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# Molecular Screening for Transfusion Transmissible *P. falciparum* in Asymptomatic Blood Donors in the Non-endemic Region

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## Abstract

Transfusion-transmitted malaria remains a critical problem for blood banks due to a symptomatic of Plasmodium infection. Malaria screening is not listed in the blood-borne pathogens screening protocol in Sudan, and the test of malaria is not routine in the blood banks for donors. We aimed to detect *Plasmodium falciparum* among asymptomatic blood donors in a blood bank in Sudan –Khartoum. 450 blood samples were collected and screened using microscopy, ICT, and PCR. Using the Guanidine Hydrochloride method, the genetic agent was extracted from the samples.

All examined samples were negative by microscopy and PCR for the 18SssrRNA *Plasmodium falciparum* gene. All donors were Sudanese males, and their ages ranged between 20-49 years old with a mean 31 ±1.45 SD. While 8 (4%) were positive by ICT, the majority of positive subjects 5 (1.1%) were in the age group between 20-29 years old, however, 192 (42.7%) of donors were in the age group 40- 49. Concerning residence, 424 (94.2%) of the participants were from Khartoum state, and 15 (3.3%) from the state of Gaziera; where one-third of them 5 (1.1%) were positive by ICT, the result was insignificant (P value=0.09). The prevalence rate of transfusion transmission malaria is 0%, although all results are negative, this does not prevent transmitted malaria through blood donors. Although the control and preventive measure for blood transfusion biosafety in Khartoum blood banks are valid and adequate, and a molecular diagnosis should run besides routine blood smear.

**Keywords:** Blood donation, Asymptomatic, PCR, ICT, *Plasmodium falciparum*

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## Introduction

As a form of medical treatment, blood transfusion is utilized in managing emergency cases like surgeries, injuries, and other conditions that are life-threatening (Farah *et al.*, 2019). It may be a potential risk for transmission of blood-borne infections even though it is a lifesaving procedure (Cheesbrough, 2006). Individuals infected with malaria carry malaria parasites in their blood and could be contacted from the blood of an infected person through blood transfusion (as *Plasmodium species* can remain viable in blood for at least 18 days when stored at +4 °C). Also, sharing syringes or needles that are contaminated with blood can be a source of infection (Di Minno *et al.*, 2016).

Annually, malaria has causes millions of morbidities and several deaths in developing countries. In 2015, the most recent World Malaria Report showed 212 million new cases of malaria worldwide, with 90% of the cases from Sub-Saharan Africa. There were an estimated 429,000 malaria deaths worldwide that same year (WHO, 2016). In Tanzania, morbidity and mortality are mainly caused by malaria. The results of a population-based survey that was done recently showed a 9% malaria prevalence among children – with Lake Victoria Regions of Geita and Kagera being the highest (TDH-MIS, 2016). Through blood transfusion, all human malaria parasites *(P. vivax, P. ovale, P. malariae, and Plasmodium falciparum*) can be transmitted. The World Health Organization (WHO) recommends the blood collected for transfusion to be screened for the presence of *Hepatitis B Virus (*HBV*), Hepatitis C Viru*s (HBV), Human Immunodeficiency Virus (HIV) and Syphilis to make sure that there is blood safety before the transfusion (Muerhoff *et al.*, 2010). Also, it is advised to screening for blood-borne parasites (BBP) like malaria before transfusion in malaria-endemic areas. Even though malaria is an endemic in Africa, most centers for blood transfusion do not screen for malaria parasites routinely (Schindler *et al.*, 2019). Moreover, the blood donated has been used for managing pregnancy-related anemia, and also, children in need. Contrary to this, it has been documented that malaria is one of the main causes of deaths among under-fives and adverse poor pregnancy outcomes in most of the resource-limited countries. This raises concern of how to control malaria and its associated adverse outcomes in a resource-limited setting. The results of previous studies done in developing countries revealed that there was a prevalence of 26.5% and 1.5% among male and female blood donors, respectively (Agboola *et al.*, 2010; Oladeinde *et al.*, 2014; Olawumi *et al.*, 2015; Wariso &

Oboro, 2015). Hence, the current study aimed to detect the molecular screen, asymptomatic blood donors in the blood bank of Khartoum, Sudan, for malaria parasites using immunochromatography and molecular techniques, as well as to evaluate two diagnostic tests: rapid diagnostic test (RDT) and molecular technique for detecting malaria parasite information, which may be important in suggesting possible interventions like introducing a policy of screening donated blood for malaria parasites

## Materials and Methods

*Study Design*

An analytical cross-sectional study was conducted in a central blood bank in Khartoum state (STAK). The study was conducted on male volunteers randomly attending the blood bank of Khartoum in the period between March 2020 to July 2020, their age ranged between (18-50), and they enrolled in the study according to the following inclusion criteria: No prior malaria attacks and no signs and symptoms of malaria were noted during the interview. A total of 450 volunteers who donated their blood to participate in the study were screened using standard blood bank protocols routinely performed that included a thick blood film technique.

*Sample Collection and Sampling Techniques*

Sampling was carried out using a non-probability sampling technique (namely convenious sampling method, we collected 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 reminder 2 ml was stored in -80 for PCR, and a survey questionnaire was used for the collection of demographic data.

*Microscopy*

Thick and thin slides were made from peripheral blood slides. After immediate collection, specimens were made on a clean, grease-free microscope slide and allowed to air dry. The films were stained with 10% Giemsa solution (Appichem, Panreac ITW Company) for 10 min and allowed to air dry. Using an oil immersion objective lens, they were subsequently examined by light microscopy. All slides were examined by microscopists and checked by senior lab specialists.

*Rapid Diagnostic Test*

The pouch was opened and the device was removed. Once opened, the device was used immediately. A large drop of blood was dispensed and four drops of diluents buffer were added into each well. After 15 minutes, the results were read and the Negative for *P. falciparum malaria*: only one pink-colored line appears in the control window (C). While, the Positive for *P. falciparum malaria* the district pink colored line, in addition to the control line, appears in the test window (T). The test should be considered invalid if no line appears or if no pink line in the control line. Repeat the test with a new device. The test was interpreted after 15 minutes.

*DNA Extraction*

2 ml of blood was transferred to a 15 ml falcon tube and completed volume until 14 ml by red cell lysis buffer (RCLB) (ammonium chloride (NH4Cl), sodium bicarbonate, 5% EDTA). it was well mixed and vortex and then spinet for 10 min at 3000 rpm in a centrifuge to pellet the white blood cells. And discarded the supernatant and repeated this step three times until clearing the pellet, then added 2ml of Wbcl or NLB (Tir, Sodium chloride (NaCl), EDTA. 10% SDS).

+ 1ml of Quinidine (extraction DNA) vortex +200_300uL of ammonium acetate and added 5ul of protease K (reduce contamination), and left to digest overnight at 37°C.in the incubator. Added 1ml of cold chloroform then well vortex and centrifuged at 2.5 rpm for 5 min. The upper layer containing the DNA was taken and put in a falcon tube, 4 ml of ice-cold absolute ethanol was added. The tube was left over the night at-20°C. The DNA was precipitated at high-speed rpm for 10 min. The supernatant was discarded and then the pellet was washed with 70% ethanol at centrifuged rpm for 10 min. The supernatant was discarded. The pellet was left to dry for 30 min at room temperature and then was suspended in 50 uL and added to water for injection and left at 4C° for 24 hrs. to dissolve. PCR is the amplification or replication of DNA to increase the sensitivity and specificity and detection of species of the parasite according to specific primers. For amplification reaction, the total volume of 20ul of master mix (Maxime PCR Permix Kit (i-taq) with1ul of primer rplu5 (5-CCTGTTGTTGCCTTAAACTTC-3), and 1ul of primer rplu 6 (5-TTAAAATTGTTGCAG-3), and 18 ml of DW was added then vortex and centrifuged, finally take 10 ul of the mixture and 1ul of the DNA template and vortex to enter the PCR machine, PCR programs were summarized in **Tables 1 and 2**.

**Table 1.** Program Rplu outer

| Step1 | Temp C | Time | Cycle |
|---|---|---|---|
| Step2 | 94 | 2 minutes | |
| Step3 | 94 | 30 seconds | 40 cycle |
| Step4 | 55 | 1 minute | 40 cycle |
| Step5 | 72 | 1 minute | 40 cycle |
| Step6 | 58 | 2 minutes | |
| Step5 | 72 | 2 minutes | |

*Nested 2 (inner)*

10 ul of mixture (master mix, 1ul primer fal1 (5-TTAAACTGGTTTGGGAAAACCAAATATATT-3), 1ul primer fal2 (5-ACACAATGAACTCAATCATGATACCCGTC-3), 18ul DW) and add 1ul of PCR product of nested 1 (outer).

**Table 2.** Program Fal nested

| Step1 | Temp C | Time | Cycle |
|---|---|---|---|
| Step2 | 94 | 2 minutes | |

| | | | |
|---|---|---|---|
| Step3 | 94 | 30 seconds | 40 cycle |
| Step4 | 58 | 1 minute | 40 cycle |
| Step5 | 72 | 1 minute | 40 cycle |
| Step6 | 58 | 2 minutes | |
| Step5 | 72 | 2 minutes | |

*Data Analysis*

Data concerning each subject were registered in a separate row in the statistical package program for Social science (SPSS).

*Ethical Consideration*

Ethical approval was obtained from Al-Neelain University and the ministry of health, Al Khartoum state.

## Results and Discussion

Out of 450 blood samples of apparently healthy volunteers were screened for malaria parasites using microscopy, rapid diagnostic test, and PCR in Khartoum central blood bank, all donors were Sudanese males, and their age ranged between 20-49 years old with a mean 31±1.45 SD. All samples examined by microscopy and PCR were insignificantly negative for the 18SssrRNA *Plasmodium falciparum* gene. While 8 (4%) were positive by ICT, the majority of positive subjects 5 (1.1%) were in the age group between 20-29 years old, however, 192 (42.7%) of donors were in the age group 40- 49. Concerning residence, 424 (94.2%) of the participants were from Khartoum state, and 15 (3.3%) from the state of Gaziera; where one-third of them 5 (1.1%) were positive by ICT and the result was insignificant (P value=0.09), all data summarized in **Table 3**. **Figure 1** displays nested PCR results of the 18S ssrRNA *Plasmodium falciparum* gene. The initial PCR results are negative for all samples**.**

**Table 3.** Results of ICT among donors according to their age and residents

| **Parameters** | **ICT n=450 (%)** | | **Total** |
|---|---|---|---|
| **Age Group** | **Positive n=8 (%)** | **Negative n=442 (%)** | |
| 20-29 Years | 5 (1.1%) | 140 (31.1%) | 145 (32.2%) |
| 30- 39 Years | 3 (0.7%) | 110 (24.4%) | 113(25.1%) |
| 40- 49 Years | 0 (0%) | 192 (42.7%) | 192 (42.7%) |
| Total | 8 (1.8%) | 442 (98.2%) | 450 (100%) |
| Mean ±SD | | 31 ±1.45 | |
| **P-value** | | 0.849 | |
| **Resident** | | | |
| Khartoum state | 2 (0.44%) | 422 (93.7%) | 424 (94.2%) |
| Gazeria state | 5 (1.11%) | 10 (2.2%) | 15 (3.3%) |
| Northern state | 0 | 4 (0.9%) | 4 (0.9%) |
| Western sate | 0 | 4 (0.9%) | 4 (0.9%) |
| Eastern state | 1 (0.22%) | 2 (0.4%) | 3 (0.7%) |
| Total | 8 (1.8%) | 442 (98.2%) | 450 (100%) |
| **P-value** | | 0.098 | |

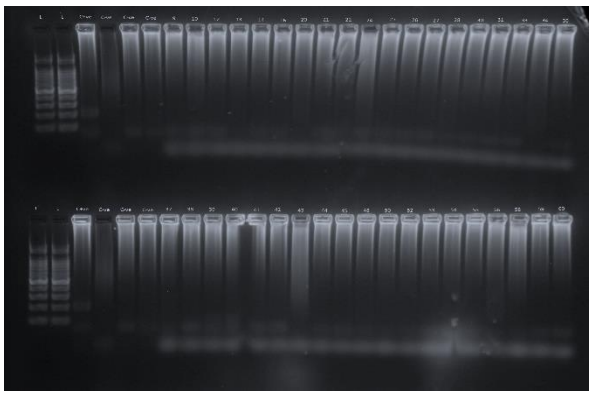

**Figure 1.** Nested PCR results for the 18S ssrRNA *Plasmodium falciparum* gene. The initial PCR results are negative for all samples

Malaria was among the first transfusion-borne infections documented and has been one of the most prevalent infections in endemic countries. So, the differentiation of natural from transfusion-borne infections is quite challenging in endemic rejoins. The current study aimed to detect malaria parasites in blood donated by healthy donors who did not manifest clinical signs of malaria in blood banks of Sudan because there is no advanced technology for detecting malaria in the volunteer. All participants recruited in this study were male, and they did not have clinical features of malaria infection (Asymptomatic). Subjects enrolled were from five states in Sudan where the majority of them from Khartoum state, followed by Gaziera state.

The study revealed that all 450 blood samples were negative for malaria parasite by microscopy (gold standard method), and by nested PCR. However, eight samples were positive by ICT, almost all of them from Gazira state 5 (1.11%). It should be noted that donors from such regions with autochthonous malaria have consideration for only a tiny fraction of the blood donor population as well as in practice, so the probability of autochthonous malaria is diluted by the majority. Our finding is acceptable when compared to the study done in 2012 by Noor *et al.* (2012) for the malaria indicator survey, which stated that regions on the boundaries of the Desert Edge considered as low stable endemic control (PfPR2–10 <1%) represent plenty of the northern, Red Sea, Nile and Khartoum states, and large areas of the states further south cover 8.2 million or 26.5% of the total population in 2011. While the hypoendemic class ≥ 1 but < 10 % of PfPR $_{2–10}$ has been estimated along with northern Darfur, northern Kordofan, the White Nile, Gezira, Kassalah, and parts of the southern states, representing 3.4 million people and 11% of the population in 2011, the mesoendemic category with pockets of hyperendemicity is chiefly found in regions between the 12° latitude and the boundary with South Sudan where is resident by approximately 3.4 million citizens.

Parasites that are under the microscopy threshold level can be detected by the PCR technique (0.004 to 1 parasite/ml of blood). However, the result directly depends on the reagents quality and the genetic material quality (DNA) of the parasite, which is gotten during extraction and amplification. More so, a review identified

both the varying complexity between malaria diagnosis and the usefulness and subjectivity of using a molecular diagnostic methodological approach in clinical and epidemiological malaria studies. The test requires a long analysis time and extensive training but it is costly, still, its sensitivity and specificity are considerable (Bharti *et al.*, 2009; Taylor *et al.*, 2010).

Our findings agreed with the study of Bakr *et al.* (2017) in Fayoum, Egypt, which revealed that all 400 blood samples were negative. Nevertheless, a conflict was found in the study done by Maselli *et al.* (2014) who revealed the subclinical malaria infection as the prevalence of *P. falciparum and P. vivax* was 5.14 and 2.26, respectively.

Siles *et al.* (2020) screened 200 donors of the blood bank of Khartoum state for malaria, their results revealed malaria parasites were detected in 6% by microscopic examination, 6.5 % by immunochromatographic test, and 18.5 by molecular technique, with variable detection sensitivity between the three different techniques. As we previously mentioned, their findings may be attributed to the endemicity of the area, the difference in transmission rate during the season of collection, the type of techniques used, and sample size (Mustafa Ali *et al.*, 2015).

Regarding eight donors who were positive by ICT and negative by PCR, this may be attributed to false-positive results by the remaining circulating antibodies after parasite clearance, as the majority of false-positive results were from a stable malaria transmission region, and it implies a high degree of exposure of this population, comprised of semi-immune inhabitants in regions where asymptomatic infections have been identified. Nevertheless, false-positive results by ICT may be observed due to cross-reaction which frequently occurs in patients with hepatitis C, rheumatoid factor (RF), schistosomiasis, Chagas disease, dengue, leishmaniasis, human African trypanosomiasis, and toxoplasmosis (Lee *et al.*, 2014). The precise mechanism underneath RF's reaction to malaria RDTs have still not been completely understood, one potential interpretation for the false-positive noted in malaria RDTs. However, is that there is a reaction between RF and specific antibodies in malaria RDT strips (Garcia, 2016). Our findings agreed with Abeku *et al.* (2008) and Elsheikh *et al.* (2019), who conclusively proved that while RDTs could be appropriate for low endemicity settings, high false-positive error rates may exist in places with moderately high transmission. Therefore, stained thick and thin blood films microscopy is still the gold standard method for malaria diagnosis; since we allow reliable, rapid, and convince techniques that permit distinguishing characteristics of the four major parasite species (Valones *et al.*, 2009; Iwuafor *et al.* 2016). Hence, our findings illustrate the importance of testing donors for malaria in transfusion services using microscopy and PCR techniques.

Fortunately, the prevalence of malaria is (0%), this changeover may be probably partially due to a significant increase in malaria intervention strategies (Since 2004, approximately 12 million long-term insecticidal nets (LLINs) were already distributed across the country, and by 2009 over than 40% of households owned at least one LLIN) in Sudan throughout the last decade.

## Conclusion

The prevalence rate of transfusion-transmitted malaria is 0%, although the results are negative, this does not prevent transmitted malaria through blood donors. Although the control and preventive measure for blood transfusion biosafety in Khartoum blood banks are valid and adequate, and a molecular diagnosis should run besides routine blood smear. Because the hazard of transmitting malaria through blood transfusion is much greater than the cost of testing donors.

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**Conflict of interest:** None

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**Ethics statement:** The study has been approved by the Ethics committee of the College of Medical Laboratory Sciences, Al-Neelain University, Khartoum, Sudan (Ethics code: AU/FM/EC. 87).

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