Plasmodium DNA Contamination between Blood Smears during Giemsa Staining and Microscopic Examination

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Giemsa-stained blood smears are mainly used for microscopic examination to diagnose malaria. However, they may also be subjected to polymerase chain reaction (PCR) to confirm diagnosis or for retrospective studies requiring the analysis of old smears. We investigated the possibility of DNA contamination occurring during automated Giemsa staining or due to the failure to clean the oil-immersion objective during microscopic examination. We tested blood smears from uninfected, *Plasmodium vivax*–infected, and *P. falciparum*–infected patients. DNA contamination was observed after both staining and microscopy, although contamination was unpredictable during staining. These results are of utmost importance when smears are used for PCR.

Plasmodium falciparum DNA from Giemsa-stained or unstained thick blood smears can be amplified by polymerase chain reaction (PCR), as has been shown elsewhere [1, 2]. As Giemsa-stained thick and thin blood smears constitute the most widespread method of malaria diagnosis in all endemic areas [3], retrospective studies involving the analysis of old smears are possible [4].

Before performing a retrospective PCR-based analysis, we studied the possibility of DNA contamination between blood smears. The blood smears for the planned retrospective analysis were collected from several health centers in French Guiana. All blood smears had previously been Giemsa stained by a staining robot (DRS-60; Sakura-Finetechnical) and examined by microscopy. The staining robot simultaneously dips 30 smears in and out of Giemsa stain for 15 min. Giemsa stain is prepared in the morning and used all day. Stained blood smears are examined by microscopy (magnification, ×100), with immersion oil. The objective is not routinely cleaned between blood smears, and immersion oil is taken directly, with a dropper, from a bottle that is used until it is empty (on average once a month). Occasionally, the dropper may come into contact with a slide. However, after the drop of immersion oil is applied, the dropper is dipped back into the bottle until the next use. After microscopy, smears are briefly turned over on absorbent paper and conserved at room temperature. Therefore, we performed experiments to assess the risk of DNA contamination during staining and microscopy.

Patients, materials, and methods. Five patients were included in our study. An initial microscopic diagnosis was performed with venous blood samples collected in EDTA-treated tubes. Parasitemia was determined by use of thin blood smears. The control patient (CP) was uninfected. Infected patients had the following parasite densities: 0.5% *P. vivax* (patient VP), 10.4% *P. falciparum* (patient FP10), 3% *P. falciparum* (patient FP3), and 1.5% *P. falciparum* (patient FP1). For each patient, several additional thick blood smears were made, and 1 mL of venous blood was used to confirm the diagnosis by PCR. In brief, erythrocytes were lysed and washed 3 times, before being resuspended in 5 vol of TNE buffer (0.15 mol/L NaCl, 10 mmol/L Tris-HCl, and 1 mmol/L EDTA [pH 8]) supplemented with 0.5% Triton X-100, 0.5% SDS, and 5 mg/mL proteinase K. After 1 h at 37°C, DNA was extracted with phenol-chloroform and precipitated with ethanol. *Plasmodium* species were detected as described elsewhere [5].

Five sets of experiments were independently performed with the staining robot before DNA extraction: (1) as negative controls, 2 blood smears from CP were stained together in the staining bath immediately after its preparation in the morning; (2) blood smears from CP, FP10, and VP were stained together in the staining bath immediately after its preparation in the morning; (3) blood smears from CP, FP10, and VP were stained together in the staining bath after it had been used all day; and (2) and (3) were repeated with blood smears from CP and FP3. Blood smears were dried before DNA extraction and PCR amplification, as described below. Three sets of microscopy experiments were also performed after blood smears had been independently stained. The blood smears were successively examined, in the following order, by microscopy (magnification, ×100), with new immersion oil (prime indicates a different blood smear from the same patient): (1) CP, FP10, VP, FP10′, VP′, and CP′; (2) FP3, CP, CP′, and CP″; and (3) FP1, CP, CP′, and CP″. The objective was washed with soap and water between each experiment but not between smears within an ex-

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periment. Each blood smear was examined for 10 min and turned over once on absorbent paper before DNA extraction. Finally, 3 drops from an old bottle of immersion oil (opened 1 month earlier) were added to a blood smear from CP that previously had been independently Giemsa stained, before DNA extraction. In brief, the sample material was scraped from the glass slide by use of a sterile scalpel and 300 μL of TNE buffer. Thirty microliters of Triton X-100, 16 μL of SDS, and 60 μL of proteinase K (5 mg/mL) were then added. After 1 h at 37°C, DNA was extracted and purified, as above. The nested PCR described elsewhere for *P. falciparum* and *P. vivax* was used to identify *Plasmodium* species [5]. DNA extracted from negative smears from CP were also amplified at the human β-globine locus to confirm that negativity was due to the absence of *Plasmodium* DNA contamination, rather than to the absence of any DNA. The sequences of the primers used were as follows: GLOB1, GAAGAGCCAAGGACAGGTAC and GLOB2, GGTGAACGTGGATGAAGTTG. The conditions used for this PCR were exactly the same as those used for the diagnosis of *Plasmodium* species [5]. The products of amplification were analyzed for size by agarose-gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide.

Results. The nested PCR with whole blood confirmed the absence of *P. falciparum* and *P. vivax* DNA in the blood smear from CP, as well as monospecific infections in FP10, FP3, FP1, and VP. After DNA extraction of negative smears, the amplification at the human β-globine locus confirmed the presence of human DNA. The results of the other experiments are presented in table 1. The 2 blood smears from CP that had been stained in a freshly prepared staining bath were found to be negative for *Plasmodium* DNA. The automated staining of blood smears from CP, FP10, and PV in a freshly prepared staining bath led to contamination of the *P. vivax* smear by *P. falciparum.* At the end of the day, both control and *P. vivax* smears were contaminated by *P. falciparum.* The *P. falciparum* smear presenting 3% parasitemia also contaminated the control smear in the morning, whereas it did not at the end of the day. When blood smears were examined successively by microscopy, with new immersion oil and without washing the objective, the blood smear containing 10.4% *P. falciparum* contaminated the next smear examined. However, the blood smears containing 0.5% *P. vivax* and 3% *P. falciparum* did not contaminate any of the next smears examined. The amplification of DNA extracted from the control blood smear, to which 1-month-old immersion oil had been added, revealed the presence of *P. falciparum* DNA in oil.

Discussion. These results show that Giemsa staining of blood smears may lead to DNA contamination between smears, although contamination events are unpredictable. Microscopic examination with immersion oil, without washing the objective between blood smears, also leads to DNA cross-contamination. Of importance, the use of immersion oil taken directly from

Table 1. Identification of *Plasmodium* species by specific nested polymerase chain reaction (PCR) from blood smears, after automated staining, microscopic examination, or addition of 1-month-old immersion oil.

Samples, process, time of process, patient	Specific nested-PCR result	
	P. falciparum	*P. vivax*
Controls, extraction from whole blood		
CP	Negative	Negative
VP	Negative	Positive
FP10	Positive	Negative
FP3	Positive	Negative
FP1	Positive	Negative
Blood smears		
Stained by robot		
Day 1 morning[a]		
CP	Negative	Negative
CP	Negative	Negative
Day 2 morning		
CP	Negative	Negative
FP10	Positive	Negative
VP	Positive	Positive
Day 2 evening[a]		
CP	Positive	Negative
FP10	Positive	Negative
VP	Positive	Positive
Day 3 morning		
CP	Positive	ND
FP3	Positive	ND
Day 3 evening		
CP	Negative	ND
FP3	Positive	ND
Successive microscopic examinations[b]		
CP	Negative	Negative
FP10	Positive	Negative
VP	Positive	Positive
FP10′	Positive	Negative
VP′	Positive	Positive
CP′	Negative	Negative
FP3	Positive	ND
CP	Negative	ND
CP′	Negative	ND
CP′	Negative	ND
FP1	Positive	ND
CP	Negative	ND
CP′	Negative	ND
CP′	Negative	ND
Giemsa-stained CP plus old immersion oil	Positive	Negative

NOTE. Unexpected results (i.e., contamination) are underlined. CP, uninfected control patient; FP10, infected patient with 10.4% *P. falciparum* parasite density; FP3, infected patient with 3% *P. falciparum* parasite density; FP1, infected patient with 1.5% *P. falciparum* parasite density; ND, not done; VP, infected patient with 0.5% *P. vivax* parasite density. Different blood smears from the same patient are indicated by prime.

[a] The staining bath was prepared in the morning and was used all day for Giemsa staining.

[b] The objective of the microscope was washed between the 2 experiments but not between smears within an experiment.

the bottle may also lead to DNA contamination. Therefore, blood smears that have been previously Giemsa stained and examined by microscopy should be analyzed with caution, since DNA contamination may have occurred. These results are highly important for retrospective studies involving PCR analysis of blood smears, especially to confirm particular cases of malarial infection.

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