Comparison of capillary and venous blood for malaria detection using two PCR based assays in febrile patients in Sierra Leone.

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Abstract

Background. Malaria parasites infect over 200 million people and cause more than 400,000 deaths each year. Rapid and sensitive diagnostics are critical tools for clinical case management and public health control efforts. Both capillary and venous blood are currently used for malaria detection and while diagnostic technologies may not be equally sensitive with both materials, the published data on this subject are scarce and not conclusive.

Methods. Paired clinical samples of venous and capillary blood from 141 febrile individuals in Bo, Sierra Leone, were obtained between January and May 2019 and tested for the presence of *Plasmodium* parasites using two multiplexed PCR assays: the FilmArray-based Global Fever Panel (GFP) and the TaqMan-based Malaria Multiplex Sample Ready (MMSR) assay.

Results. We observed no significant differences in *Plasmodium* parasite detection between capillary and venous blood for both assays. The GFP assay was more sensitive than MMSR for all markers that could be compared (*Plasmodium* spp. and *P. falciparum*) in both venous and capillary blood.

Conclusions. No difference was found in malaria detection between venous and capillary blood using two different PCR-based detection assays. This data gives support for use of capillary blood, a material which can be obtained easier by less invasive methods, for PCR-based malaria diagnostics, independent of the platform.

Background

Malaria remains one of the most deadly infectious diseases in the world, disproportionately affecting lower-income countries in Africa and South Asia. According to current World Health Organization (WHO) estimates, more than 200 million malaria cases and more than 400,000 deaths are expected to occur this year, with the highest burden falling on young children in sub-Saharan Africa [1].

Sensitive and accurate diagnosis of malaria is the key to effective clinical case management and is an essential component of public health prevention and control strategies. Although diagnostic technologies using saliva and urine are being developed [2, 3], most current technologies continue to use whole blood collected via venipuncture or finger stick, and these materials are used interchangeably [4]. However, the chemical and cytological composition between blood from capillary and venous compartments differs [5-8], and these differences may potentially affect the sensitivity of malaria diagnostics. Sequestration of *Plasmodium*-infected erythrocytes in small capillaries of certain organs (e.g., brain, lungs) — a documented feature of malaria pathology [9] — may lead to unequal distribution of malaria parasites between capillary and venous compartments. That, in turn, may lead to different diagnostic results from these sample types.

Several previous studies assessed parasite density and the sensitivity of malaria detection in paired capillary and venous blood samples from asymptomatic carriers and symptomatic malaria patients in

sub-Saharan Africa [4, 10-14]. Some comparisons using standard microscopic techniques and PCR showed no difference between capillary and venous samples [10, 12, 13], but others showed slightly higher parasite numbers in capillary specimens [4, 11, 14]. Additionally, one PCR-based study documented a greater diversity of strains detected in venous blood samples [10]. Because of the lack of consensus about whether sensitivity is different for capillary and venous samples, this area of diagnostic research merits further study. In this study, we compared the sensitivity of malaria detection in paired capillary and venous samples from symptomatic hospital patients in Bo, Sierra Leone, using two different PCR-based assays capable of detecting multiple *Plasmodium* species: the FilmArray-based Global Fever Panel (GFP) and the TaqMan-based Malaria Multiplex Sample Ready (MMSR) assay.

Materials And Methods

Study population and sample collection.

The study population was recruited from among persons seeking care at Mercy Hospital in Bo, Sierra Leone. All persons with clinically confirmed or self-reported fever with onset within the 10 days before the enrollment date were invited to participate in the study. Informed consent from patients (or, for children, consent from their parents) was obtained and documented prior to collection of clinical data and biological specimens. In total, 141 volunteers were enrolled between 28 January and 20 May 2019. Paired samples of venous and capillary blood were collected into EDTA-containing vacutainers or microtainers from each participant by venipuncture and finger stick, respectively.

The study protocol was approved by the Institutional Review Boards at the US Naval Research Laboratory and George Mason University, and by the Sierra Leone Ministry of Health and Sanitation.

PCR-based analysis.

Two PCR-based methods were used in this study: the FilmArray Global Fever Panel (GFP, BioFire Defense, Salt Lake City, UT, USA) and the Malaria Multiplex Sample Ready assay (MMSR, BioGX, Birmingham, AL, USA). The same volume (200 µL) of blood specimen was used in both systems and from both specimen types.

GFP is a fully automated, nested PCR assay capable of automated extraction of nucleic acids from a blood sample and rapid (one hour) detection of nineteen targets using the FilmArray platform, including *P. falciparum*, *P. vivax*/*P. ovale*, and *Plasmodium* spp. [15]. Briefly, within two hours of sample collection, 200 µL of capillary or venous blood was diluted with GFP sample buffer, loaded into GFP pouches, and analyzed on the BioFire Film Array 2.0 instrument according to the manufacturer's instructions. Detection and identification of the target is made automatically based on the melting temperature of the obtained amplicon. The GFP has three different assays for *Plasmodium*: one species-level assay that detects *P. falciparum*, one species-level assay that detects both *P. vivax* and/or *P. ovale*, and one genus-level assay (*Plasmodium* spp.) that detects all *Plasmodium* species known to cause malaria in humans. The limits of detection (LOD) for GFP are 180, 150, and 240 genomic copies per mL blood for *P. falciparum*, *P. vivax*,

and *P. ovale* respectively (Bio Fire, unpublished data). The FilmArray system calculates semi-quantitative crossing point (Cp) values, however the customer software does not use the Cp values to provide GFP qualitative results. While the FilmArray system generates crossing point (Cp) values not crossing threshold (Ct) values for the purposes of the discussion in this paper, “Ct” is used for both amplification systems.

The MMSR assay is a room temperature-stabilized TaqMan-based real-time PCR assay [16, 17], designed to detect *P. falciparum*, *P. vivax*, *Plasmodium* spp., and RNaseP (sample extraction control) in a single assay. While this assay was not specifically designed to detect *P. ovale* and other less common malaria species, samples containing these parasites may be identified with *Plasmodium* spp.-positive and *P. falciparum*-negative test outcome [18]. Within two hours of collection, DNA was extracted from 200 µL capillary or venous blood using QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA); the final volume of extracted DNA was the same as the initial sample volume – 200 µL. Five microliters of the extracted DNA was added to each MMSR tube, previously rehydrated with 5 µL water. Tubes were then subjected to a thermal cycling program as follows: initial incubation at 95°C for 2 minutes; 45 cycles of denaturation at 95°C for 10 seconds and annealing/elongation at 59°C for 1 minute. Fluorescence levels were measured at the end of each cycle. A sample was considered positive for a particular target in the MMSR assay if a sigmoidal amplification curve with Ct value < 40 was observed. The reported LODs for *P. falciparum* in the genus-specific assay (*Plasmodium* spp.) were 244-390 parasites per mL DNA solution, depending on whether the lyophilized or “wet” format was used [17]. The reported LOD in the *P. falciparum*-specific assay was similar in the lyophilized test (244 copies/mL), but was about 10-fold higher in the “wet” assay. The LOD for *P. vivax* was not reported for the lyophilized, “sample ready” format, but was previously determined for the “wet” assay as 127 parasites/mL [16].

Data from capillary or venous samples with negative RNaseP results (MMSR assay) or invalid FilmArray results were not included in the comparison. However, if the matched partner of the invalid sample showed valid results, data from the matched partner were included in statistical analyses of the population as a whole.

Sample populations returning valid results were compared using McNemar’s chi-square analysis of (self-)paired samples, corrected for continuity. Ct values for positive (matched) samples were compared using paired t-tests and for unmatched populations using unpaired t-tests, assuming unequal variances.

Results

The median age of the 141 participants that provided blood samples was 27 years (range: 5 to 78 years), and 60% were female. Depending on the assay and material tested, at least one malaria marker was detected in 36% to 61% of the tested samples (Table 1). These results were in line with previous reports of malaria positivity among febrile individuals in this location as detected by PCR-based methods [18, 19].

Table 1. Malaria marker prevalence as detected by GFP and MMSR assays.

	Number (%) of positive samples	
	GFP (n=66)	MMSR (n=110)
Capillary		
Plasmodium spp.	37 (56%)	29 (26%)
P. falciparum	32 (48%)	37 (34%)
P. vivax/P. ovale[1]	4 (6%)	0
any malaria marker	38 (58%)	40 (36%)
Venous		
Plasmodium spp.	40 (61%)	35 (32%)
P. falciparum	30 (45%)	37 (34%)
P. vivax/P. ovale[1]	4 (6%)	0
any malaria marker	40 (61%)	40 (36%)

[1] GFP assay detects both *P. vivax* and *P. ovale* while MMSR assay detects only *P. vivax*

The study was designed to return four test results for each participant (two platforms, both venous and capillary samples tested on each). However approximately 63% of the participants had one or more missing data points due to failed runs, invalid results, or insufficient sample volumes to perform both tests on both platforms. Consequently, valid results were returned for 110 paired capillary/venous blood samples using MMSR and for 66 paired samples using GFP. For cross-platform comparisons, venous samples from 136 individuals and capillary samples from 54 individuals were successfully tested on both platforms. The demographic and clinical characteristics of included and excluded volunteers were similar.

We observed no significant age-specific differences for any marker on either platform (Supplemental Table 1). Although no gender-specific differences were noted on the GFP platform, a significantly higher proportion of male participants were positive for *Plasmodium* spp. in the MMSR assay ($p = 0.011$); this result may simply represent an artifact of the large relative difference between male and female sample sets within a small total population ($n = 136$).

Comparison of detection platforms.

A total of 54 matched capillary and 136 venous blood samples returned valid results for both GFP and MMSR. The results for both detection platforms were compared in Table 2 for capillary and Table 3 for

venous samples. GFP assays detected a significantly higher proportion of positive samples across all detected markers for both capillary and venous blood. The difference was particularly evident with the genus-level marker (*Plasmodium* spp.). The GFP platform detected *Plasmodium* spp. in over half of the samples (both sample types), whereas the same marker was detected in 22% fewer samples analyzed by MMSR. Fewer samples were positive for *P. falciparum* marker than *Plasmodium* spp. marker on the GFP, but cross-platform differences were still significant (*P. falciparum* was detected in 23/54 capillary samples by GFP compared to only 15/54 by MMSR, and 58/136 venous samples by GFP compared to only 47/136 by MMSR).

Table 2. Comparison of malaria detection by GFP and MMSR assays - **capillary blood (n=54)**; GFP is more sensitive than MMSR

ırker					p-value for McNemar's c^2 test
***ısmodium* spp.**		MMSR			
		Positive	Negative	Total	**< 0.001**
;FP	Positive	10	18	28	
	Negative	1	25	26	
	Total	11	43	54	
falciparum		MMSR			
		Positive	Negative	Total	
;FP	Positive	13	10	23	**0.043**
	Negative	2	29	31	
	Total	15	39	54	
y *Plasmodium* marker		MMSR			
		Positive	Negative	Total	
;FP	Positive	14	14	28	**0.006**
	Negative	2	24	26	
	Total	16	38	54	

Table 3. Comparison of malaria detection by GFP and MMSR assays - **venous blood (n=136)**; GFP is more sensitive than MMSR

Marker					*p*-value for McNemar's c^2 test
***Plasmodium* spp.**		MMSR			**< 0.001**
		Positive	Negative	Total	
GFP	Positive	44	27	71	
	Negative	0	65	65	
	Total	44	92	136	
P. falciparum		MMSR			
		Positive	Negative	Total	
GFP	Positive	43	15	58	**0.022**
	Negative	4	74	78	
	Total	47	89	136	
Any malaria marker		MMSR			
		Positive	Negative	Total	
GFP	Positive	49	22	71	**< 0.001**
	Negative	1	64	65	
	Total	50	86	136	

The GFP assay detected *P. vivax*/*P. ovale* in six out of 136 matched venous samples (4%) and in four out of 54 matched capillary samples (7%) - Tables 2 and 3. The four capillary samples positive for *P. vivax*/*P. ovale* were simultaneously positive for this marker in venous samples. However, none of these samples were deemed positive for *P. vivax* in the MMSR assay, suggesting that the *P. ovale* may have been responsible for the *P. vivax*/*P. ovale*-positives in the GFP assay.

We have previously observed that *P. ovale* (and *P. malariae*) may be detected when a sample is *Plasmodium* spp.-positive and negative for the species-specific markers in the MMSR assay [18]; only one of the samples positive for *P. vivax*/*P. ovale* demonstrated this behavior in the MMSR assay. Presumptively, samples harboring *P. malariae* and other less-common species can be identified similarly by the MMSR assay (negative for *P. vivax* and positive for *Plasmodium* spp.). This does not have to be true, however, for GFP because the *Plasmodium* spp. assay is slightly more sensitive than the GFP species-level assays (BioFire, unpublished data). We identified an additional nine venous and four capillary samples that were *Plasmodium* spp.-positive but negative in the species-specific assays in GFP assays; all but two were negative for all markers in the corresponding MMSR assays. Interestingly, only three had identical results in both venous and capillary samples. All eleven of these samples that were

only positive for *Plasmodium* spp. had late Ct values, suggesting analyte levels near LOD where it is expected that the *Plasmodium* spp. assay will slightly outperform the species-level assays on the GFP

Comparison of venous and capillary samples.

Results from matched sets of capillary and venous samples using GFP (Table 4) showed no significant differences in the proportion of samples testing positive for any of the malaria markers between venous and capillary samples. Fewer than 10% of the 66 matched samples on GFP gave discordant results where a marker was positive in one sample type and negative in the other. Comparisons of Ct values between venous and capillary samples (Figure 1) show a high degree of correlation when tested using GFP. There was no significant difference in Ct values between matched venous and capillary samples for any marker ($p > 0.13$).

Table 4. Comparison of malaria detection in capillary and venous blood – **GFP assay (n=66).**

Marker					*p*-value for McNemar's c^2 test
***Plasmodium* spp.**		Capillary			0.371
		Positive	Negative	Total	
Venous	Positive	36	4	40	
	Negative	1	25	26	
	Total	37	29	66	
P. falciparum		Capillary			
		Positive	Negative	Total	0.617
Venous	Positive	29	1	30	
	Negative	3	33	36	
	Total	32	34	66	
P. vivax/P. ovale		Capillary			
		Positive	Negative	Total	null
Venous	Positive	4	0	4	
	Negative	0	62	62	
	Total	4	62	66	
Any malaria marker		Capillary			
		Positive	Negative	Total	0.617
Venous	Positive	37	3	40	
	Negative	1	25	26	
	Total	38	28	66	

Similarly, no significant differences between positive results were observed for any marker when using MMSR ($p > 0.24$, $n = 110$; Table 5). There was lower concordance between the two sample types in MMSR assays than in the GFP assays – 82% for genus specific marker (*Plasmodium* spp.), 84% for *P. falciparum* and 84% for any malaria marker versus >92% for GFP. A lower correlation was observed when comparing Ct values for matched samples in MMSR assays (Figure 2), although no significant differences in Ct values were observed, independent of the assay ($p > 0.4$). However, when comparing concordant populations (both sample types positive) and discordant populations (one sample type positive, one sample type negative), Ct values from the discordant populations were indeed higher ($p < 0.015$), supporting the hypothesis that samples with discordant results had lower target concentrations.

Table 5. Comparison of malaria detection in capillary and venous blood – **MMSR assay (n=110).**

Marker					*p*-value for McNemar's c^2 test
***Plasmodium* spp.**		Capillary			0.264
		Positive	Negative	Total	
Venous	Positive	22	13	35	
	Negative	7	68	75	
	Total	29	81	110	
P. falciparum		Capillary			
		Positive	Negative	Total	0.814
Venous	Positive	28	9	37	
	Negative	9	64	73	
	Total	37	73	110	
P. vivax		Capillary			null
		Positive	Negative	Total	
Venous	Positive	0	0	0	
	Negative	0	110	110	
	Total	0	110	110	
Any malaria marker		Capillary			
		Positive	Negative	Total	0.814
Venous	Positive	31	9	40	
	Negative	9	61	70	
	Total	40	70	110	

Discussion

The goal of this study was to compare the sensitivity of DNA-based malaria diagnostic assays using capillary and venous blood. In contrast to most of the previous studies comparing these two sample types [4, 10-13], we assessed the sensitivity of two PCR assays capable of detecting multiple species of

Plasmodium instead of determining the parasite density by microscopy. Our analysis documented no significant differences in malaria detection between these two sample types.

These results are in line with several previous studies that also found no differences in parasite density or malaria detection between capillary and venous samples [10, 12, 13]. Comparisons of Ct values obtained using GFP and MMSR further support the hypothesis that there are no significant differences in average parasite densities between these two sample types.

The higher malaria detection rate observed for GFP compared to MMSR for all detected markers reflects fundamental and comprehensive differences between the two systems: different sample processing (nucleic acid extraction), different PCR chemistry, nested PCR (GFP) versus single-step PCR (MMSR), and different gene targets. The differences in assay sensitivities are especially pronounced in the case of genus-level targets (*Plasmodium* spp.). Although the previously determined LODs for the two platforms are within the same range (~150-400 parasites or target copies per milliliter), a five-fold larger MMSR sample volume was used in the current study than in the MMSR LOD study (5 µL versus 1 µL [17]); it is possible that the increased sample volume, combined with a different method for DNA extraction, interfered with the MMSR assays performed here. The differences in sensitivity can be also a consequence of using different diagnostic targets; the effect is especially pronounced in case of the genus-specific targets (*Plasmodium* spp.). The samples with discordant results between sample types had significantly higher Ct values on both platforms; these results suggest that discordant results may arise when testing samples with parasite concentrations close to the LOD.

We could not unambiguously assess the sensitivity differences in detecting *P. vivax* (MMSR) and *P. vivax*/*P. ovale* (GFP) due to the different specificities of the two platforms and the low prevalence of *P. vivax* within West Africa [1, 20, 21]. Importantly, two of the samples that were only positive for *Plasmodium* spp. by GFP in venous blood had relatively early Ct values that, if the infection were *P. falciparum, P. ovale,* or *P. vivax*, should also have been detected by one of the species-level assays; it is therefore likely that these samples contained *P. malariae*. The presence of small but significant numbers of samples positive for *P. vivax*/*P. ovale* in the GFP assay or *Plasmodium* spp. only in both assays could be due to co-circulation of *P. ovale* and *P. malariae* in the tested population as previously reported [18].

Three studies have previously reported higher parasite densities or improved malaria detection sensitivities in capillary blood [4, 11, 12]. The hypothesis that capillary blood may contain higher concentrations of malaria parasites is based on the well-documented feature of malaria pathology – increased adherence of *Plasmodium*-infected erythrocytes to vascular endothelial cells – which leads to their sequestration in certain organs [9]. However, while it is known that malaria-infected erythrocytes can sequester in specific organs (e.g., brain, lungs), it is not clear if significant sequestration occurs in capillaries used for diagnostic sample collection. The results obtained in this study do not provide support to the hypothesis that *Plasmodium* markers are present in higher concentrations in capillary blood, but rather, adds to the body of literature showing no documented difference between sample types.

A notable difference between the current and previous studies is the population age distribution. Whereas most other studies emphasized testing of children or young teens [4, 10, 11, 13, 14], our study population was significantly older, with a median of 27 years and 25% of the tested individuals between 21 and 25 years of age. It is possible that age-related physiological differences may affect the parasite's lifecycle, its clinical presentation, and potentially even its prevalence within various compartments [22-24]. An age-controlled study with a greater number of participants and longer collection period would be needed to explore these variables.

Conclusions

In summary, we found no difference in *Plasmodium* parasite detection sensitivity between capillary and venous blood. While accepting the limitations of the current study (small sampled population with a different age distribution from previous studies), our results provide additional supporting evidence that PCR-based methods can produce equally satisfactory results using capillary and venous blood, in spite of the higher diagnostic sensitivity of capillary blood suggested by some recent studies. The implication of this finding is that capillary blood — typically less cumbersome and invasive to obtain — can be used without a loss of the assay sensitivity. Additional, larger-scale studies are needed to support these conclusions.

Abbreviations

Cp – crossing point

Ct – crossing threshold

GFP – Global Fever Panel

MMSR – Multiplex Malaria Sample Ready assay

LOD – limit of detection

PCR – polymerase chain reaction

WHO – World Health Organization

Declarations

Ethics approval and consent to participate.

The research protocol used in this study was approved by the Sierra Leone Ethics and Scientific Review Committee and the institutional review boards of the US Naval Research Laboratory and George Mason University.

Consent for publication

Not applicable.

Availability of data and material.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests.

Brian Jones, Olivia Jackson and Cynthia Phillips are employees of BioFire Defense, the manufacturer of the instrument (FilmArray) and assay (Global Fever Panel) used in this study.

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Authors' contributions.

TAL, **CRT**, **RA**, **DAS** conceived the study and designed the experimental protocols; **RA**, **UB, JL, JML, VB**, **RA** performed the laboratory testing; **AGS** processed the experimental data and developed the database; **TAL**, **CRT**, **KHJ** developed human subjects protocol; **OJ, BWJ, CLP** analyzed the GFP assay data; **CRT** performed the statistical analyses; **TAL** and **CRT** wrote the first version of the manuscript; **UB**, **OJ, BWJ, CLP, RA**, **KHJ, DAS** made edits to the manuscript. All authors read and approved the final manuscript.

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