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Low sensitivity of nested PCR using *Plasmodium* DNA extracted from stained thick blood smears: an epidemiological retrospective study among subjects with low parasitaemia in an endemic area of the Brazilian Amazon region

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Abstract

Background: The success of PCR technique depends on many factors, such as high quality DNA pellets obtained from blood samples, good reagents and adequate conditions of amplification. Taking these limitations into account, a retrospective epidemiological study for malaria diagnosis was conducted in a mesoendemic area in the Brazilian Amazon.

Methods: A nested PCR protocol with DNA extracted from two blood storage devices obtained from Giemsa-stained thick blood smears and filter-papers was used for malaria diagnosis. The extracted DNA was used as a template to amplify approximately 100 bp species-specific sequences of the small subunit of the ribosomal RNA (18S SSU rRNA) of *Plasmodium* sp. The prevalence of single and mixed infections was examined in a cross-sectional survey carried out among 369 miners living in the district of Apiacás, Mato Grosso State. The parasitemia levels detected by microscopic examination were compared to the PCR results.

Results: DNA samples isolated from blood on filter-paper allowed the detection and identification of *Plasmodium* in 165 (44.7%) of the 369 individuals evaluated, while only 62 (16.8%) had positive results using DNA obtained from thick smears, a similar rate observed by microscopic examination. The sensitivities of PCR using DNA from blood smears and filter-papers were 65% and 73.0%, respectively. Low parasite infections (below 20 parasites/μL blood) were not detected when thick blood smears were used as a DNA source.

Conclusions: Although the blood preserved as thick blood smears provides an alternative and useful tool for malaria molecular diagnosis, its relatively poor performance at low level parasitemias impairs the correct determination of malaria prevalence in epidemiological studies. However, the results obtained in the present study confirm that the use of filter-paper to collect blood is useful for field studies.

Background

The standard method for detecting *Plasmodium* infection is the microscopic examination of Giemsa-stained thick blood smears (TBS). Although effective and inexpensive, this method is laborious and time-consuming, and its sensitivity drops with the decrease of parasitemia [1-3].

Recently, alternative diagnostic methods, such as PCR, have been used for the detection and identification of malaria parasites. The PCR method successfully detects parasites in mixed and low level infections, being more sensitive when compared to microscopic examination [3-6], validating its use in malaria epidemiological studies.

It is well known that the success of the PCR technique depends on a variety of factors such as high quality DNA obtained from blood samples, good reagents and adequate conditions of amplification. Whole blood has been shown to be a reliable source of high-quality DNA [7,8] when it is carefully transported, handled and stored to prevent contamination or degradation of the DNA. Giemsa-stained or unstained TBS and, particularly, blood conserved on filter-papers have been used as a source of DNA in molecular and epidemiological studies [9-14].

Considering the influence of the quality of biological material in malaria molecular diagnosis, the efficacy of PCR in detecting malaria infection using DNA from blood conserved as Giemsa-stained TBS or on filter-paper, was evaluated. Analysed data from a cross-sectional survey carried out among miners living in the district of Apiacàs, northern Mato Grosso State in the Brazilian Amazon, are presented here. The influence of parasitemia levels on PCR positivity using two different blood-storage blood-storage methods, Giemsa-stained TBS and filter-paper, was also evaluated.

Methods

Study area, individuals and blood sample collection

The population studied consists of migrant miners who had lived for approximately 20 years in a Brazilian malaria endemic area. At the time of blood collection (1996) these individuals were living in the district of Apiacàs in the Mato Grosso state. Consent for drawing blood was obtained from each individual according to the Universidade Federal de Mato Grosso and Universidade Federal de Minas Gerais ethic committee rules. Venous blood samples (1 ml/individual) were drawn in EDTA Vacutainer® tubes (Becton Dickson, Oxnard, CA). Ten microlitres of blood were used to prepare the TBS and an equal volume was conserved on filter-paper. The Giemsa-stained TBS were kept at room temperature and the filter-papers were air-dried and placed individually in plastic bags until required for DNA extraction. The parasite density was quantified after examination of 200 microscopic fields at l.000× magnification under oil-immersion. All slides were examined by three well-trained microscopists from the Brazilian Ministry of Health. The malaria prevalence as detected by microscopic examination of TBS was 17.3% (95% confidence interval [CI]; range, 14.2–20.8%) in the general population (527 individuals evaluated) indicating a mesoendemic epidemiological pattern of transmission of *Plasmodium falciparum* and *Plasmodium vivax*. In the present study we have selected 62 infected individuals and 307 individuals who were negative for any *Plasmodium* species as assessed by TBS microscopic examination. These 369 individuals had their DNA samples isolated from both stained thick blood smears and filter-paper.

DNA template preparation

Blood samples conserved as TBS or on filter-paper were used in 2002 for extraction of *Plasmodium* DNA as previously described in references 15 and 16, respectively. Briefly, the Giemsa-stained TBS containing 10 µL of blood were destained and scraped using a clean scalpel blade and collected in a microfuge tube containing 4 M guanidine. Alternatively, filter-papers containing 10 µL blood samples were punched, placed in a microfuge tube containing 250 µL of lysis buffer (50 mM NaCl, 50 mM Tris HC1 [pH 7.4], l0 mM EDTA; 1% [vol/vol] Triton X-100, 200 µg of proteinase K/mL) and incubated overnight at 60°C. All the samples were extracted with phenol-chloroform and DNA was precipitated with isopropanol. DNA was dissolved in 30 µL of sterile water and kept at -20°C until use. DNA from *P. falciparum*, *P. vivax* and *Plasmodium malariae* were used as positive controls. They were randomly included to check inhibition during extraction or amplification processes.

Nested PCR assay

DNA samples were processed by nested PCR to amplify species-specific sequences of the small subunit ribosomal ribonucleic acid (18S SSU rRNA) genes of *P. falciparum*, *P. vivax* and *P. malariae*, as described [15] with modifications. In the first step, a 130 bp fragment of the *Plasmodium* sp 18S genes was amplified using 10 pmoles of each genus-specific primer named P1 (5' ACGATCAGATACCGRCGTAATCTT 3') and P2 (5' GAACCCAAAGACTTTGATTTCTCAT 3'). Twenty microliters of a PCR mixture (200 µM each dNTPs, 25 mM $MgCl_2$, *taq* buffer (l0 mM Tris-HCl [pH 9.0], 50 mM KC1, 0.1% Triton X-100 and 1.25 U of *Taq* polymerase) were added to 5 µl of isolated DNA. The DNA amplification was carried out under the following conditions: 92°C for two min, 35 cycles at 92°C for 30 s and at 60°C for one min, followed by a final extension at 60°C for five min.

The first amplified PCR product was diluted 40-fold in sterile water. Five µL of this solution were used in the

second amplification. The PCR procedure was performed using P1 in combination with each species-specific primer to *P. falciparum* (5'-AATCTAAAAAGTCACCTCGAAAGATG-3'), *P. vivax* (5'-CAATCTAAGAATAAACTCCGAAGAGAAA-3') and *P. malariae* (5'-GAAGCTATCTAAAAGAAACACTCATAT-3') in individual tubes. The conditions of DNA amplification used was 92°C for 2 min; 25 cycles at 92°C for 30 s and 60°C for one min, 60°C for five min to final extension.

The amplified products were visualized in 1.5% agarose gel stained with ethidium bromide. The expected band sizes were 100 bp for *P. falciparum* and *P. vivax* and 110–115 bp for *P. malariae*.

In order to prevent cross-contamination, different sets of pipetes and distinct work areas for DNA template and mix preparation and DNA amplification were used. Moreover, one uninfected blood sample was included for every ten samples processed. Twenty percent of positive-PCR samples were re-tested to confirm the presence of plasmodial DNA.

Amplification of human DNA

To check for the presence of DNA or inhibitory effects of components found in blood, all negatives samples of *Plasmodium* sp. were amplified with a oligonucleotide primer specific for human β-globin as described [17], with modifications. This amplification was carried out in the following conditions: 95°C for four min, 30 cycles at 55°C for 30 s, 72°C for 30 s, and 95°C for one min followed by a final extension at 72°C for two min. The fragment amplified (280 bp) was analysed by 1.5% agarose gel electrophoresis stained with ethidium bromide.

Statistical analysis

Individual data points were stored in a data base using Epi-Info package version 6.03 (Centers for Disease control and Prevention, Atlanta, GA). Differences in proportions were evaluated by the chi-square test. The nonparametric Mann-Whitney test was used to compare median of parasitaemia. The values for sensitivity and specificity were estimated using microscopy examination as gold standard. The *k* statistics was used to measure agreement between the PCR results obtained using two difference sources of DNA. *P* values <0.05 were considered significant.

Results

In the present study, to each nested PCR reaction one positive and one negative control were used. The *Plasmodium* sp. negative test samples were assayed for DNA quality and the presence of inhibitors using the human β-globin specific primers. All samples showed a fragment of 280 bp, specific for this gene.

Agreement of PCR results using DNA extracted from two different blood storage devices in comparison to microscopic examination

The nested PCR using DNA from TBS was positive in 62 of 369 samples (16.8%), the same proportion observed by microscopy. However, the PCR detected malarial species not identified by microscopy in 22 samples. On the other hand, for unknown reasons, *Plasmodium* DNA was not detected in other 22 individuals who had microscopically detectable parasites on their blood smears (Table 1). The PCR which used blood stored on filter-paper detected 165 (44.7%) individuals positive for *Plasmodium* DNA, 120 of which were undetected by microscopy. Similar to the PCR made on TBS, the filter-paper PCR did not detect 17 cases that were detected by microscopy. Thirteen samples that were positive by microscopic examination but negative by PCR from TBS were also negative by PCR using DNA from filter-paper.

Table 1: Sensitivity and specificity of nested PCR to *Plasmodium* detection according to the different blood storage conditions.

	Blood Storage Device			
Microscopic examination	**Thick Blood Smears**		**Filter-papers**	
	Positive	**Negative**	**Positive**	**Negative**
Positive	40	22	45	17
Negative	22	285	120	187
Total	62	307	165	204
Sensitivity	65%		73%	
Specificity	93%		61%	
Observed concordance	88%		63%	
Kappa coefficient (95%)	0.57		0.20	

Table 2: *Plasmodium* species as detected by nested PCR using DNA extracted from thick smears and filter-papers

Species detected	Positive samples according to the blood storage device		Statistical Analysis		Positive samples by microscopic examination of TBS(%)
X	TBS (%)	FP (%)	X^{2*}	*p*	
P. falciparum	34 (9.2)	78 (21.1)	19.46	<0.001	27 (7.3)
P. vivax	11 (3.0)	36 (9.8)	5.99	<0.05	28 (7.6)
P. malariae	11 (3.0)	14 (3.8)	0.17	>0.05	0
P. falciparum/P. vivax	0	10 (2.7)	8.21	<0.05	07 (1.9)
P. falciparum/P. malariae	06 (1.6)	10 (2.7)	0.57	>0.05	0
P. vivax/P. malariae	0	13 (3.5)	11.28	<0.001	0
P. falciparum/P. vivax/P. malariae	0	04 (1.1)	4.02**	>0.05	0
Total of positive samples	**62 (16.8)**	**165 (44.7)**	**66.19**	**<0.001**	**62 (16.8)**

*Chi-square test Yates correction **Fisher exact test

The sensitivity and the specificity of PCR using DNA from blood smears were 65% (95% CI; range, 51.0 to 76.0%) and 93.0% (95% CI; range, 89.0 to 95.0%), respectively. The agreement between this method and microscopic examination was moderate (*Kappa* = 0.57; [95% CI; range:0.46 to 0.69]). Using DNA from blood stored on filter-paper, the agreement was even lower than with the previous storage method (*Kappa* = 0.20 [95% CI; range, 0.07 to 0.33]). Among the 307 microscopy-negative samples, this PCR method detected as many as 120 samples containing *Plasmodium* DNA. The sensitivity and the specificity of the nested PCR using filter-paper samples were 73.0% (95% CI; range, 60.0 to 83.0%) and 61.0% (95% CI; range, 55.0 to 66.0%), respectively.

Prevalence of Plasmodium species detected by nested PCR according to the DNA storage device

Plasmodium sp. was detected in 62 (16.8%) and 165 (44.7%) of 369 DNA samples extracted from scrapes of TBS or filter-paper, respectively (Table 2). The number of malaria positive cases detected using DNA extracted from blood conserved on filter-paper was statistically higher when compared to the method which used DNA obtained from scraped TBS ($p < 0.001$). This is also true when the prevalence of *P. falciparum*, *P. vivax* and mixed infections (*P. falciparum*/*P. vivax* and *P. vivax*/*P. malariae*) were analysed separately.

Effect of blood parasite density on PCR positivity

The nested PCR results using DNA from TBS and blood on filter-paper were analysed in relation to the level of circulating parasites detected by microscopic examination. For this analysis, only the 62 patients with parasites detected by TBS microscopic examination were included. Forty five of those patients, showing a mean parasitemia of 598 parasites/μL of blood (range: 10–4000, median 100 parasites/μL), were PCR-positive with DNA from filter-paper, whereas the 17 individuals that were PCR-negative had a mean parasitemia of 12 parasites/μL (range: 10–200, median 30 parasites/μL), a statistically lower number when compared to the former group ($p < 0.05$). For samples amplified using DNA from TBS, there was no statistical difference between the mean parasitemias from patients who were PCR-positive or PCR-negative [470 parasites/μL (range: 10–4000, median 130 parasites/μL) and 225 parasites/μL (range: 20–800, median 85 parasites/μL), respectively]. Interestingly, PCR-negative individuals whose DNA samples were amplified from filter-paper showed a statistically lower mean number of parasites when compared to TBS-PCR-negative results. From the 307 samples that were considered negative by microscopic examination, 22 (7.1%) and 120 (39%) were positive using DNA from TBS and blood on filter-paper, respectively ($p < 0.05$).

Discussion

Although the microscopic examination of TBS remains the method of choice for the diagnosis of human malaria, in recent years, considerable attention has been given to molecular methods, including the PCR technique. In malaria studies, this method is considered to have a promising future, especially due to the identification of parasites in areas where four *Plasmodium* species occur simultaneously [3]. Nevertheless, it has been recognized that the success of the technique depends on the quality of DNA. It has been observed that intrinsic (as DNA amount or a high content of human DNA or haemoglobin) and extrinsic (use of heparin or inadequate conditions of blood collecting, storage and amplification of samples) factors can inhibit the PCR assay [3,18,19]. This retrospective study has evaluated the sensitivity and specificity of PCR to detect malaria parasites according to the blood conservation devices used for DNA extraction. Considering the two alternatives for DNA preparation, the

best results (44.7% of prevalence) were obtained for nested PCR analysis of the 18 SSU rRNA genes when target DNA was isolated from blood on filter-papers. The study showed that the sensitivity and specificity of nested PCR were 73% and 61%, respectively, when target DNA was extracted from filter-paper and 65% and 93% when DNA was obtained from thick smears. This is probably due to the conservation of the biological material used as a source of DNA, which directly affects the quality of DNA and, consequently, the sensitivity and specificity of the PCR assay. The sensitivity rates below 80% observed for both DNA preparation methods could be explained by the low level of circulating parasites in the blood of the individuals who were part of our study. The number of false negative results observed with PCR using DNA from thick smears could be an effect of a reduced number of parasites present in the samples, as some could have been lost during the process of scraping the slides. Furthermore, factors involved in preparing slides for microscopic examination may contribute to the stability of DNA template. Classical methods for fixing and storage of cell may be crucial factors in determining the rate of DNA degradation. For example, methanol fixation may affect the dissociation of protein-nucleic acid complexes within the cell. Thus, Giemsa-stain and excessive manipulation of samples could act negatively on DNA integrity. Thus, TBS should be used as a DNA source mainly when a large number of parasites is present on the slides, which will reduce the risk of false negative results enabling the success of the technique.

In contrast to the results of the present study, the use of DNA from thick or thin smears has produced good results by PCR assay in the different studies involving *Plasmodium* [9,12-15,20]. All these studies demonstrated that *Plasmodium* DNA might be successfully isolated from TBS indicating that this method of DNA preservation could be considered adequate and convenient for epidemiological studies. A possible explanation for the differences between our results and the above mentioned is the relatively reduced number of parasites present in our samples. On the other hand, the results obtained by PCR using isolated DNA from filter-papers indicate its great usefulness in field studies. Although false negative results have occurred in our study, the use of isolated DNA from filter-paper allowed the detection of *Plasmodium* in several samples previously negative by microscopic examination. *Plasmodium falciparum* is the most prevalent species in the study area. A higher number of mixed infections involving this species was observed when PCR was performed using DNA from filter-paper. In this case, delayed or missed diagnosis increases the possibility of complicated or severe malaria. Moreover, the results of this study demonstrated that the *P. malariae* (11%) and the prevalence of mixed infections (10%) may be substantially higher than previously reported in Brazil. The false negative results observed using DNA from filter-paper may reflect trapping of parasite DNA in the filter-paper, as previosuly reported [21]. The present study corroborates previous results [10,22], which showed that material on filter-paper appears to keep its characteristics and that this method is a simple, low cost way for preserving blood, with less risk of contamination and/or DNA degradation due to handling, transport and storage.

An important point to be discussed is that routine microscopy failed to detect very low parasite densities in Apiacás population studied. However, malaria prevalence as diagnosed by PCR using filter-paper DNA showed a high number of subclinical parasitaemia (32.5%) mainly among symptomless subjects. In the Apiacás epidemiological setting, the detection of low-level parasitaemia may not be clinically relevant or represent an indication of treatment since the development of clinical immunity has been reported for this population [6,23,24] and others [25] living in the Brazilian Amazon. Whether those individuals with negative thick blood smears but positive PCR may act as reservoirs of the parasite remains unclear. Although in a malaria endemic area it is most probable that the PCR actually detects infection, a prospective study performed in the symptomless individuals would be advisable to confirm the infection.

Conclusions

1- The blood storage on thick smears and filter-paper is useful to DNA isolation which could be used in nested PCR to amplify the 18 SSU rRNA gene of *Plasmodium* species in retrospective studies.

2- Best results were obtained for nested PCR when target DNA obtained from blood on filter-papers was used. The sensitivity of malaria parasite detection by PCR may be influenced by the way blood is collected and stored.

3- The PCR assay, using DNA from filter-paper indicates that the malaria infection prevalence in Apiacás is 44%, higher than previously diagnosed microscopically (17%), indicating that the malaria epidemiology of Apiacás must be re-evaluated.

4- Giemsa-stained thick blood smears, commonly the only available material, may be used to carry out epidemiological retrospective studies, but one limitation to its use is that samples containing a low number of parasites may produce false negative results by PCR.

5- When blood samples cannot be collected conventionally, collection of blood on filter- paper rather than stained thick smears should be preferred method for sample storage for PCR analysis.

Authors' contributions

KKGS carried out all the experiments of this work. CJFF was the physician responsible for the clinical assistance of the blood donors who participated in the design of the study and also performed the statistical analysis. ACN participated in the planning and execution of the PCR assays as well as in the analysis of the results. MFH participated in the analysis of the results and in the manuscript preparation. EMB conceived, designed and coordinated this study. All authors read and approved the final manuscript.

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