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MOLECULAR DETECTION OF *PLASMODIUM VIVAX* ON DRY BLOOD SPOT IN EASTERN SUDAN

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Plasmodium vivax nowadays is emerging as one of the common causative species of malaria mainly in Sudan. Laboratory studies based on genomic approaches provide an alternative to identify the increased frequency of recurrent relapses of malaria infections and cases of low parasitemia such as *P. vivax*. The main objective of this study was to compare the performance of PCR and RDT to the gold standard diagnostics microscopy as a mean of detecting *Plasmodium vivax* parasites during active malaria. A total of 572 febrile patients were enrolled in the present study from Kassala, Halfa, and Eastern Nile area of Sudan. The sample was diagnosed by quality, insured microscopy, ICT (Immune−Chromatography Test) and PCR methods. The results indicated that the incidence of *P. vivax* infections among suspected malaria cases was relatively high. The total positive samples number of *P. vivax* by three methods was 164; while the three methods detected 71 (28.7%), 70 (28.3%), and 123 (38.8%), respectively.

The study findings indicated the changing *Plasmodium vivax* distribution pattern which seemly attributed to the recent demographic movement and high rate of immigration from neighboring countries to the east region in the recent years; ending with such rising trend of *P.vivax* malaria in eastern Sudan due to which management of the dormant hypnozoite stage when treating the cases of relapsing malaria. In conclusion, detection of *Plasmodium vivax* gene showed superior capability to identify cases of low parasitemia compared to the gold standard diagnostic microscope methods and reliable mean for adequate detection and primarily tool for eliminating *Plasmodium vivax* malaria.

Keywords: Malaria, *Plasmodium vivax*, Molecular ICT, Eastern Sudan

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Malaria is an infectious febrile disease of humans and other animal species; it's caused by *Plasmodium* parasites (Centers for Disease Control, 2015). According to the WHO estimates, there were about 228 million cases of malaria in 2018 in the world (WHO, 2019). Reduction of malaria mortality rates varied from 533 000 in 2010 to 380 000 in 2018 in the WHO African Region (WHO, 2019). Although malaria case incidence has fallen globally since 2010, the rate of decline has stalled and even reversed in some regions since 2014. Globally, the total malaria deaths reached 445 000 deaths, about the same number was reported in 2015. In WHO report 2016, 91 countries reported a total of 216 million cases of malaria, with an increase of 5 million cases over the previous year. The African Region continues to account for about 90% of malaria cases and deaths worldwidewhile fifteen countries – all but one in sub-Saharan Africa – carry 80% of the global malaria burden (Mbacham etal., 2019).

Plasmodium vivax is the most frequent and widely distributed cause of recurring (benign tertian) malaria. *P. vivax* is one of the five species of malaria parasites that commonly infect humans. It is less virulent than *Plasmodium falciparum*, the deadliest of the five, but *vivax* malaria can also lead to severe disease and death (Anstey et al., 2012).

Plasmodium vivax infection is becominga major health problem in Sudan. This parasite species has the broadest geographic distribution of the five malaria species known to infect humans (Guerra et al., 2009). There are about 2.5 billion people at risk of malaria and an estimated 80 to 300 million clinical cases of *P. vivax* annually. Although *P. vivax* is mainly endemic in Southeast Asia and Latin America, it has recently been observed in Ethiopia and Sudan (Mahgoub et al., 2012). However, in recent years many clinicians observed recurrent relapses of malaria infections in different areas in Sudan suggesting perhaps a higher than expected transmission of non–falciparum malaria parasites most likely *P. vivax* since it is the second most important malaria parasite species in Sudan. The objective of this study was to compare the reliability of the diagnostics methods for the detection of *P. vivax*and to recommend the best diagnostic option for detection this spices and co-infection.

MATERIALS AND METHODS

Ethical Considerations

The study was approved by the ethical research committee of the Faculty of Medicine, the University of Ribat, Khartoum, Sudan.

Study Area and Sample Collection

This was a cross-sectional study carried out in eastern area of Sudan. It is the region of Sudan lying to the west and south of Gedaref state to the Eritrean border. The area is considered mesoendemic for malaria; transmission follows mainly the vector breeding in the rainy season (July to OctoberWhole–blood samples were collected from patients with malaria – like symptoms, including fever and/or

chills, sweats, headaches, muscle pains, nausea and vomiting. About 3ml of venous blood samples were collected into an EDTA anticoagulant tube. Additionally, the venous peripheral blood was prepared as dried blood spots: two 50 µl aliquots of blood from the same patient were applied to filter paper Whatman Grade No. 3 (Whatman plc, Maidstone, UK), air-dried immediately, placed individually in sealed plastic bags and the specimens were transported for molecular detection by PCR in the National center for tropical medicine research,Department of molecular epidemiology.

Lab Diagnosis of Malaria

Thick and thin blood smears were made in the same slide and the rapid diagnostic test (ICT) were performed immediately.

The collected fresh blood samples were diagnosed for malaria using blood film microscopy and ICT and confirmed with PCR. Microscopic examination was performed on both thick and thin blood films, microscopic fields were read at least twice, and the procedure was followed according to quality control guidelines of WHO. PCR was performed for *P. vivax*with positive and negative control included. Genomic DNA was extracted from whole blood samples using Chelex method. A fragment of the plasmodial 18S rRNA gene with 121 bp size was amplified by PCR and species identification was performed with species-specific oligoprobes using the following *P. vivax*primers; rVIV1 (CGCTTCTAGCTTAATCCACAT AACTGATAC), and rVIV2 (ACTTCCAAGCCGAAGCAAAGA AAGTCCTTA), using the following PCR cycling steps: 95°C for 5 min. Initial denaturation, 94°C for 1 min. Denaturation, 64°C for 2 min. Annealing, 72°C for 2 min. Extension, according to the protocol adopted from Snounou, Singh (2002).

Statistical Analysis

Data were analyzed using SPSS (statistical package for the social sciences) version twentieth software.

RESULTS

The participant gender distribution in the study was as follows: more males were affected by malaria; however, the percentage of females was 53.1% while the percentage of males was 46.9%.

Comparison of the rapid diagnostic test (RDT) and polymerase chain reaction (PCR) with the microscopic gold-standard method demonstrated the following. Out of 572 samples, the total positive malaria patients revealed by microscopy in the three areas of the study resulted in 71 positive samples (12.4%) due to *Plasmodium vivax*, in different areas (Table 1). When the RDT was used, among total number of positive samples, 70(12.3 %) were positive for *Plasmodium vivax* (Table 1). On the other hand, among the total number of the positive samples revealed by polymerase chain reaction (*PCR),* 123(23.3%) were positive for *Plasmodium vivax* (Table 1). According to the method of gold standard microscopy, the fraction of *P. vivax* in Halfa, Kassala, and Eastern Nile constituted 12.4,14.1, and 10.9%, respectively (Table 2).

In the present study, males were found to be the gender more affected with malaria (53.1%).

Throughout the entire studied area, the fraction of *P. vivax* infections among suspected malaria cases was relatively high (about 38.8 % by PCR). This result is similar to that previously done in Aljabalain area located in the White Nile state in central Sudan. The most remarkable result in this study was the unexpected high proportion (about 40% by PCR) of *P. vivax* infections among suspected malaria cases, eight times more than that previously reported in Sudan (Makarim et al., 2016). These results suggest that the change in the infection pattern is most likely explained by the recent changed composition of the community resulting from several migrations of people from several Asian and African countries to work at petroleum and new sugar companies in White Nile area. This can be true especially for migrants from Ethiopia, where high prevalence of *P. vivax* infection (31%) among malaria cases was found (Lo et al., 2015).

The prevalence of *P. vivax* had been estimated in this work for three areas of study. The results showed that no statistically significant differences between the three areas of the study (P-value >0.05) were revealed. This comes with an agreement with a study performed in relation to the epidemiology and distribution of *Plasmodium vivax* malaria in Sudan, where the overall fraction of *P. vivax* among the malaria cases constituted 26.6% (Amandaet al., 2017). The prevalence showed significant variations between the states ($p<0.001$), which could be explained by differences in population movement, the presence of refugees, and proximity to *Plasmodium vivax* endemic neighboring countries. It also varied significantly with residence status ($p<0.001$), reflecting the stability of transmission (Amanda et al., 2017).

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 as in Sudan. The present study compared microscopy and ICT with PCR. It was found that results obtained by PCR method were superior to those obtained by microscopy. Sensitivity of microscopy, ICT and PCR were evaluated in this study in order to determine the most sensitive method that detects more positive cases. The result proved that PCR was the most sensitive technique (detected 47.0% of the total positive samples) (P-value <0.001). These results are in agreement with many studies done worldwide for different sero-prevalence studies comparing the sensitivity of different techniques.

As diagnostic resources are limited in the study area, without a reference laboratory, the gold standard microscopy remains the reliable, affordable and applicable laboratory method for diagnosing malaria. PCR diagnosis for malaria is accurate especially for differentiating between plasmodia species, but it is more expensive and needs well-trained personnel.

Table 1. Prevalence of P. vivax infections detected in malaria cases

Detection	Microscopy, %	RDT, %	PCR, %
P. vivax	71(12.4)	70(12.2)	123(21.5)
Negative	501(87.6)	502 (87.8)	449 (78.5)

Table 2. Prevalence of *P. vivax* infections detected by microscopy from different sites

Sites	*P. vivax*	
	Positive *n*, %	Negative *n*, %
Halfa	24(12.4)	170(87.6)
Kassala	28(14.1)	170(85.9)
Eastern Nile	19(10.9)	161(89.4)
Total	71(12.4)	501(87.6)

In all the cases, *P*-value<0.05 statistical significantly different.

Currently, the Sudanese National Malaria Control Program recommends the use of RDT in those settings where no expert microscopy is available, and maintains microscopic examination in those places where microscopy is of an adequate level (Elmardi et al., 2009). This RDT strategy was investigated earlier in Sudan for the home management of malaria using artemisinin-based combination therapy (Elmardi et al., 2009). This is in agreement with a study performed at Gadarif teaching hospital in eastern Sudan. Based on the findings of his study, it appears likely that implementation of malaria RDT in Sudan in settings where microscopic expertise is available should not be recommended (Awadalla et al., 2013).

CONCLUSION

We can conclude that *Plasmodium vivax* malaria remains a major public health problem in eastern Sudan. The possibility of low parisitemia infections is increasing and seems to be more prevalent in future. PCR detects more cases than has been revealed by microscopy, while RDT reveals similar cases of malaria parasitic infections. The results indicate the superior capacity of PCR in detection of more cases and raise queries about the possibility of asymptomatic carrier, recurrent infections, or presence of drug resistance of local or newly imported resistant strains.

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Conflict of interests: all authors declare no conflict of interest.

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ВЫЯВЛЕНИЕ PLASMODIUM VIVAX В ПЯТНАХ СУХОЙ КРОВИ МОЛЕКУЛЯРНЫМИ МЕТОДАМИ

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Ключевые слова: малярия, *Plasmodium vivax*, молекулярные методы, Восточный Судан

РЕЗЮМЕ

В настоящее время *Plasmodium vivax* становится одним из самых распространенных возбудителей малярии в Судане. Лабораторные исследования, основанные на геномных подходах, служат альтернативой при изучении возросшей частоты повторных рецидивов малярийных инфекций и случаев пониженной паразитемии, наблюдаемых у *P. vivax*. Целью настоящей работы было сравнение методов ПЦР и RDT (rapid diagnostic test) со стандартными методами светооптической диагностики *Plasmodium vivax*. Были исследованы 572 пациента с явно выраженными признаками лихорадки из Кассалы, Халфы и территории Восточного Нила (Судан). Было проведено сравнение стандартных методов с методами иммунной хроматографии и методами ПЦР. Результаты показали, что заражение *P. vivax* среди всех обнаруженных случаев малярии было относительно высоким. Общее количество положительных реакций на *P. vivax* всеми методами составило 164, при этом различные методики определили 71(28.7%), 70 (28.3%) и 23 (38.8%).

В нашем исследовании были обнаружены изменения в характере распространения *Plasmodium vivax* в Судане, что, возможно, объясняется последними демографическими изменениями. Эти изменения связаны с эмиграцией в Судан жителей соседних африканских стран, усилившейся в последние годы. Определение гипнозоитов, покоящейся стадии малярийного плазмодия, является насущной задачей в выявлении рецидивов малярии. Наше исследование показало, что выявление генов *Plasmodium viva* продемонстрировало свое преимущество при определении плазмодия при низких уровнях паразитемии в сравнении со стандартными светооптическими методами и является адекватным методом для выявления и последующей ликвидации малярии.