Original Article

Prevalence of Plasmodium Falciparum Gametocytes among Malaria Positive Cases at Korogwe District Hospital: The Use of Molecular Techniques in Comparison with Light Microscopy

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Abstract

Background
Monitoring gametocytes in the population can inform about the human infective reservoir, which greatly aids malaria transmission, and provide relevant data for transmission models. Using molecular techniques in preference to light microscopy to detect gametocytes may lead to most reliable results. Effective determination of gametocytes is inevitable to achieve the transmission-blocking interventions as a prime target to end malaria. We aimed to determine the prevalence of P. falciparum gametocytes in malaria-positive cases from Korogwe district hospital

Methodology
Archived DNA samples collected from Korogwe district hospital collected in a cross-sectional study were used to determine the prevalence of P. falciparum gametocytes using specific primers for cPCR targeting a Pfg27 gene. Demographic data, including blood slides data were retrieved from the database for statistical analysis.

Results
With light microscopy, prevalence of P. falciparum gametocytes was 9.8%; sensitivity and specificity were 35.6% and 99.2% respectively. The cPCR gave a prevalence of 25.9%, with a sensitivity of 94.1% and specificity of 81.5%. The cPCR was diagnostically found to be significantly superior over light microscopy technique (X^2=45.780, $P < 0.001$).

Conclusion
cPCR is superior to light microscopy technique in detecting P. falciparum gametocytes when one considers a successive malaria transmission-blocking intervention.

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Keywords: Pfg27 , Conventional PCR , Light microscopy , Pf gametocytes

Introduction

Malaria is transmitted through the bites of female Anopheles mosquitoes infected with Plasmodium falciparum, which is the most prevalent species in sub-Saharan Africa.[1] In 2018, Plasmodium falciparum accounted for 99.7% of the estimated malaria cases in the WHO African region, 50% in the WHO south East Asia region, 71% of cases in the Eastern Mediterranean, and 65% in the Western Pacific.[2] Infection by Plasmodium falciparum can result in asymptomatic carriage, uncomplicated or severe malaria.[3] Therefore, in-depth understanding on the survival and replication of Plasmodium falciparum is crucial to fighting malaria in Africa and particularly in sub-Saharan Africa. Whereas the development cycle of the Plasmodium falciparum contains asexual and sexual stages, the sexual stage is the most important stage in malaria transmission. Gametocytes are the sexual precursor cells of the malaria parasite that mediate the transmission of the parasite from its mammalian host to the Anopheles mosquito.[4]

According to the WHO Malaria report of 2021, about 241 million cases were reported from 85 endemic countries globally. In 2019, nearly half of the World's population was at risk of malaria,[1] with the low- and middle-income countries greatly bearing the most burden. Sub-Saharan Africa alone possesses about 95% of the global burden.[5] There has been a subsequent decline of malaria in many places over the past decade but still, deaths due to malaria remain high with over 400,000 annually. In 2020 malaria deaths rose to 627,000 with an increase of 12% in deaths due to severe disruptions caused by the COVID-19 pandemic.[1] In Tanzania, 10 to 12 million people contract malaria annually. The current situation of malaria compels the need for more studies to block the transmission models so that the communities can effectively attain the goal of zero malaria.[6]

There are several techniques to detect gametocytes in the population studies such as light microscopy, magnetic fractionation, and molecular techniques. Light microscopy has been the most commonly used technique in the clinical settings to detect gametocytes. Unfortunately, there has been a great underreporting of the presence of gametocytes among malaria positive cases while malaria continues to spread among people across the communities.[7] Models, which include only moderate to high gametocytemia detectable by light microscopy, predict finite eradication times after the introduction of LLIN. Models that include low gametocytaemia reservoir requiring polymerase chain reaction or magnetic fractionation detection, predict much more stable and persistent transmission.[8]
A study conducted in Malawi reported that the prevalence of gametocyte carriage by molecular testing was 3.5% during the dry season and 8.6% during the rainy season, and by microscopy 0.8% and 3.3%, respectively among school-age children. Age and housing conditions were highly associated with high odds of gametocytes carriage.[9] Another study conducted in Kimwanga district of Bagamoyo Tanzania noted that the higher gametocytaemia observed in asymptomatic children indicates the reservoir infections and points to the need for detection and treatment of both asymptomatic and symptomatic malaria.[10]

This study utilized archived samples of malaria cases from Korogwe district hospital in Tanga region where malaria prevalence is 3.1%,[11] to detect Plasmodium falciparum gametocytes using molecular techniques targeting Pfg27 protein gene that is essential for gametocytes production. Furthermore, light microscopy technique was conducted to allow comparison between the two techniques. The study also supported and consolidated findings from other studies conducted in malaria-endemic countries including Tanzania, Malawi, Ghana, and Ethiopia.

Proper understanding of these processes deepens our knowledge on gametocytes and malaria transmission models hence opening up a way towards the development and strengthening of malaria transmission-blocking intervention strategies.[4]

Methodology

Study site and Population

The cross-sectional study was conducted in Korogwe district hospital in Tanga region, Tanzania. Malaria is one of the main causes of death in Korogwe.[13] Korogwe district is a town with a population of 242,038 according to 2012 national census. Korogwe district hospital where the samples were obtained is located at the center of Korogwe district. Children aged 2 to 10 years had been used in the previous study to collect samples for microscopy and molecular techniques. This study utilized secondary data of results from the previous work for the microscopy and a molecular technique known as the Malaria Rapid Diagnostic Test (MRDT). For the molecular technique known as conventional PCR, samples stored in the form of DNA from a previous study on the same population were used. The sample were previous collected from Kororgwe District Hospital and stored at -20oC. During the initiation of this study, samples were transported to Kilimanjaro Clinical Research Institute (KCRI) Biotechnology laboratory and stored at the same storage temperature (-20oC). All the data and samples with enough clinical information required for this study were conveniently selected and retrieved.

Inclusion and exclusion Criteria

All samples previously collected from children aged between 2 to 10 years and archived at the Korogwe district hospital laboratory and later transported to KCRI with enough demographic information were used for this study. These samples were checked for malaria by using the MRDT. Any sample with some missing information and all samples from older children (>10years) were excluded.

Laboratory procedures

Malaria Rapid diagnostic tests and Microscopy done in the previous study

All blood samples obtained from study participants in Korogwe district hospital had been investigated for malaria following malaria diagnosis algorithm firstly by MRDTs kits (MRDTs_SD Bioline Malaria Ag Pf, Abbott Diagnostics Korea Inc., lot 05FK50). The data of the results of this part, which was done in the previous study, was used as inclusion, and exclusion criteria as earlier explained. Blood smears from cases confirmed to be malaria positive on MRDT had been prepared and examined on microscope under x100 high power resolution lens with oil emulsion. The blood smears had been examined for sexual and asexual stages of Plasmodium falciparum and reported (parasites/200WBCs or parasites/500WBC); the microscope techniques had been performed by two expert laboratory technician, qualified and certified by the board of Tanzania Medical Health laboratory Practisionals. Parasites had been reported as per parasite quantification protocol (P/ul).[1]

Molecular techniques (Conventional PCR)

For this study, DNA samples were retrieved from the -20C freezers, thawed and quality checked for degradation/fragmentation by nanodrog machine using 1ul from the total volume. DNA were amplified using conventional PCR under the following procedures: -

Heat stable DNA polymerase (AHPolHS-ready to load 7.5mM AZ21.0075.1 5x master mix) and Pfg27specific primers together with other PCR components were used.

More briefly: each sample contained: - 2µL of AHpol (DNA polymerase), 1.6µL primer mix (Forward primer: 5 ′ - C G G G G T A C C A T G A G T A A G G T A C A A A A G 3′ and Reverse primer: 5′-CGGGGTACCGTAGTCTTCCGTAACTTC 3′ and 5.4 µL of DNase/RNase free water (VWR Life science, Molecular Biology Grad . 17F275304) were mixed making a total of 9µL.

The master mix was vortexed, and 9μL of master mix were added to each well on PCR plate (Enduraplate optical 96wellss plate clear reaction_Applied biosystem-Life technology. Lot. I04A9Q714) and 2μL of DNA samples were then added to the wells making a total reaction volume of 11μL per sample. The PCR plates were sealed with sealing films and processed in conventional PCR machine (SimpliAmp_Thermocycler AppliedBiosystems_Thermofisher Scientific) for amplification with the following conditions. Initial denaturation at 95˚C for 15 minutes one cycle, followed by second denaturation at 95˚C for 20 seconds, annealing at 54˚C for 2 minutes, Extension at 72˚C for 2 minutes (all were run for 35 cycles). Final extension at 72˚C for 10 minutes one cycle.

Gel Electrophoresis
For detection of the PCR positive Plasmodium falciparum gametocytes, the gel was prepared from 1X TBE buffer using agarose powder (Molecular grade-Bioline, Cat no. BIO-41025, USA). 3μl of the sample was loaded in a gel, alongside a ladder marker (Gene ruler 100bp, Thermofisher Scientific_molecular biology_00867370), and allowed to run at 100V for 1hour. Gel visualization was done by a gel doc machine (Vilber Bioprint).

Statistical Analysis
Data cleaning and analysis were performed by IBM SPSS Statistics for Windows version 26.0 (IBM Corp, Armonk, NY, USA). Sensitivity of both techniques were calculated to allow comparison. Data from parent study was retrieved and used to allow for further comparison. Descriptive statistics was used to summarize the data; Chi square test was computed to make comparison of the two techniques.

Results

General characteristics of participants
A total of 174 participants were enrolled in the study, of whom 51.1% (89) and 48.9% (85) were male and female children respectively. The children < 5 years comprised 43.7% (76), and 56.3% (98) were > 5 years.
Participants who had fever were 94.3% (164); and majority of them were positively diagnosed with malaria infection. Hemoglobin level was found to be normal in 59.8(104), moderate in 21.3% (37) and severely low in 19% (33) of the study participants. Of all participants, 83.3% (145) were bed net users, 69% (120) were malaria positive and 31% (54) were malaria negative by MRDT while 43.7% (76) and 56.3% (98) were positive and negative respectively by microscopy technique (Table 1).

Table 1. Characteristics of study participants (N=174)

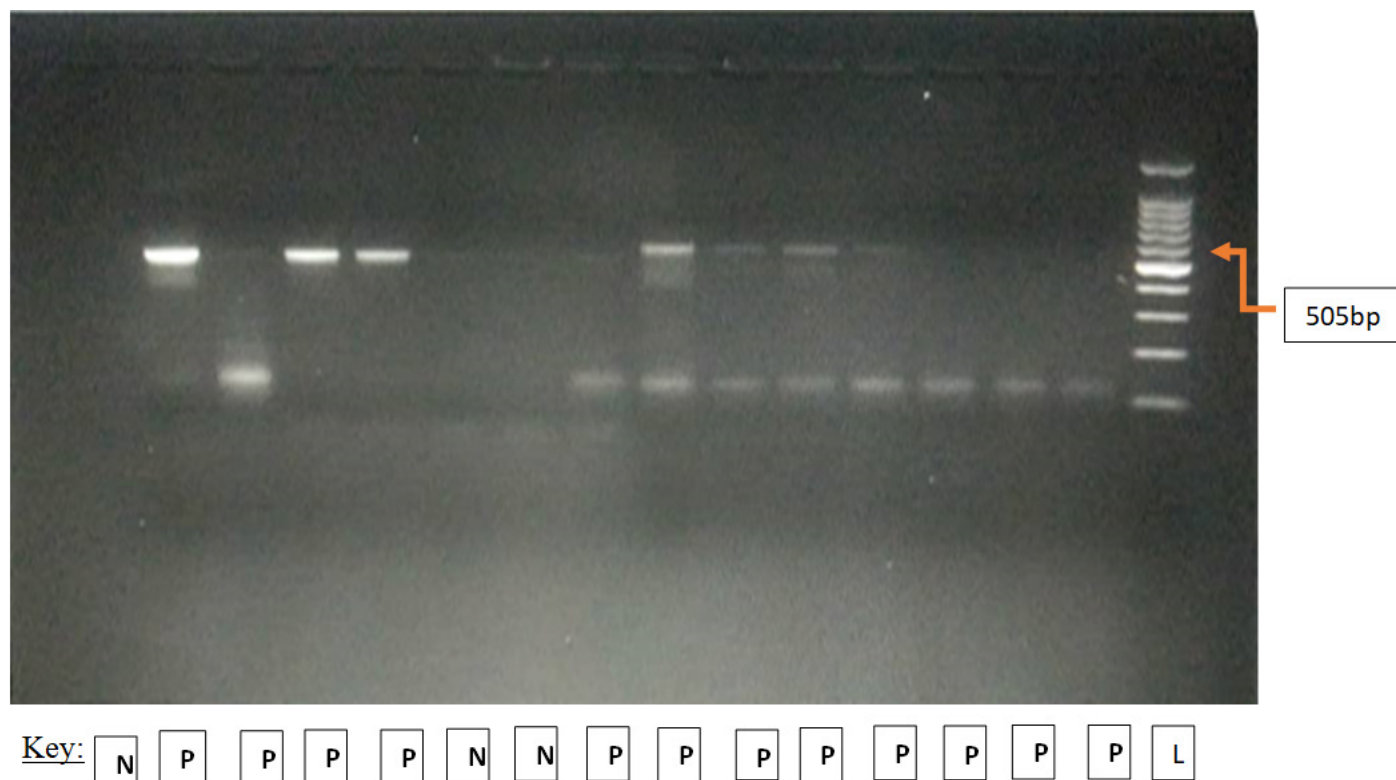
Variable		Frequency (n)	Percentage (%)
Sex	Male	89	51.1
	Female	85	48.9
Age	<5 years of age	76	43.7
	≥ 5 years of age	98	56.3
Body Temperature	No fever (less than 37.5˚C)	10	5.7
	With fever (≥37.5˚)	164	94.3
Hemoglobin level	Severely low (less than 5mg/dL)	33	19
	Moderate(5-8mg/dL)	37	21.3
	Normal(≥8mg/dL)	104	59.8
Bednet use	In Use	145	83.3
	Not in use	8	4.6
	Rarely use	21	12.1
Malaria Status	**MRDT**		
	Positive	120	69
	Negative	54	31
	MICROSCOPY		
	Positive	76	43.7
	Negative	98	56.3

Prevalence of Plasmodium falciparum gametocytes by microscopy and molecular (Conventional PCR) technique. Among the 174 participants, 25.9% (45) were Plasmodium falciparum gametocytes positive by conventional PCR, while 9.8% (17) were Plasmodium falciparum gametocytes positive by microscopy.

The prevalence of Plasmodium falciparum gametocytes was more than 2-fold higher by conventional PCR than by microscopy technique. Chi-square value was 45.780 and p < 0.001 (Table 2).

Table 2. Plasmodium gametocytes prevalence by conventional PCR and microscopy (N=174)

Technique	Frequency (%)		Total	Chi- square	P value
	Positive (%)	Negative (%)			
Conventional PCR	45 (25.9)	129 (74.1)	174	45.78	<0.001
Microscopy	17 (9.8)	157 (90.2)	174		



Key: P, Positive (Pfg27 gene was detected); L, Ladder; N, Negative (Pfg27 gene was not detected)

Figure 1. Pictorial presentation of DNA bands (Pfg27 gene) upon conventional PCR detection.

Comparison of Molecular Technique (Conventional PCR) with Microscopy in detecting P. falciparum gametocyte

In this study the peripheral blood smear for microscopy was considered as the reference, and thus we found that the sensitivity and specificity of molecular (conventional PCR) technique were 94.1% and 81.5% respectively, while the positive and negative predictive values were 35.6% and 99.2% respectively (Table 3). When the conventional PCR was used as a reference, the sensitivity for gametocytes detection by microscopy technique was found to be as low as 35.6% but the specificity was 99.2%.

Table 3. Cross-tabulation for sensitivity and specificity of conventional PCR

Technique		**Microscopy**		**Total**	**PPV**	**NPV**
Positive (%)		**Negative (%)**			**(%)**	**(%)**
PCR (Conventional)	Positive	16 (94.1)	29 (18.5)	45	35.6	
	Negative	1 (5.9)	128 (81.5)	129		99.2
		17 (100)	157 (100)	174		

The microscopy positive and negative predictive values were 94.1% and 81.5% respectively (Table 4).

In comparison, conventional PCR was significantly superior to the microscopy technique in the determination of Plasmodium falciparum gametocytes. (X2 = 45.780, $P < 0.001$).

Table 4. Cross-tabulation for sensitivity and specificity of Microscopy compared with Conventional PCR

Technique		**Conventional PCR**		**Total**	**PPV**	**NPV**
		Positive (%)	**Negative (%)**		**(%)**	**(%)**
Microscopy	Positive	16 (**35.6**)	1 (**0.8**)	17	94.1	
	Negative	29 (64.4)	128 (**99.2**)	157		81.5
Total		45 (100)	129 (100)	174		

Key: PPV, Positive Predictive Value – proportion of the truly (conventional PCR) positive in microscopy positive results; NPV, Negative predictive value – proportion of the truly (conventional PCR) negative in microscopy negative results.

Discussion

Malaria continues to be a global health challenge disproportionately affecting sub-Saharan Africa where over 94% of the burden lies.[14] Gametocyte carriers contribute largely to the increase in transmission of malaria,[15] as studies have shown that even low levels of gametocytes may lead to effective transmission of malaria.[16] Therefore, accurate diagnosis for malaria is not only important in choosing the correct treatment regimen but also for applying effective malaria control strategies in endemic regions where species like P. falciparum contributes to considerable suffering, and death.[17]

This study assessed Plasmodium falciparum gametocytes using microscopy and molecular whereby Pfg27 was the targeted protein during detection of gametocytes presence using the conventional PCR. It was found that Plasmodium falciparum gametocyte prevalence by molecular technique (conventional PCR) targeting Pfg27 gene was 25.9% (45/174) which is more than two-fold higher than that of light microscopy, which was 9.8% (17/174). A study conducted in Ghana found the prevalence among children to be 39.5% (15/38) by molecular technique,[16] which is higher than what we found in our current study. Another study conducted in Cameroon,[18] found the prevalence of gametocytes by molecular technique (Real-Time PCR) to be three-fold (24.1%) higher compared to the microscopy technique (8.9%), which is closely similar to our study result. The slight difference may be attributed to different sample sizes used in the two studies and nature of the study areas. The high gametocyte carriage suggests that children may therefore, be efficient gametocyte reservoirs and hence contribute significantly to malaria transmission, considering that main participants of this study were children with age ranging between 2 and 10 years. The microscopy technique is considered as a gold standard test in malaria diagnosis, however, several studies have proven molecular techniques as the most sensitive technique for malaria diagnosis.[18]

In this study we found the sensitivity and specificity of conventional PCR with reference to microscopy technique in detection of Plasmodium falciparum gametocytes to be 94.1% (16/17) and 81.5% (128/157) respectively. This indicates that conventional PCR is a more reliable tool in detecting the cases which truly carry gametocytes and those which truly do not carry gametocytes. On the other hand, the sensitivity and specificity of the microscopy technique with respect to conventional PCR was found to be 35.6% (16/45) and 99.2% (128/129) respectively. This shows that microscopy is more specific but less sensitive in detecting those that truly carry gametocytes as compared to conventional PCR. This is further indicated by the fact that the conventional PCR positive predictive value with respect to microscopy is only 35.6%, suggesting that most positive cases had been identified as negative by microscopy. Conversely, the positive predictive value of microscopy with respect to conventional PCR was as high as 94.1%, indicating that most positive cases had indeed been identified by conventional PCR.

These results are closely similar to other studies conducted in Tanzania in which sensitivity and specificity of microscopy technique with reference to PCR were 63.8% (95% CI = 50.1–76.0) and 95.7% (95% CI = 92.5–97.9), respectively, suggesting that PCR and other molecular methods should be considered for use in Plasmodium falciparum gametocytes detection in preference to microscopy technique.[19] Therefore, to accurately report gametocytes prevalence, molecular techniques are recommended over light microscopy. However, in peripheral settings there are some challenges in performing the PCR procedures due to a number of infrastructural limitations including inconsistent electricity supply; limited funds for procurement of PCR supplies including reagents; and insufficient trained and skilled personnel to undertake such molecular techniques. Thus, while the conventional PCR is far better than microscopy, the procedure of sending some samples to a well-equipped laboratory for confirmatory processing will continue until such time as the challenges highlighted have been resolved.

Study limitations

Use of archived samples from another study had a risk of selecting some samples which had thawed and frozen again, which could affect molecular technique results. Small sample size than expected was used due missing information of some study participants and because of limited funding for the study.

Conclusion

The study reports that conventional PCR is superior to light microscopy technique in the diagnosis of Plasmodium falciparum gametocytes. Correctly detecting P. falciparum gametocyte carriers in a population.which is crucial to ensure success in malaria transmission-blocking interventions.

Aauthor's contribution

APU and MPK conducted the study under the supervision of SM. R K and DK assisted the review of the final report and manuscript.

Conflict of interest

All of the authors declare no conflict of interest in this study.

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The study was self-funded by author's contributions

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