STORAGE DURATION AND POLYMERASE CHAIN REACTION DETECTION OF
PLASMODIUM FALCIPARUM FROM BLOOD SPOTS ON FILTER PAPER

Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; Shoklo Malaria Research Unit, Mae Sot, Thailand

Abstract. To evaluate the effect of long-term storage of sample filters on the sensitivity of polymerase chain reaction (PCR) detection of malaria, 252 blood spots from patients with microscopically confirmed Plasmodium falciparum malaria were analyzed and stratified by storage duration. The spots were collected between 1996 and 2000 on filter paper and stored at room temperature. A Chelex-based method was used to extract the DNA. Unexpectedly, after the first purification, the sensitivity of the PCR from recently stored samples was low and showed progressively increased with time storage ($P = 0.003$, by chi-square test for linear trend). This suggested that PCR inhibitors were easier to dissolve from the more recent blood spots ($< 4$ years old) than from blood spots $\geq 4$ years old, thus leading to a time-dependent increase in PCR sensitivity. However, if DNA was purified again (when the first PCR result was negative), the cumulative sensitivity was not influenced by storage duration. This indicated that length of storage is not a critical issue providing purification is sufficient.

INTRODUCTION

Accurate diagnosis and epidemiologic surveillance are essential elements in the management of malaria for controlling the spread of drug resistance. Various polymerase chain reaction (PCR)–based methods have been described for the diagnosis of malaria,¹ for species identification,² and for the study of parasite genetic diversity.³ Most optimized methods use venous blood samples that are then frozen. However, a simplified method of collection and storage has been developed. This has proved cheaper, practical, safe, and does not require venipuncture, since it can be performed on capillary blood samples. Because of its practicality for field-based studies, blood collection on filter paper has been widely used,⁴–⁷ although a 10–100-fold reduction in sensitivity compared with frozen blood has been reported.⁸

Collecting blood spots on filter paper is a convenient way to keep parasite DNA for epidemiologic investigations. Unfortunately, storage in the first 30 days shows a decreased sensitivity in a PCR for different types of samples.⁹ Little is known regarding the effect of long-term storage (> 30 days) on the sensitivity of a PCR in detecting malaria. Thus, the aim of this study was to evaluate the effect of long-term storage of blood samples from patients with Plasmodium falciparum malaria collected on filter paper on the sensitivity of a PCR.

MATERIALS AND METHODS

Study population. Between August 1996 and May 2000, a consecutive cross section of 262 children (mean $\pm$ SD age = 8.6 $\pm$ 4 years) with microscopically confirmed P. falciparum malaria were recruited at the outpatient clinics of the Shoklo Malaria Research Unit in Maela, a camp for Karen refugees on the Thailand-Myanmar border. After informed consent was obtained, blood spots were collected on filter paper for PCR genotyping.³ The samples were stored at room temperature. The patients also had a stool examination. The project was reviewed and approved by the ethical committee of the Faculty of Tropical Medicine, Mahidol University (Bangkok, Thailand). The initial study objective was to determine whether helminths had any association with the multiplicity of infection. However, a low PCR positivity rate after the first purification of DNA led to question the role of storage duration in this observation.

Preparation of DNA, PCR amplification, and product analysis. The PCR was performed in 2001. DNA was extracted from blood spots using a modified method with the InstaGene Whole Blood kit (Bio-Rad Laboratories, Hercules, CA). Briefly, blood spots were soaked overnight in a tube containing 100 $\mu$L of phosphate-buffered saline (PBS) at 4°C. We used overnight soaking in PBS instead of saponin because soaking in saponin for a few minutes was very inefficient for desiccated blood spots, and because overnight soaking in saponin might be harmful to the DNA preparation. The tube was centrifuged at 13,000 rpm for two minutes. After discarding the supernatant, 100 $\mu$L of PBS was added to the tube and the sample was centrifuged at 13,000 rpm for two minutes to wash the sediment. After discarding the supernatant, 60 $\mu$L of InstaGene Matrix was added. The tube was incubated for eight minutes at 70°C, vortexed for 15 seconds, boiled for four minutes, and vortexed for 15 seconds. Finally, the tube was centrifuged at 13,000 rpm for one minute and the supernatant was aspirated and purified again with InstaGene Matrix as described. The supernatant was then used for the PCR. If the PCR result was negative, the supernatant was purified again up to three times.

Nested PCR amplification of the P. falciparum merozoite surface protein-2 (MSP-2) gene was performed using two pairs of primers.⁴ The PCR was performed in a final volume of 10 $\mu$L containing 1.5 $\mu$L of extracted DNA or the first PCR product, 1 $\mu$M of each primer, 200 $\mu$M of each dNTP, 0.3 units of Taq polymerase, 2.5 mM MgCl₂ in 10 mM Tris-HCl, pH 8.3, and 50 mM KCl buffer. The DNA was denatured at 96°C for five minutes, following by 30 cycles of amplification (96°C for 30 seconds for denaturation, 58°C for 45 seconds for annealing, and 72°C for 30 seconds for extension). At the end of 30 cycles, extension was completed by incubation at 72°C for seven minutes. The MSP-2 amplification products were analyzed by electrophoresis on a 2.5% agarose gel (agarose: NuSieve = 3:1; FMC Bioproducts, Rockland, ME).

Microscopic misdiagnoses and MSP-2 variants might have accounted for the 14 negative results. Therefore, nested PCR amplification of small subunit ribosomal RNA (ssrRNA) for species identification was performed. This showed that four samples were still negative, but five samples were positive for...
P. falciparum and five samples were positive for P. vivax. Assuming that samples that were positive for P. falciparum ssrRNA and negative for P. falciparum MSP-2 might have had variant MSP-2 alleles that were not annealed by our primer, we excluded these samples from analysis. The samples that contained P. vivax were also excluded from analysis. Therefore, of the 262 blood spots, only 252 were retained for the analysis.

**Statistical analysis.** Analysis was performed using SPSS 10.0 for Windows (SPSS, Inc., Chicago, IL). The chi-square test and chi-square test for trend were used to explore the relationship between the year when samples were collected and the PCR result for first, second, and third purifications of the DNA. A positive PCR result was defined as one or more bands of MSP-2. Using microscopy as the reference (all cases were positive), we defined the sensitivity of the PCR as the number of positive PCR samples divided by the number of positive PCR samples plus the number of false-negative PCR samples (expressed as a percentage). This was done for the first, second, and third purifications.

**RESULTS**

For the first purification, 31% (79 of 252) of the samples were false negative for MSP-2. When this result was stratified by year, the sensitivity of the PCR in reference to blood smears showed an increase with time (P = 0.003, by chi-square test for linear trend) (Table 1). Notably, sensitivity increased markedly after four years. However, when negative samples were re-purified further, the sensitivity of the PCR increased gradually to 98% (248 of 252) after the third purification; thus, there were no difference of sensitivity in the samples from each year (P = 0.4, by chi-square test).

After the second purification, in contrast to the first purification, the percentage of samples that became positive decrease with time (P = 0.03, by chi-square test for linear trend) (Table 1).

**DISCUSSION**

After three purifications of DNA, there was no effect of storage duration on the sensitivity of an MSP-2 PCR on blood spots stored at room temperature. However, an unexpected finding was that for the first purification, the sensitivity was lowest for the most recent samples, and that it increased with time storage. It was expected that sensitivity would decrease with time because it is more difficult to extract DNA from dry blood on filter paper stored for a long time. However, apart from the quantity of the DNA, the sensitivity of the PCR is also affected by the impurities in the sample, notably heme, which interferes with the PCR. An explanation for our finding was that the longer the samples were stored, the drier the blood spots became and the stronger the fixation of hemoglobin and other blood proteins on the filter paper. Therefore, for the first purification, in samples stored for less than four years, heme and proteins on the filter paper might have inhibited the PCR, resulting in high numbers of false-negative results. Our interpretation was supported by the cumulative increase in PCR sensitivity after repeated purifications. It is possible that each purification removed the remaining PCR inhibitors, as suggested by the switch from a positive correlation between PCR positivity and storage duration after the first purification to a negative correlation between PCR positivity and storage duration after the second purification. This study has shown that length of storage of blood spots for DNA extraction was not a critical issue, and retrospective epidemiologic studies are possible with such samples obtained from endemic areas over long periods of time provided that sufficiently purified DNA is used.

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