Detection and Quantification of *Plasmodium* DNA in Dried Blood Spots Using a Commercial Real-Time PCR Assay and Filter Card System

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Abstract: Blood collected on FTA® filter cards was analysed qualitatively and quantitatively with real-time PCR and the results compared with expert microscopy performed on-site in Gabon. There was 100% concordance for fresh blood samples. However, correlation of quantitative PCR-results was rather weak (0.52) and reproducibility showed coefficients of variation ranging from 10%–90%.

Keywords: real-time PCR, *Plasmodium*, detection and quantification, filter paper

Molecular methods have been shown to be superior in the detection of *Plasmodium* spp when compared to microscopy.[1–3] Recently, real-time PCR has shown the added advantage to allow quantification of microbial DNA/RNA[4] including plasmodial DNA.[1,5,6] In general, PCR-based methods are not routine laboratory tests for malaria diagnosis in most endemic areas,[7] yet they are of use in many other contexts, such as research, epidemiology or detection of resistance. PCR is often performed centrally or even abroad, which requires blood sample storage and transport.[7] Whereas dried blood spots on filter paper are a convenient way to do so,[2] several issues remain unclear when real-time PCR is used. First, nucleic acids have to be extracted from the filter paper and inhibiting substances have to be removed; and second, it remains unclear if quantification of plasmodial DNA from dry blood spots is reliable and reproducible.

A commercial real-time PCR has been shown to perform well in the detection of *Plasmodium* spp in returning travellers.[8] Contrary to conventional filter papers, the Flinders Technology Associates system (FTA® filter cards, Maidstone, U.K) uses filter cards impregnated with proprietary reagents that lyse erythrocytes, degrade proteins and entraps nucleic acids in a matrix. This allows longterm preservation[9] as well as a simple elution procedure. The objective of this study was to evaluate this novel filter card system and compare the quantitative and qualitative real-time PCR results with expert microscopy, which was performed on-site in Gabon.

Samples were collected at the Albert Schweitzer Hospital in Lambaréné, Gabon. Samples were from pregnant women and from children that participated in various clinical studies on malaria. They either attended the study site routinely as part of study visits, or because they felt unwell. Fifty-one samples from pregnant women consisted in thawed erythrocyte pellets that had been routinely prepared as part of other studies, by centrifuging EDTA-anticolagulated blood and discarding the plasma. Forty-seven fresh EDTA anticolagulated blood samples from children who had a Full-Blood-Count (FBC) performed were also included. Ethical approval was obtained from the ethics committee of the International Foundation for the Albert Schweitzer Hospital in Lambaréné. Giemsa-stained thick smears were prepared according to the Lambaréné method.[10] Thirty μL of the whole EDTA-anticoagulated

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blood or erythrocyte pellets were spotted on FTA® filter cards, starting in the centre, in an outward spiral motion. Samples were processed 12 months later in Portugal, using a Micropunch (Whatman®, Maidstone, U.K) to obtain a two-millimetre disk from the centre of the spot. To investigate the reproducibility, in 10 filter cards of fresh pediatric blood, four punches were made on a virtual circle, 3–4 mm from the centre. The purification and elution procedure of the filter card manufacturer was used (Whatman, Maidstone, U.K), including the FTA® purification reagent (Whatman, Maidstone, U.K), as well as a buffer solution (10 mM of Tris-HCl and 0,1mM of EDTA at a pH of 8), elution reagent one (0.1 N NaOH and 0.3 mM of EDTA, at a pH 13) and two (0.1 molar Tris-HCl, at pH 7) (all reagents from Sigma St. Louis, MO, U.S.A). The samples were analysed on a Rotor-Gene® 6000 machine (Corbett Research, Sydney, Australia) using the RealArt® Malaria PCR kit (Artus, Hamburg, Germany). The kit amplifies a 163 bp region of the genome, present in all four human *Plasmodium* species. To 5 µL of the purified and eluted sample, 15 µL of the master mix 1 were added and than processed in the Rotor-Gene® 6000 according to the instruction of the manufacturer of the PCR kit (http://www1.qiagen.com/HB/artusMalariaRGPCRKit_EN, accessed 19 of May 2008).

Forty-seven samples from children were analysed. Microscopy showed 26 cases of *P. falciparum*, 2 *P. malariae*, 5 mixed infections with *P. falciparum* and *P. malariae*, and 13 samples were negative. Of all samples from pregnant women, 14 were positive by microscopy (*P. falciparum*) and 37 samples were negative. The overall results of the real-time PCR are shown in Table 1. In the samples from the children, the sensitivity and specificity for the RT-PCR was 100%. In the samples from pregnant women, three microscopy-positive samples were repeatedly negative by RT-PCR. Contrary to this, in five microscopy negative samples, the RT-PCR result was positive, although the number of DNA copies per microliter was low in four of these cases. The parasitaemia in the positive samples from the children ranged from 180–300,000 parasites/µL (mean: 27,792 parasites/µL, median: 6,600 parasites/µL). The correlation of the parasitaemia and the quantitative RT-PCR result from the central filter paper disk was 0.52 ($P < 0.01$).

The results of the reproducibility are shown in Table 2. Although the quantitative results for the RT-PCR tended to be approximately in the same decimal order, the coefficient of variation showed values ranging from 10% to 90%.

This study confirms the good performance of the commercial real-time PCR for the detection of *Plasmodium* spp, with 100% agreement in fresh blood samples. The five "false positive" PCR results in the pregnant women are most likely true positives, supported by the fact that a flow-cytometric method used to determine the FBC, indicated the presence of hemozoin-containing monocytes as described elsewhere.[11] This is in keeping with studies that show that both PCR methods[1] and hemozoin detection[12] allow identification of more malaria cases in pregnant women than microscopy of peripheral blood smears do. The three "false negatives" may have been caused by the nature of the sample (erythrocyte pellets) or the storage conditions (freezing) of the blood sample. However, the internal amplification control of the kit makes the presence of inhibitors a rather unlikely explanation.

Table 1. Overall results of microscopy and real-time PCR.

Children (n = 47)	**RT-PCR positive**	**RT-PCR negative**	**Total**
Microscopy positive	34	0	34*
Microscopy negative	0	13	13
Total	34	13	47
Women (n = 51)			
Microscopy positive	11	3	14
Microscopy negative	5	32	37
Total	16	35	51

*Microscopy showed 26 cases of *P. falciparum*, two *P. malariae* and 5 mixed infections with *P. falciparum* and *P. malariae.* All 14 microscopy positive samples in pregnant women were *P. falciparum.*

Table 2. Quantification and reproducibility of RT-PCR results.

Sample	Microscopy: (parasites/μl)	RT-PCR (DNA copies/μl)					CV, (%)
		Centre	Circle: 1	Circle: 2	Circle: 3	Circle: 4	
1	180	1,590	1,720	1,360	1,670	709	29,5
2	530	77,4	49,1	45,6	33,4	42,1	13,7
3	1,460	30	60,9	30,7	7,86	3,21	89,0
4	5,000	450	133	175	146	159	10,2
5	6,600	133	104	18,5	143	121	48,8
6	14,000	441	96,8	185	98	110	29,7
7	46,000	20,3	29,6	19,7	35,8	22,3	23,5
8	50,000	3,710	9,620	16,700	12,000	10,600	22,2
9	150,000	29,800	4,800	3,530	3,890	3,940	11,5
10	300,000	7,270	6,090	3,840	4,950	5,520	16,3

In ten samples from children the RT-PCR was performed using 5 disks, one from a punch in the centre of the blood spot (centre) and four from punches on a virtual circle 3 mm from the centre circle 1, 2, 3 and 4). CV: coefficient of variation.

The FTA® filter cards were easy to use and the elution procedure was simple and efficient. However, the correlation between parasitaemia and number of copies obtained by real-time PCR was rather low. It appears that the application of whole blood on the filter paper leads to an uneven distribution of the blood components. In fact, when whole blood was only spotted in the centre of the circle, liquid blood components diffused to the outside, while RBCs remained rather in the centre. This may be the reason why the manufacturer recommends to apply the samples in a spiral-like motion to the outside. However, the results of the reproducibility indicate that the distribution of RBC and consequently plasmodial DNA remains rather uneven. This is in contrast with some studies on quantification of viral DNA/RNA that showed good performance and reproducibility.[13,14] However, viral particles in blood or serum may diffuse more homogeneously when spotted on filter paper.

Overall, we found that the DNA extraction and the real-time PCR method were very easy to perform and the whole process could be completed in around 4 hours. Nonetheless, one drawback may be the rather high cost for both, the filterpapers and the commercial assay.

Finally, it might have been interesting to evaluate the RT-PCR, by using fresh blood directly as well as spotting it on filtercards, however, the performance of the used RT-PCR using fresh blood directly has been reported before (6).

In conclusion, the real-time PCR method as well as the filter card system were easy and robust, yielding 100% concordance with microscopy, when fresh blood was used. However, the filter cards may not be the most appropriate solution when highly reliable and reproducible quantitative results are required apart from the rather high price (~2–3 US$ each).

Disclosure

The authors report no conflicts of interest.

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