore, a rapid, sensitive and reliable diagnosis followed by effective treatment could help to interrupt the malaria transmission and reduce malaria burden. To achieve these goals, all malarious countries share a common need for reliable laboratory diagnostic techniques to ensure early and rational treatment. However, transmission pattern

of malaria parasite could not be the same in each endemic area, rendering the need for providing precise data on transmission pattern, applicable in designing a more efficient control programme. Although light microscopy can characterize and quantify Plasmodium parasites rapidly and is the gold standard in control and elimination settings [19], the asymptomatic and mixed-infections are beyond its detective ability [20-22]. As a result, PCR has been introduced as the reliable and sensitive method for malaria diagnosis.

In the present study, based on microscopic method, the level of mixed - Infection reported from Sudanese malaria endemic areas was 0.2% in 2012 (Sudan, Federal Ministry of Health, unpublished data). However, highly sensitive PCR assays detected a much higher prevalence of coexisting mixed-species infection (4.2.%) than does examination with light microscopy. This suggests that a proportion of patients with microscopically confirmed *P. vivax* mono-infection in this region actually have subpatent *P. falciparum* parasitemia, which could be hampered preventive measures for accurate diagnosis of Plasmodium species and treatment of cases in these parts of the world. In addition, presence of the mixed - Infections in an individual person could be the cause of important alterations in the pathogenicity as showed in several studies, including in Ivory Coast [23], Sri Lanka [24] and Thailand [25]. In a study carried out in Vanuatu, *P. falciparum* appeared to be associated with a reduced *P. vivax* parasitaemia [26,27], but the reverse effect has been observed in another study [28].

Moreover, in the present investigation, the sensitivity and specificity of microscopy in the detection of *P. vivax* infection when compared with nested-PCR were 97% and 50%, respectively, that confirm previous studies [6,29 ,30]. Interestingly, the level of the mixed - Infections detected in the present study (4.2%) was similar to previous reported from Kingdom Saudi Arabia 2.4%, [29]. However, it was less than reported from Iran 22% [18], Pakistan 23.5%, [18], Afghanistan 6.5%, [18], Yemen 11.6%, [31], Bangladesh 27.5% [32] and from Brazil Amazon Regions 26.9% [10], but it was higher than reported from Ethiopia 2.3% [30]. Therefore, it should be noted that because of the specific therapy required both for *P. falciparum* and for radical cure of *P. vivax* the miss diagnosed parasite species may continue to serve as a source of infection for vector mosquitoes, complicating control measures.

The morphologic characteristics of malaria parasites by microscopy method can determine parasite species, but in this study, 2 of 71 cases (2.8%) of mono-infection of *P. falciparum* infection were detected by nested-PCR. The reason could be that under microscopy, at early stage the size of *P. falciparum* parasite resembles the size of *P. vivax*. Therefore, there is possibility that microscopists may occasionally fail to differentiate between species in cases when morphologic characteristics overlaps or in cases that parasite morphology has been altered by drug treatment, or improper storage of the sample and the blood films processes. As found by this study, the nested-PCR were able to detect more mixed - Infections that lead to prompt diagnosis and correct treatment.

8. CONCLUSION

In conclusion, the present data point to the need of improving microscopy diagnosis method in malaria endemic areas of Sudan and also suggest that the clinicians should have careful clinical observation, along with the reports on Giemsa-stained thick blood films. Miss-diagnose of the parasite species may hamper elimination efforts, since they are asymptomatic and would not be efficiently detected by conventional diagnosis methods. Although molecular techniques are not practical in rural areas for diagnosis of *P. vivax* and

P. falciparum mixed infections; These could be used to collect epidemiological facts for control and eliminate of the disease in Sudan.

In this regard, accurate detection of mixed - Infections is important for both clinical and epidemiological reasons.

However, additional comprehensive studies are needed, with a larger sample size collected from different malaria-endemic areas in Sudan and its neighboring countries, to determine the real pattern of Plasmodium transmission in this region. The result of this study could help the malaria control programme in Sudan to prevent the epidemic of both *P. vivax* or *P. falciparum* in this areas.

CONSENT

The participants were only enrolled in this study after a written informed consent was obtained from them or their guadians,

ETHICAL APPROVAL

The Ethical approval for this study was obtained from the Research Ethical Committee from the Gezira State Ministry of Health, and permission obtained from the local health authorities in the three states included in the study in Sudan, also ethical approval was obtained from the Rasearch Committee of Pasteur Research Institute of Iran.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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