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Original Article

Plasmodium falciparum Allelic Diversity: A Comparison of DNA Extraction from Isolates Collected on Rapid Diagnostic Tests (Rdts) and Filter Paper

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Abstract

Background: To perform molecular epidemiologic studies based on large cohorts, material such as RDTs or filter papers are useful for biological sample collection and extraction of RNA or DNA of good quality. Thus, we aimed to assess the quality of DNA extracted from malaria rapid diagnostic tests (RDTs) stored at various temperatures for the analysis of *Plasmodium falciparum* genetic diversity.

Methods: Febrile patients benefitted from free malaria diagnosis using microscopy in a malaria sentinel site, at the Regional Hospital Estuaire-Melen, in Gabon, in 2015. *P. falciparum* isolates were collected onto one filter paper and 2 similar RDTs devices (Acon®) per patient. Nucleic acids were extracted with QiAmp Qiagen kit from paper and RDTs and the quality of the DNA was analyzed by *msp1* gene amplification.

Results: *Msp1* gene amplification was achieved in nucleic acids extracted from all filter papers and RDTs devices (n = 45, 100%). K1 alleles were detected in 93.3% (n = 42/45) of the samples and Mad20 alleles in 73.3% (n = 33/45). The number and the intensity of K1 and/or Mad20 fragments were comparable according to the sample collection material and the storage conditions (room temperature *vs* -20°C) of the samples. The size of the fragments indicating allelic diversity was comparable in 80% (n=36) of the samples.

Conclusion: These data show that RDTs are a valuable source of DNA for malaria parasite genetic polymorphism analysis. Storage conditions of the devices did not influence the quality of DNA extracted from RDTs device, although some alleles may be missed.



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Introduction

Molecular epidemiologic studies performed in malaria endemic areas provided accurate data on *Plasmodium falciparum* genetic diversity and on spread of drug resistant molecular markers. For such studies, large sample collections of *P. falciparum* isolates are required. The use of a simplified blood collection method could help to achieve large cohorts; without altering the quality of data generated.

Basically molecular analyses requires nucleic acids, RNA or DNA, of good quality. Indeed, the success of tools such as PCR, Real Time PCR or sequencing, depends on an extraction of high quality DNA. Thus, stained thick blood films have been successfully tested as source of DNA for real-time PCR species-specific-*Plasmodium* amplification (1-2).

Nevertheless, blood collection on slides cannot be performed in all endemic areas. Likewise, *P. falciparum* detection by genotyping, after DNA extraction from Dried Blood spots is rapid and simple. However, its sensitivity depends on filter paper type (3). Nevertheless, highly sensitive method for detecting *Plasmodium* 18S ribosomal RNA have been developed from DBS samples dried and stored under simulated field conditions to facilitate the molecular surveillance (4). Rapid diagnostic tests (RDTs), widely used as an alternative tool to microscopy for malaria diagnosis (5), constitute a valuable resource for population-based studies. They were shown to be suitable material for genetic studies; as a source of DNA for *Plasmodium* detection and for drug resistance molecular markers characterization (6-9). The type of RDT brands should be considered as well as the storage conditions of the samples, all are factors that will influence the molecular analyses notably the nucleic acids amplification and the data interpretation. The aim of this pilot study was to assess the quality of DNA extracted from malaria rapid diagnostic tests

(RDTs) stored at various temperatures for the analysis of *P. falciparum* genetic diversity.

Materials and Methods

Blood samples were collected by fingerpick from children consulting for fever at the clinical and operational research unit, located at the Regional Hospital of Melen, a malaria sentinel site during one month. This hospital is located at 11 km of Libreville, the capital city of Gabon where malaria transmission is perennial throughout the year. Patients benefitted from a free malaria diagnosis based on microscopy according to Lambaréné's method and Malaria Pf Rapid Test Device® RDTs (Acon Labs, San Diego, CA) in 2015 (10-11). For each patient, blood was collected onto filter paper (903 Saver Whatman) and on two RDTs devices. RDTs devices used were similar and from the same manufacturer. The filter paper and one RDT device were dried and stored at ambient temperature. The second device was kept at -20°C. Samples were kept until molecular analysis was performed at the end of the sample collection period.

RDTs are stored between 19 °C–25 °C within the recommended temperature ranges (4 °C–30 °C) and used within the indicated shelf life. *P. falciparum* RDT positive samples were used. Nucleic acids were extracted from filter paper and RDTs using QiAmp Qiagen kit according to the manufacturer instructions. *Msp1* gene amplification using nested-PCR was done to detect *P. falciparum* and to compare the allelic diversity as previously described (12). Positive and negative controls have been included. Positive controls were *P. falciparum* reference DNA extracted from whole blood as well as RDTs and Filter papers. Negative controls were uninfected blood samples collected on RDTs and Filter papers.

Ethical consideration

The study conducted in the sentinel site was authorized by the Ministry of Health. The Department of Parasitology is the laboratory of reference for malaria diagnosis, malaria tools diagnosis evaluation as well as drug resistance assessment for Malaria National Control Program. All febrile patients benefit from free malaria diagnosis and treatment is prescribed according to national guidelines.

Results

Overall, 15 *P. falciparum* infected patients were randomly selected. The parasite density

ranged from 140p/μl to 74400p/μl; with a median of 37270p/μl. For each patient, 3 samples were collected for molecular analysis. *Msp1* gene amplification was achieved in 45 samples (n = 45, 100%). Two allelic families have been detected: K1 and Mad20 alleles. K1 alleles were found in 93.3% (n = 42/45) of the samples onto filter paper and RDTs. Similarly, Mad20 alleles was detected in 73.3% (n = 33) of the 45 samples (Table 1). Mad20 and K1 amplification rate did not vary according to the storage conditions.

Table 1: Allelic diversity and rate of amplification according to the type of sample blood collection holder

Variable	all RDT		RDT at -20°C		RDT at RT*		Filter paper	
	N ^z	n ^y	N ^z	n ^y	N ^z	n ^y	N ^z	n ^y
K1 allelic family, n (%)	14(93.3)	6(100.0)	14(93.3)	6(100.0)	28(93.3)	6(100.0)	14(93.3)	6(100.0)
Mad20 allelic family, n (%)	11(73.3)	4(80.0)	11(73.3)	4(80.0)	22(73.3)	4(80.0)	11(73.3)	5(100.0)

*RT: room temperature; ^zN: number of samples amplified; ^yn: number of detected alleles

Among *P. falciparum* isolates, 10 carried at least 2 alleles of which 7 had at least 3 alleles. For 80% (n=36) of the samples, the number, the size and intensity of K1 and/or Mad20 alleles detected were comparable whatever the sample holder and the storage conditions (room temperature *vs.* -20°C). Amplification rate and number of alleles detected did not vary according to the parasitemia in RDTs collected isolates (P=0.2).

Within K1 allelic family, six alleles were found: 170 bp, 190 bp, 200 bp, 210 bp, 250 bp,

300 bp in samples realized from isolates n° 1, 2 and 4 to 15. Mad20 alleles, 150 bp, 190 bp, 200 bp, 210 bp and 250 bp, were detected in samples realized from isolates No. 3-6, No. 8-12 and No. 14 and 15. K1 and Mad20 allelic profiles were different in three isolates (n°5, 9 and 12) according to the sample blood collection method (filter paper *vs.* RDTs) (Table 2). For these isolates, the number of K1 or Mad20 alleles detected was lower after DNA extraction from RDTs.

Table 2: Difference of detected alleles within each sample according to the type of sample blood collection holder

Isolates	Number of alleles (K1 and or Mad20) per isolate			
	Filter paper		RDT	
	K1	Mad20	K1	Mad20
N° 5	3(190bp, 200bp, 210 bp)	1(210 bp)	1 (200 bp)	1 (210 bp)
N° 9	2(200bp, 300bp)	2(150bp, 210 bp)	2(200bp, 300 bp)	1(210 bp)
N° 12	2(200bp, 210 bp)	2(150bp, 210 bp)	1(200 bp)	1(210 bp)

Discussion

Extended genetic diversity of *P. falciparum* strains and high multiplicity of infections based on *msp1* gene analysis, a molecular marker of *P. falciparum* polymorphism are observed in Gabonese symptomatic children (12-13). Allelic polymorphism in *msp1* gene Block 2 is represented by three main variants: K1, MAD20, and RO33.

In Gabon, the highest allelic diversity is found in K1 and Mad20 families, therefore the present study focused on their analysis (12-13). *Msp1* gene amplification was achieved in all isolates collected on RDTs and filter papers highlighting successful DNA extraction.

A high rate of amplification of *msp1* and *msp2* genes, as well as, a successful genotyping of malaria drug resistance molecular markers after DNA extraction were reported in RDTs collected isolates from Senegal and Comoros islands (9). In the present study, a high multiplicity of infections was found with more than 70% of isolates carrying at least 2 alleles. Moreover, allelic diversity was comparable in 80% of the isolates. Detection of 11 alleles in 15 isolates indicated a high polymorphism in circulating strains.

These results corroborate data obtained previously in Gabon within K1 and Mad20 allelic families (12-15). Many factors such as storage conditions could affect the efficacy of extracting DNA from field-based RDTs. However, here, no influence of temperature on the quality of DNA extracted from RDTs stored at room temperature or at -20 ° C was observed. The limit of this study can be the determination of multiple infections in isolates since an under estimation of the diversity within isolates can occur. Indeed, in 3 isolates *msp1* amplification was achieved however the number of alleles was different according to the material sample collection. Nested PCR targeting the *Plasmodium* *ssrRNA* gene was used to distinguish *P. falcipa-*

rum, *P. malariae* and *P. ovale*. *Plasmodium falciparum* in previous study but was not required in the present study (16). Indeed, all samples amplified. In addition, the aim of the current study was not to perform molecular surveillance of malaria as reported by others (17-18).

Conclusion

RDTs are good tools for malaria diagnosis but would also be useful for molecular epidemiologic studies and for *Plasmodium* genetic diversity analysis.

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Conflict of interest

The authors declare none conflict of interest whatever the aspect financial and personal that could bias their work. They also declare that all the data presented in the manuscript are their own original work and they take complete responsibility for the integrity of the data and the accuracy of the data analysis.

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