ORIGINAL RESEARCH PAPER

Biological Science

EVALUATION OF PERFORMANCE OF TWO MONOPLEX QUANTITATIVE REAL TIME PCR ASSAYS IN COMPARISON TO MICROSCOPY FOR IDENTIFICATION OF MALARIA PARASITES.

KEY WORDS: Malaria, PCR, performance, correlation, concordance

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ABSTRACT

Background: Microscopy is the gold standard for Malaria diagnosis with shortcomings such as false positives, false negatives, errors in species identification, and errors in enumeration of parasites. Quantitative real-time PCR (qPCR) has improved submicroscopic malaria diagnosis. This study evaluated the performance, concordance, correlation and methods agreement of two monoplex qPCR assays against expert malaria microscopy for the detection and enumeration of malaria parasites.

Methods: This was a cross sectional study utilizing 127 archived blood samples collected from five provinces in Kenya. Malaria microscopy was conducted by two independent microscopists then 18S-rRNA-qPCR and non-18S-rRNA-qPCR assays were done to identify and quantify the infecting species. The sensitivity, specificity, and predictive values. Cohen Kappa value was used to quantify the method agreement and Bland Altman test was used to assess the bias and limits of agreement. Correlation between microscopy and qPCR parasite densities was determined by the Spearman's rank test. Statistical significance was taken at $p<0.05$.

Results: A higher sensitivity and a lower specificity were observed in all the three plasmodium species in non 18S-rRNA-qPCR compared to 18S-rRNA-qPCR. The sensitivity and specificity of 18S-rRNA-qPCR was 91.3% and 75% in detection of P. falciparum, 67.6% and 88.1% in detection of P. malariae, and 55.8% and 91.4% in detection of P. ovale. The sensitivity and specificity of non 18S-rRNA-qPCR was 99.1% and 66.7% in detection of P. falciparum, 77.9% and 88.1% in detection of P. malariae, and 79.4% and 90.3% in detection of P. ovale. All the positive and negative predictive values were above 70% except the negative predictive value for 18S-rRNA-qPCR (47.4%). Kappa of more than 0.5 was observed between microscopy and both18S-rRNA-qPCR and non-18S-rRNA-qPCR in the detection of all three malaria parasites. The non-18S-rRNA-qPCR method had higher kappa > 0.65, in all the three species compared to 18S-rRNA-qPCR method (kappa < 0.55). There was a clear positive correlation between microscopy parasite density and the parasite densities estimated by the 18S-rRNA-qPCR and Non-18S-rRNA-qPCR ($P<0.001$).

Conclusion: The results showed that both monoplex realtime PCR methods demonstrated a high performance compared to microscopy proving to be better methods in the identification and speciation of malaria parasites especially of low parasitemia. The realtime PCR methods also had a positive correlation with parasite density and hence can be used in accurate determination of parasite densities when compared to microscopy. Therefore, this study recommends the utilization of realtime PCR methods in the detection, speciation and quantification of both microscopic and submicroscopic malaria parasites.

BACKGROUND

Malaria continues to be a major public health concern as a leading cause of morbidity and mortality, especially in children and pregnant women [1]. The disease is caused by protozoan parasites belonging to the genus *plasmodium*, transmitted by the female *Anopheles* mosquito vector [2]. *Plasmodium falciparum* is the most virulent with severe clinical manifestation contributing to a larger extent the malarial deaths in Africa [3]. The World Health Organization (WHO) World Malaria Report 2013 and the Global Malaria Action Plan states that 3.4 billion people live in areas at risk of malaria transmission . The need for more sensitive, specific, accurate and reliable field diagnostic methods is imperative as malaria control programs intensify toward malaria eradication [4, 5]. In addition, in order to better understand transmission dynamics, detection of submicroscopic parasites and effective development of drugs and vaccines for treatment of malaria, more effective diagnostic methods have to be developed. [6].

Microscopy is the gold standard for malaria diagnosis, despite its many inherent limitations, including the need for highly experienced and motivated technicians, variability in smear quality, the inability to determine malaria species at low parasitemia, and the loss of slide quality with time [7]. Malaria rapid diagnostic tests (RDTs) have been developed to improve timeliness, sensitivity and objectivity of malaria diagnosis [8]. Contrary, these kits are faced with a host of limitations such as inability to quantify parasites, inability to distinguish mixed species, persistent antigenemia of target antigens, false negative results, false positive results, and inability to detect submicroscopic parasites [8,9].

Polymerase chain reaction (PCR) methods offers an alternative or complementary tool to microscopy for confirmatory identification of *Plasmodium* spp. in clinical specimens [6]. The PCR methods are highly sensitive with a very low parasite detection limit between 0.7 -0.02 parasites/µl as compared to microscopy which detects >50 parasites/µl [4]. PCR increases the sensitivity and specificity of malaria detection, identification of mixed infections, accurate quantification of the parasite load [10]. quantitative real-time PCR (qPCR) methods use fluorescent binding dyes, such as SYBR® Green, or fluorescent probes for continuous monitoring of amplicon formation throughout the reaction [11]. In the PCR reaction the probes anneal on the target sequence and its degraded during the extension of the sequence by the 5' exonuclease activity of the Taq

polymerase resulting in an increase in reporter fluorescence emission. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation [12].

The ribosomal RNA genes in *Plasmodium* are four to eight copies per haploid genome and are scattered on different chromosomes, with two distinct subgroups whose expression is regulated both by type A and type B genes that are expressed in the asexual and sexual stages in the vertebrate host, respectively. The nucleotide sequence of the SSUrRNA is largely conserved between *Plasmodium species* and different species reveal genetic heterogeneity in their respective ribosomal regions [13]. Several qPCR methods have been developed with 18S-rRNA-qPCR being the method of preference, but re-examination of the sequence revealed presence of only 4 to 8 divergent, non-tandem copies and non-identical sequences hence influencing PCR sensitivity [14]. Despite the wide application of 18S rRNA gene for malaria diagnosis, other non-18S-rRNA-qPCR methods are now available and they could prove useful in the final stages of malaria elimination strategies.

In the non-18S-rRNA-qPCR, the species specific primers and probes are selected based on the presence of only one copy of the gene and minor homology that existed between the selected gene in one species and human DNA or other *Plasmodium* spp. DNA. The following genes for *P. ovale*, the P25 ookinete surface protein (Pos25) gene (Ab074973); and for *P. malariae*, the circumsporozoite (CS) gene (S69014)[15] and the aquaglyceroporin (AQP) gene for *P.falciparum*, [16] were selected. The aquaglyceroporin (AQP) gene is located on chromosome 11 of the parasite. The sequence contains an open reading frame of 774 bp with an A-T content of 70.5% and has no indication of introns. Stretches with an A-T content are 85%, which are typical for intergenic regions in *P.falciparum*, on either side or an in-frame stop codon 54 bp upstream of the predicted start-methionine mark the boundaries of the coding region [16]. The *P. ovale* ookinete surface proteins (*Pfs*25) is a 25-kDa protein with a hydrophobic signal peptide sequence at the N-terminus, followed by four epidermal growth factor (EGF)-like cysteine-rich domains, and a hydrophobic glycosylphosphatidylinositol (GPI) anchor signal sequence at the C-terminus[17]. The *P. malariae* CS gene is present in a single copy per haploid genome without introns. It is divided into three regions: the 5' non-repeat region*;* a central repeat region, consisting of one or two short motifs and the 3'nonrepeat (3'NR) region [18].

The non-18S-rRNA-qPCR methods have not been evaluated in Kenya, in order to check on their diagnostic performance, agreement levels with microscopy and their capability to correlate well with microscopy in quantification of malaria parasites. Most real-time PCR assays are performed using primers and probes of the conserved region of the *Plasmodium* but various studies have shown that they have different performances in the detection of the four malaria species. In addition, the 18S-rRNA genes are shared by many organisms, hence malaria negative individuals sometimes cross-hybridize with the other genes to give a false positive result. This study will evaluate the performance, concordance, correlation and methods agreement of two singplex qPCR assays against expert malaria microscopy for the detection and enumeration of malaria parasites.

METHODS

Study Design

A cross sectional study involving archived blood samples were collected from adults and children within five malaria epidemiological zones: Nyanza, Western, Rift Valley, Eastern and Coast provinces. Samples from visiting scientists coming from non-malaria endemic areas in United States of America and Europe were used to evaluate the performance of two qPCR methods with Malaria Microscopy as the gold standard. A total of 127 samples were used in the study. Scientific and ethical approval for this study were obtained from KEMRI Ethical Review committee (KEMRI Protocol SSC# 1111) and the Walter Reed Institute of Army Research Human use review committee (WRAIR-Human Use and Review Committee Protocol # 1306)

Malaria Microscopy

Blood smears were prepared with the aid of a paper template by placing 6 µL (for thick smear) towards the labeled end of the slide and 2 µL (for thin smear) in the central part of the slide where thick and thin film are immediately prepared respectively [20]. Slides from the same donor were stained in the same batch with 3% Giemsa for one hour. Slides were read by expert microscopists, where five blinded slides from each sample were read five times. Malaria parasites were counted against 200 white blood cells (WBCs) from the thick film if the parasite WBC ratio was $\leq$2. Slides with parasite WBC ratio >2 was counted against 2000 red blood cells (RBC) on the thin smear. The parasite density was obtained by assuming a total WBC count of 8000/µL and 4.5 million RBC/µL and at least 1000 fields was examined before a score of negative result was entered. Parasite speciation was based on morphology [21].

Nucleic Acids Isolation And Purification

Nucleic acid (DNA and RNA) was isolated from EDTA blood samples using QIAamp MinElute© Virus Spin as per the manufacturer's guidelines. Nucleic Acid was eluted from the spin column using 200 µL of Buffer AVE©. The purity and the concentration of the Nucleic Acid were tested using uv based absorbance on the Nanodrop spectrophotometer (Thermo scientific Inc, USA) and stored at – 20°C until required.

Polymerase Chain Reaction (PCR)

Amplification and real-time measurements was performed in the Applied Biosystems 7300© analytical PCR system (Applied Biosystem) and each sample was assayed in duplicate. The following species specific primers and probe sequences were used;

Table1 Primers and Probes sequences for 18S-rRNA-qPCR and non-18S-rRNA-qPCR assays for the three malaria species.

Species	Species primer and Probe Sequences
Non – 18S rRNA P.falciparum (AQP gene)	
Forward	5' CCA TCA AGA GAT TTA GGA TCC AGA TT 3'
Reverse	5' GCT ACA AGA GGT ACC CAA AAA TAA AAA3'
Probe	FAM 5' TTG CAT ATG GAA AAG ATA CCT MGB'
Non – 18S rRNA P.malariae (CS gene)	
Forward	5' CTC AAA TTC CAC CAA GTC AAG AAA 3'
Reverse	5'GAT TCG TGC TAT ATC TGA CTT CTA ACT CA 3'
Probe	FAM 5'AGT GAG TTG TGT TAC AAT AA MGB
Non – 18S rRNA P. ovale (P25 gene)	
Forward	5' CCC AAG CCC AGA TAA TAA GGA A 3'
Reverse	5' TTG TCC TCT GGG TTT GGA ACT T 3'
Probe	5' TAG ATG CTC ATG TAA TAT AG MGB
18S rRNA *P. falciparum*	
Forward	FAL3F : 5'AGT ACA CTA TAT TCT TAT TTG AAA TTG AA

Reverse	FAL3R: 5'TG CCT TAA ACT TCC TTG TGT TAG
Probe	6FAM 5' CTC TTC TTT TAA GAA TGT ACT TGC TTG ATT TAMRA
18S rRNA *P. malariae*	
Forward	MAL4F: TT TGT ATA ATT TTT TAT GCA TGG GAA TTT TG
Reverse	MAL5R:ATGCTGTAGTATTCAAACAC AGAAAC
Probe	MAL3P: 6FAM 5'TGTTCAAAGCAAACAGTTAAAACA 3' TAMRA
18S rRNA *P. ovale*	
Forward	OVA3F:5' TAT AGC TGA ATT TGC TTA TTT TGA AG3'
Reverse	OVA3P:5' ATA CAA TTA ATG TGT CCT TTT CCC TA 3'
Probe	VIC 5'G CTT TAC AAT CAA ACG AAT ACA TTC3'TAMRA

In the Non -18S rRNA qPCR assays, the thermal profile was: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. For the reaction, 2 µl of template was added to 23 µl of reaction master mix containing 12.5µL 2X Taqman Master Mix (Applied BioSystems), 0.8 µM each primer, 0.5 µM probe, and 8.45 µL water. Each reactions plate included Water as non-template control (NTC), serially diluted standards and human DNA (hDNA) for negative control.

For 18S rRNA qPCR assays thermal profile was : Reverse Transcription step of 1 minute at 50°C, Denaturation step of 15 minutes at 95°C and 40 cycles of Amplification for 15 seconds at 95°C, and annealing for 1 minute at 60°C. For the reaction, 1 µl of template was added to 9 µl of reaction master mix containing 5µL 2X Quantitect Mix (QIAgen), 0.4 µM each primer, 0.2 µM probe, 1.1 µL water, 4mM $MgCl_2$ and 0.1 µL Reverse Transcription mix. Each reaction plate included controls as above. In both assays, Ct > 40 was regarded as negative for Malaria parasites.

P. falciparum Standard curves was developed from cultured highly synchronized ring stage 3D7 parasites in order to emulate infected human blood samples. The percent parasitemia of the ring stage was determined by flow cytometry and microscopy.To determine the number of parasites/µl in culture material, the percent parasitemia was multiplied by the number of red blood cells (RBCs)/µl, which was counted by Coulter analysis (Coulter AC µ T 5 diff CP; Beckman Coulter, Inc., Miami, FL). When analyzing and quantifying samples, each 96-well plate was run with the standard 3D7 DNA, which was serially 10-fold diluted from 10,000 parasites to 0.1 parasite/µl using Human DNA of malaria naïve adults from non-malaria endemic area. For *P. malariae* and *P.ovale,* a standard was obtained from field samples whose percent parasitaemia was determined by expert microscopy following 5X5 reads (five blinded slides from the same samples are read five times by five expert microscopists). The extracted total nucleic acid was diluted with human DNA as explained above.

qPCR Parasite Densities

In both assays, the standards were validated to determine the gradient of the slope and the correlation coefficient between the parasite 10-fold serial dilution and their respective cycle thresholds [11]. An assay results was accepted if no signal was obtained from non-template control and human DNA well. The samples were considered positive if fluorescence of reporter dye rose above the default threshold(Ct value < 40) of the thermocycler [22]. The parasites densities of the samples were extrapolated from the standards curve using the following equation: $Y=Mx+C$ Where,Y= Sample Ct; M= Slope of standard curve; C= y intercept of standard curve; X = Log_{10} (Parasite densities) Log_{10}(Parasite densities) = (Sample Ct – y intercept)/ Slope, Parasite Densities = 10^ Log_{10} (Parasite densities) PCR results was accepted when the slope of the linear regression line was between -3.58 and -3.10, correlation coefficient (R^2) is greater than 0.980,Pearson's correlation coefficient (r) greater than |-0.990| and a PCR efficiency of 90 – 110%.

Data Storage & Analysis

The generated data was stored in a Microsoft excel spread sheet. All the data analysis was done using Graph Pad Prism V 5.0 and STATA V 14.2 statistical software. The diagnostic performance of the two qPCR methods was evaluated using the following standard measures of diagnostic accuracy: Sensitivity, specificity, the positive predictive value (PPV), the negative predictive value (NPV), and the likelihood ratios. [23,25]. Cohen's Kappa coefficient (κ) scores with 95% confidence intervals was used to quantify agreement between the two qPCR methods and microscopy on a scale of 0-1. [23, 24]. The correlation of qPCR estimates of parasite density with microscopy results for blood were analyzed using scatter plots and Spearman's rank correlation coefficients [25]. The evaluation of parasite densities agreement between microscopy and the two qPCR methods was done using Bland-Altman plots [26,27].

RESULTS

Demographic

The study evaluated 18S rRNA qPCR and non-18S rRNA qPCR malaria detection assays against microscopy using 127 samples. Microscopy showed that 35% of the samples were positive *for P. falciparum* as mono infection. In mixed infection, 31% were of *P. falciparum* and *P. malariae*, and 23% were of *P. falciparum*, *P ovale* and *P. malariae*. The 18S rRNA qPCR method showed that: *P. falciparum* (43%) in mono infection; 24% were of *P.falciparum* and *P.malariae*, and 12% were of *P.falciparum*, *P ovale* and *P.malariae* in mixed infections. The non 18S rRNA qPCR method showed that: *P. falciparum* (36%) in mono infection; 32% were of *P.falciparum* and *P.malariae*, and 16% were of *P.falciparum*, *P ovale* and *P.malariae* in mixed infections. The study found that the non-18S-rRNA-qPCR method detected more mixed infections than the microscopy and 18S rRNA qPCR methods (Table 4).

Table 2: Microscopy, 18S-rRNA-qPCR and non-18S-rRNA-qPCR malaria detection results

		Microscopy		18S rRNA qPCR		Non 18S rRNA qPCR	
No infection	Negative	11	9%	10	8%	3	2%
Mono Infections	*P. falciparum*	44	35%	54	43%	46	36%
	P. malariae	0	0%	5	4%	1	1%
	P. ovale	1	1%	1	1%	1	1%
Mixed infections	*P. falciparum/ malariae*	39	31%	30	24%	41	32%
	P. falciparum/ ovale	3	2%	9	7%	15	12%
	P.falciparum/ malariae/ ovale	29	23%	15	12%	20	16%
	P. malariae/ ovale	0	0%	3	2%	0	0%

Prevalence of Malaria Species

Microscopy, the gold standard for malaria diagnosis, showed a prevalence of 90% for *P. falciparum*, 54% for *P. malariae*, and 27% for *P. ovale*. The 18S-rRNA-qPCR method showed a prevalence of 86% for *P. falciparum*, 54% for *P. malariae*, and 21% for *P. ovale*. Finally the Non-18S-rRNA-qPCR method reported a prevalence of *P.falciparum* at 96%, *P.malariae* at 49%, and *P.ovale* at 29%.

Table 3: Prevalence Of Malaria By Technique And Species

Species	Microscopy	18s	N18S
P. falciparum (95% CI)	90% (85.46 - 95.64%)	86% (80.88 - 97.72%)	96% (92.68 - 99.44%)

P. malariae (95% CI)	54% (45.33 - 62.67%)	54% (45.33 - 62.67%)	49% (40.31 - 57.69 %)
P. ovale (95% CI)	27% (19.28 - 34.72%)	21% (13.92 - 28.08%)	29% (21.11 - 36.89%)

Diagnostic performance of 18S rRNA qPCR and non-18S rRNA qPCR

The diagnostic performance of the two methods (18S rRNA qPCR and non-18S rRNA qPCR) were compared against microscopy for all Plasmodium species. In the detection of *P. falciparum*, non-18S-rRNA-qPCR method had a higher sensitivity of 99.1% compared to 18S-rRNA-qPCR method (91.3%). However, the specificity of 18S-rRNA-qPCR method was higher (75%) than non-18S-rRNA-qPCR method (66.7%) hence a higher positive predictive value (97.2%). A similar trend of a higher Sensitivity and a lower specificity was observed in *P. malariae* and *P. ovale* for non 18S-rRNA-qPCR method compared to 18S-rRNA-qPCR method. Of note, for *P. malaria* both methods had the same specificity (88.1%). In all the Plasmodium Species, 18S-rRNA-qPCR method had a higher Positive predictive values compared to non18S-rRNA-qPCR method (Table 4).

Table 4: The Diagnostic Performance Of 18S-rRNA-qPCR And Non-18S-rRNA-qPCR In The Detection Of Plasmodium Species Compared To Microscopy.

Plasmodium Species	qPCR Methods	Sensitivity (%)	Specificity (%)	Predictive Value	
		(95%CI)	(95% CI)	Positive (95%CI)	Negative (95%CI)
P. falciparum	18S qPCR	91.3	75	97.2	47.4
		(84.6% - 95.8%)	(42.8% - 94.5%)	(92.1% - 99.4%)	(24.5% - 71.1%)
	Non 18S qPCR	99.1	66.7	96.6	88.9
		(95.2% - 99.9%)	(34.9% - 90.1%)	(91.6% - 99.1%)	(51.8% - 99.7%)
P.malariae	18S qPCR	67.6	88.1	86.8	70.27
		(55.2% - 78.5%)	(77.1% - 95.1%)	(74.7% - 94.5%)	(58.5% - 80.3%)
	Non 18S qPCR	77.9	88.1	88.3	77.6
		(66.2% - 87.1%)	(77.1% - 95.1%)	(77.4% - 95.2%)	(65.8% - 86.9%)
P.ovale	18S qPCR	55.8	91.4	86.8	70.4
		(37.8% - 72.8%)	(83.8% - 96.2%)	(74.7% - 94.5%)	(49.8% - 86.3%)
	Non 18S qPCR	79.4	90.3	75	92.3
		(62.1% - 91.3%)	(82.4% - 95.5%)	(57.8% - 87.8%)	(84.8% - 96.8%)

Percentage method agreement and Kappa values

In non-18S-rRNA-qPCR method the Kappa values were higher (kappa > 0.65) in all the three species compared to 18S-rRNA-qPCR method (kappa< 0.55) (Table 5)

Table 5: Method Agreement And Kappa Values

	Plasmodium falciparum		*Plasmodium malariae*		*Plasmodium ovale*	
	Method Agreement (95%CI)	Kappa (95%CI)	Method Agreement (95%CI)	Kappa (95%CI)	Method Agreement (95%CI)	Kappa (95%CI)
18S qPCR	57.3	0.549	57.1	0.549	55.3	0.506
	(34.9 - 79.8)	(0.408-0.689)	(37.5 - 74.7)	(0.408-0.689)	(36.5-74.2)	(0.332-0.680)
Non 18S qPCR	43.9	0.655	65.9	0.655	67.3	0.685
	(14.2 - 73.7)	(0.525-0.785)	(14.2 - 73.7)	(0.525-0.785)	(50.2 - 84.4)	(0.542-0.827)

Concordance between 18S-rRNA-qPCR, Non-18S-rRNA-qPCR and Microscopy

Using parasite densities to show the limits of agreement

Bland Altman test was used to assess the bias and 95% limits of agreement (LoA) between parasite densities. Microscopy versus 18S-rRNA-qPCR produced a bias (limits of agreement) of 0.9236 (-1.353, 2.668) for detection *P. falciparum* though most of the results were spread above and below the average. The bias was higher for detection of *P. malariae at* 1.448. The bias and limits of agreement for f *P.ovale* was -0.1432 (-2.668-2.381) and all of the results lied within the limits of agreement though they were widely spread out.

Microscopy versus non-18S-rRNA-qPCR showed a much lower bias for detection of *P. falciparum* (0.66) with a 95% limits of agreement of -0.5601-2.407. The plot shows a close spread of the results within the average. The bias for detection of *P. malariae* was 0.5068 (-1.397, 2.410) and most of the results lied within the LoA and the average. The the detection of *P.ovale* had a higher bias and most of the results were widely spread away from the average though within the limits of agreement.

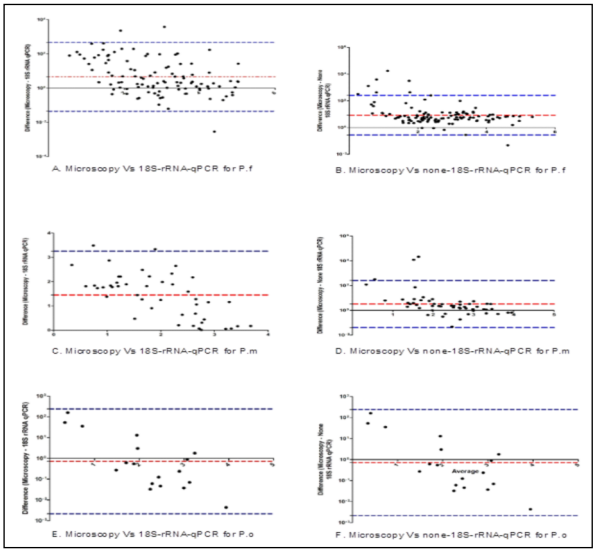

Figure 1: Bland-Altman plots of agreement between determinations of parasite density by microscopy and 18SrRNA-qPCR and Non-18S-rRNA-qPCR.

Solid horizontal lines indicate the mean difference in parasite density estimates between methods; dotted lines indicate 95% limits of agreement (mean + 2 SDs) for the methods.

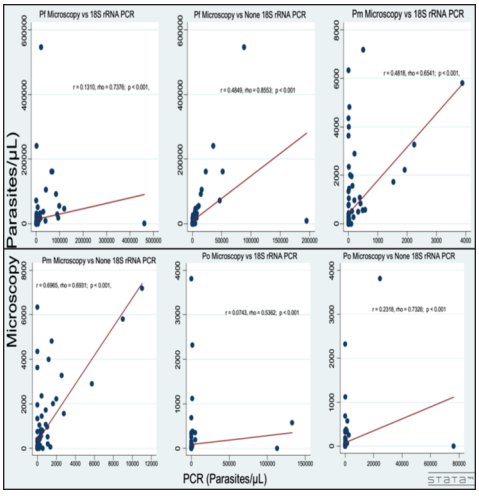

Figure 2. Scatter Plot Showing Correlation Line Of Parasite Density Obtained By Microscopy Counts Compared To 18SrRNA-qPCR And Non-18S-rRNA-qPCR.

Correlation between 18S-rRNA-qPCR, Non-18S-rRNA-qPCR and Microscopy

The scatter plots showed that there was a clear association of parasite densities between microscopy and the 18S-rRNA-qPCR and the Non-18S-rRNA-qPCR methods. The parasite densities were not normally distributed hence the Spearman's rank coefficient was used to quantify the level of the correlations. There was significant differences between the ranks ($P<0.001$) hence indicating that the correlations were due to random chance. The Spearman's coefficient between Microscopy and 18S-rRNA-qPCR was lower ($\rho = 0.7376$, $P<0.001$) as compared to non-18S-rRNA-qPCR ($\rho = 0.8553$, $P<0.001$) in *P. falciparum* parasite densities. The 18S-rRNA-qPCR coefficient was also lower ($\rho = 0.6541$, $P<0.001$) in the *P. malariae* parasite densities as compared to non-18S-rRNA-qPCR coefficient ($\rho = 0.6931$, $P<0.001$). The difference between the method coeffients was higher in correlating the *P. ovale* parasite densities. For 18S-rRNA-qPCR the coefficient was ($\rho = 0.5362$, $P<0.001$) while for non-18S-rRNA-qPCR, the coefficient was ($\rho = 0.7326$, $P<0.001$) Figure 1.

DISCUSSION

The study has indicated that 18S-rRNA-qPCR and Non-18S-rRNA-qPCR have a high performance index in the diagnosis of malarial species. However, the sensitivity for the detection of *P. falciparum* for both methods was indicated to be >90% as compared to the detection of the other *P. malariae* and *P. ovale*. A study by Swan showed that the sensitivity of PCR to detect *P. falciparum* was 97% when compared to microscopy while our study indicates that Non-18S-rRNA-qPCR has a higher sensitivity of 99.1% [30]. The use qPCR has also been shown by other studies that it is more sensitive and specific in the detection of the four plasmodium species [31]. A study by Morrasin *et al* showed that PCR when used routinely for 12 months maintained higher inter-serial sensitivity and specificity than microscopy [32]. It is also noted that the specificity of the two methods in the detection of *P. malariae* and *P. ovale* was higher (>88.1%) than the detection *P. falciparum*. Nucleic acid tests developed for malaria diagnosis have repeatedly shown better performance and accuracy in detecting malaria parasites [3].

Demographic analysis show that for mono infections; 18S-rRNA-qPCR described 60 plasmodium positive patients, non-18S-rRNA-qPCR described 48 plasmodium positive patients, while microscopy described 45 plasmodium positive infections. This is an indicator that microscopy missed this infections probably because they were at very low densities. Previous studies have shown that microscopically detectable plasmodium species in pregnant women is a poor test because the host immunity is able to regulate the parasite density to low levels [33]. A study by Delhaes *et al* pointed out that microcopy misses out on a number of imported malaria infections in travelers returning from malaria endemic regions and recommend the use of qPCR methods to detect malaria[34].

The results also highlight the ability of non-18S-rRNA-qPCR (n=56) to pick more mixed infection (*P. falciparum/malariae, P. falciparum/ovale)* than microscopy (n=42) or 18S-rRNA-qPCR (n=39). Many methods are molecular based methods have been developed to detect mixed infections [3] but the present study shows that the non-18S-rRNA-qPCR is more efficient technique. Furthermore, the 18S-rRNA target gene has been pointed in previous studies that it is not fully satisfactory because of its ability to cross-hybridize with human DNA for P. malariae [35]. It has also been shown that PCR assay are able to detect malaria 4-7 days post microscopy detection, this is important for patient who self-treat themselves but they go to the hospital after persistence of the disease [31].

The study showed that the prevalence of the non -18s PCR method was higher for *P. falciparum* as compared to microscopy. Similar findings of a higher PCR prevalence than microscopy was observed by a study done by Alfredo et al, 2009, who showed that the prevalence of malaria was 5.3% by microscopy and 23.2% by RT-PCR [33]. This can be relevant in malaria elimination programs because it clearly indicates that microscopy appears to miss out on a proportion of infections. The implication of this is that treatment based on microscopy alone would lead to under treatment of the population and submicroscopic infections can persist in the body system for a while as they aid malaria transmission [36]. The study has also indicated that the qPCR methods have a relatively higher method agreement with microscopy with moderately strong kappa values. Both the 18S-rRNA-qPCR and Non-18S-rRNA-qPCR can be relied upon in the detection of the three malarial species. However, as noted earlier non-18s rRNA can be relied in detection of P. malariae than the 18S rRNA PCR method because of false-positives due to cross-hybridisation with human DNA [9, 35]. Even though, microscopy is still the most widely used technique for malaria species identification [37, 38], the errors associated with the method make it an imperfect gold standard [5] [23]. This means there is likelihood that microscopy could miss out on proper species identification makes qPCR the better alternative because it does not rely on staff proficiency to identify all the malaria species. This is so because malaria qPCR assays are mostly performed using conserved region of the Plasmodium 18S rRNA of all four human malaria species as the target sequence [39]

Using Bland-Altman, both *P. falciparum* qPCR Methods agreed with microscopy with very low bias levels. However, microscopy underestimates parasite density relative to qPCR [9, 40] due to the use of a multiplication factor to derive parasite density from a small amount of screened blood (usually about 0.2 μL blood). In this study, we also observed discrepancy between microscopy counts and qPCR parasite density estimates for *P. falciparum* and *P. malariae*; microscopy gave a slightly higher estimate, consistent with reports by Rougemont *et al* hence suggesting microscopy results slightly overestimated parasite counts[9].

The Plus-system reporting system is used in hospitals and health centers to estimate the levels of infection. This method is limited in when it comes to proper case management, drug and vaccine clinical trials, and drug efficacy trials [41]. The use parasite count against white blood cell is the better option for most laboratories but lack of properly trained or proficient personnel makes the implementation of exact parasite count and reporting a challenge [42].The use of qPCR eliminates the human error factor and because the parasite densities are estimated by standard controls. This study showed significant positive correlations in the estimation of parasite densities for all the three plasmodium species. The non-18S-rRNA-qPCR showed higher correlation coefficients in determining the levels of infection than the 18S-rRNA-qPCR. A previous study by Delhaes *et al,* also indicated that qPCR method had a good correlation in quantifying parasitemia. This implies that the use of qPCR methods would be a powerful tool in the estimation of the levels of parasite during drug trials and proper patient management[34].

CONCLUSIONS

This study showed that the 18S-rRNA-qPCR and Non-18S-rRNA-qPCR have demonstrated a high performance compared to microscopy proving to be better methods in the identification and speciation of malaria parasites especially of low parasitemia. High agreement between the two qPCR methods with microscopy was observed and therefore, these two qPCR methods can be relied upon in detection of plasmodium parasites. The two qPCR methods had a positive correlation with parasite density and hence both qPCR methods can be utilized in accurate determination of parasite densities when compared to microscopy.

We can recommend the use of 18S-rRNA-qPCR and Non-18S-rRNA-qPCR methods as the primary identification method for

the three malaria species.

Declarations

Consent For Publication

This study reports no individual data. This work is to be published with permission of the Director, Kenya Medical Research Institute. Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. All authors have read and approved the manuscript for publication.

Availability Of Data And Materials

The data supporting the conclusions of this paper are included within the paper. Raw data may be obtained from the corresponding author upon request.

Issues relating to Journal policies

None

Competing interests

All authors declare that they have no competing interests.

Funding

This work was supported by the Armed Forces Health Surveillance Center -Global Emerging Infections Surveillance and Response System funding (AFHSC-GEIS, Proposal # 0046_14_KY).

Authors' contributions

CO substantially contributed to conception and design of the study, data acquisition and drafting manuscript. CM contributed in data analysis and data interpretation, and manuscript drafting. All authors read and approved the manuscript.

Publication/submission To Another Journal

This manuscript has not been published or submitted to another journal for publication.

Disclaimer Statement

The views expressed in this work are those of the authors and do not represent those of the Walter Reed Army Institute of Research, US Army Medical Department, or US Department of the Army or the Department of Defense.

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