DNA recovery from used malaria RDT to detect *Plasmodium* species and to assess *Plasmodium falciparum* genetic diversity: A pilot study in Madagascar

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Abstract

Background: Rapid diagnostic tests (mRDT) are widely used for malaria diagnosis in Madagascar, where *Plasmodium falciparum* is the predominant species. Molecular diagnosis is essential for malaria surveillance, but requires additional blood samples for DNA extraction. Used mRDTs is an attractive alternative that can be used as a source of DNA. *P. falciparum* genetic diversity and multiplicity of infection, usually determined by the genotyping of polymorphic regions of merozoite surface proteins 1 and 2 genes *(msp1, msp2),* and the repeated region RII of the *glutamate-rich protein gene (glurp)* have been associated with malaria transmission levels and subsequently with the impact of the deployed control strategies.

Thus, the study aims using mRDT as DNA source to detect *Plasmodium* species, to characterize *P. falciparum* genetic diversity and determine the multiplicity of infection.

Methods: A pilot study was conducted in two sites with different epidemiological patterns: Ankazomborona (low transmission area) and Matanga (high transmission area). On May 2018, used mRDT (SD BIOLINE Malaria Ag P.f/Pan, 05FK63) were collected as DNA source. *Plasmodium* DNA was extracted by simple elution with nuclease free water. Nested-PCR were performed to confirm *Plasmodium* species and to analyze *P. falciparum msp1*, *msp2* and *glurp* genes polymorphisms.

Results: Amongst the 170 obtained samples (N=74 from Ankazomborona and N=96 from Matanga), *Plasmodium* positivity rate was 23.5% (40/170) [95%CI: 17.5 – 30.8%] by nested-PCR with 92.2% (37/40) positive to *P. falciparum*, 5% (2/40) to *P. vivax* and 2.5% (1/40) to *P. falciparum/P. vivax* mixed infection. Results showed high polymorphisms in *P. falciparum msp1*, *msp2* and *glurp* genes. Multiple infection rate was 28.6% [95% CI: 12.2 - 52.3%]. The mean of MOI was 1.79 ± 0.74.

Conclusion: This pilot study highlighted that malaria diagnosis and molecular analysis are possible by using used malaria RDT. A large-scale study needs to be conducted to assess more comprehensively malaria parasites transmission levels and provide new data for guiding the implementation of local strategies for malaria control and elimination.

Trial registration: retrospectively registered

Background

Despite the deployment of considerable efforts and various strategies to control and eliminate malaria, this disease remains one of the main cause of morbidity and mortality worldwide [1]. In 2020, 241 million cases were reported leading to 627,000 deaths [2]. Sub-Saharan Africa including Madagascar still remains the most affected region [2]. Among the five *Plasmodium* species infecting humans, *P. falciparum* is the most prevalent malaria species in Sub-Saharan Africa and the most virulent species leading severe malaria [3].

Rapid Diagnostic Tests for malaria (mRDT) are commonly used for malaria diagnosis in field by community health workers to promptly manage malaria cases. Malaria molecular diagnosis are essential for epidemiological surveillance, but requires to collect additional blood samples for DNA extraction. Used mRDTs is an attractive alternative that can be used as a source of DNA [4-6].

P. falciparum genotyping remains an important tool for studying types and numbers of parasite clones present in an infection. Currently, this approach is mainly used to investigate the genetic diversity of parasites in human infections and subsequently estimate the malaria transmission intensity [1, 7-10]. Moreover, numerous studies have demonstrated an association between high multiplicity of infection (MOI) and malaria severity, especially in high malaria transmission areas [11]. Usually, *P. falciparum* genetic diversity is determined by the genotyping of the polymorphic regions of the block 2 of merozoite surface protein-1 (*msp1*), block 3 of merozoite surface protein-2 (*msp2*) and the RII repeated region of the glutamic rich protein genes (*glurp*) [12-15].

In Madagascar, four to eight malaria ecozones are described according to their different epidemiological profile that range from low to high transmission [16, 17]. Regular molecular and epidemiological monitoring over time and space of the genetic diversity of *P. falciparum* populations in association with malaria phenotypes (uncomplicated malaria, severe malaria and asymptomatic malaria cases) is crucial to evaluate the impact of malaria control interventions and to guide the deployment of local tailored strategies for elimination [1, 11, 18, 19].

Hence, this study aims to valorize blood samples collected onto mRDT as DNA source for detecting *Plasmodium* infections, assessing the genetic diversity of *P. falciparum* populations and estimating the MOI in *P. falciparum* isolates collected from symptomatic patients seen in health centres in two sites with different epidemiological patterns: Ankazomborona, located in a low transmission area and Matanga in a high transmission area.

Materials And Methods

Study sites and sample collection. The study was conducted on May 2018 in two sites. In Ankazomborona, district region of Marovoay, located on the west coast of Madagascar (16° 07’ 00” S and 46° 45’ 00” E), a seasonal, endemic and low transmission area and in Matanga, district region of Vangaindrano, located on the east coast of Madagascar (23° 31’ 00” S and 47° 33’ 00” E), a perennial endemic and high transmission area (Figure1). *P. falciparum*-positive mRDT were collected. They were obtained from children aged 6 months to 15 years suffering to uncomplicated malaria. These mRDT containing blood samples were conserved at room temperature before DNA extraction.

DNA extraction. To improve the protocol aiming at extracting parasite DNA from mRDT, EDTA tubes containing whole blood infected by *P. falciparum* and *P. vivax* were used. Blood smears were read to identify *Plasmodium* species and estimate the parasite density (parasites/µL). Blood samples were then diluted with non-infected blood to obtain aliquots containing parasitemia ranging from 1500 parasites/ µL to 5 parasites/µL. Four mRDT were tested for each dilution. The cassettes of mRDT were opened

laterally and the strips were taken out and cut for DNA extraction. For each parasite densities, four different parts of the strip were used to estimate the best yield of DNA extract: (A) distal part, (B) central part, (C) proximal part and (D) all parts (Figure 2).

Two methods of DNA extraction were applied: the Instagene Matrix© method (BioRad™), according to the manufacturer's instructions and a simple elution method in water as previously described [4].

P. falciparum **and** P. vivax **detection by nested-PCR.** Nested-PCR [20] was performed to detect *P. falciparum* and *P. vivax* DNA and estimate which DNA extraction method and which part of mRDT strip provide the more reliable results. Nested-PCR assays showed that all parts of mRDT strip and simple elution method in water were the best approaches (see Results section). These methods were then selected for all further experiments.

Plasmodium falciparum msp1, msp2 and glurp genotyping. The polymorphic region of *msp1*, *msp2* and *glurp* were genotyped using nested-PCR. Primers targeting the block 2 region of *msp1*, the block 3 region of *msp2*, and the RII repeated region of *glurp* were used for primary PCR (Table 1). All PCR reactions were carried out in a total volume of 25μL, containing 200nM dNTP mix, 2mM $MgCl_2$, 200nM each of forward and reverse primers for both *msp1*, *msp2* and *glurp*, 0.5 U of Taq DNA Polymerase (Bioline) and 3μL of extracted DNA, used as template. PCR amplification of *msp1*, *msp2* and *glurp* genes comprised an initial step of 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min 30 s, and a final extension of 72°C for 5 min.

Table 1
Primary and secondary PCR primers

Gene	Allelic type	PCR round	Primer sequence (5'–3')	Fragment size (bp)
Msp1		**Primary PCR**	CACATGAAAGTTATCAAGAACTTGTC	633
			GTACCGCTAATTCATATTCTATTGCTAG	
	MAD20	**Nested PCR**	GAACAAGTSGAACAGCTGTTA	120-250
			TGAATTATCTGAAGGATTTGTACGCCT	
	K1	**Nested PCR**	GAAATTACTACAAAAGGTGCCAAGTG	160-300
			AGATGAAGTATTTGAACGAGCTAAAGT	
	RO33	**Nested PCR**	GCAAATACTCAAGTTGTTGCAAAGC	100 – 160
			AGGATTTGCAGCAYCCTGGAGATCT	
Msp2		**Primary PCR**	ATGAAGGTAATTAAAACATTGTCTATTAAT	811
			ATATGGCAAAAGATAAAACAAGTGTTGCTG	
	3D7	**Nested PCR**	GCAGAAAGTAAKGCCTYTCTACTGGTGCT	150-350
			AGATGAAGTATTTGAACGAGGTAAAGTG	
	FC27	**Nested PCR**	GCAAATGAAGGTTCTAATACTAATAG	300-600
			GCTTTGGGTCCTTCTTCAGTTGATTC	
Glurp		**Primary PCR**	ATG AAT TYG AAG ATG TTC ACA CTG AAC	1200
			ATG AAT TYG AAG ATG TTC ACA CTG AAC	
		Nested PCR	CTG AAC CAA ATCA AAA TAA CG	600 – 1000
			TTC TTC TGG TTT TAT AGT TTC	

For the nested-PCR, specific primers to allelic families of *msp1* (MAD20, K1, and RO33), and *msp2* (3D7 and FC27) were used. For *glurp* amplification, inner primers were used to amplify generated amplicons (Table 1). This secondary reaction contained the same reagents as the primary reaction except primers (Table 1) and 2μL of the primary PCR product was used as DNA template. The cycling profile for the secondary PCR was similar to the primary PCR for *glurp* while for *msp1* and *msp2* the annealing temperature was increased from 55°C to 60°C. DNA from non-infected blood and from reference *P. falciparum* strains (3D7, Dd2 and 7G8) were included in each set of PCR reactions as a negative and positive controls.

Eight microliters of nested PCR products were loaded on 2% agarose gel stained with ethidium bromide and separated by electrophoresis for an average of 60 min at 120 V. After electrophoresis, the gels were visualized under UV trans-illumination using Image Lab gel doc system and then analyzed to estimate the bands sizes. PCR products size were estimated by Image Lab software using 100bp DNA ladder marker.

Polyclonal infection was defined by the presence of more than one allele for a given gene [21, 22]. Multiplicity of infection (MOI) was defined by the number of genotypes per infection [23].

Results

Optimization of DNA extraction. Out of 16 mRDT samples (8 tested with *P. falciparum* infected bloods and 8 by *P. vivax* infected bloods), DNA extracted from all parts of mRDT strip and simple elution method in water gave successful results by PCR amplification (Figure 3).

P. falciparum **and** P. vivax **infections by nested PCR.** Out of 170 samples of used mRDT (74 from Ankazomborona and 96 from Matanga) analyzed, nested-PCR allowed to detect 40 positives samples (23.5%, 95%CI: 17.5 – 30.8%) of which 37 were *P. falciparum* (92.5%, 95%CI: 78.5 – 98.0%), 2 *P. vivax* (5%, 95%CI: 0.9 – 18.2%) and 1 *P. falciparum*/*P. vivax* mixed infection (2.5%, 95%CI: 0.1 – 14.7%) (Table 2).

Table 2
Positivity of P. falciparum and P. vivax by nested PCR

	Ankazomborona (n = 74)		**Matanga (n = 96)**		**Total (N = 170)**	
	n	**%**	**N**	**%**	**n**	**%**
P. falciparum	16	21.6	21	21.9	37	21.8
P. vivax	2	2.7			2	1.2
P. falciparum + P. vivax	1	1.4			1	0.6
Negative	55	74.3	75	76.5	130	76.5

Frequency of msp1 and msp2 allelic families. Out of all 38 *P. falciparum* positive samples, 21 were successfully amplified for *msp1* (55.3%, 95%CI: 38.5 – 71.0%) and 9 for *msp2* (23.7%, 95%CI: 12.0 – 40.6%). For *msp1*, MAD20 was the most frequent allelic family and detected in 52.4% (95%CI: 30.3 – 73.6%) of the samples, followed by RO33 (47.6%; 95%CI: 26.4 – 69.7%) and K1 (28.6%; 95%CI: 12.2 – 52.3%). A total of 28.6% of the positive samples for *msp1* contained polyclonal infection with MAD20/K1, MAD20/R033 and K1/R033. For *msp2*, all positive samples (9/9) belonged to the FC27 allelic family and were classified as monoclonal infection. None of the 3D7 allelic family was detected.

Genetic diversity, allelic frequency and multiplicity of infection. Generated alleles were classified according to their fragment sizes for *msp1*, *msp2* and *glurp*. Allelic frequency for an allele was defined as being its proportion compared to the total number of all detected alleles in the whole isolates. *P. falciparum* isolates in both sites were highly polymorphic, since almost alleles detected were once.

For *msp1* gene, 14 different alleles were observed: 4 alleles for MAD20 (size range 150-250 bp), 1 allele for RO33 (size range 120–150 bp) and for 6 alleles for K1 (size range 140–300 bp) (Table 2). Only one sample from Matanga presented polyclonal infection for MAD20 allelic family (Table 3).

Table 3
Alleles distribution for Msp1

Allele	Fragment size (bp)	Ankazomborona n (%)	Matanga n (%)	Total n (%)
MAD20 allelic family				
MAD20-A	150	1 (9)	0 (0)	1 (9)
MAD20-B	200	5 (46)	2 (18)	7 (64)
MAD20-C	250	0 (0)	2 (18)	2 (18)
MAD20-D	200 + 250	0 (0)	1 (9)	1 (9)
Total for MAD20		**6 (55)**	**5 (45)**	**11 (100)**
RO33 allelic family				
RO33	120 - 150	5 (50)	5 (50)	10 (100)
Total for RO33		**5 (50)**	**5 (50)**	**10 (100)**
K1 allelic family				
K1-A	140	0 (0)	1 (≈17)	1 (≈17)
K1-B	160	1 (≈17)	0 (0)	1 (≈17)
K1-C	180	0 (0)	1 (≈17)	1 (≈17)
K1-D	220	1 (≈17)	0 (0)	1 (≈17)
K1-E	240	0 (0)	1 (≈17)	1 (≈17)
K1-F	300	0 (0)	1 (≈17)	1 (≈17)
Total for K1		**2 (33.3)**	**4 (67.7)**	**6 (100)**

For *msp2* gene, only the FC27 allelic family was observed but the genetic diversity was high: 6 different alleles were detected among the 9 positive samples (Table 4).

Table 4
Allele distribution for Msp2 (FC27 allelic family)

Allele	Fragment size (bp)	Ankazomborona n (%)	Matanga n (%)	Total n (%)
FC27-A	420	1 (11)	0 (0)	1 (11)
FC27-B	440	1 (11)	2 (22)	3 (33)
FC27-C	460	2 (22)	0 (0)	2 (22)
FC27-D	500	1 (11)	0 (0)	1 (11)
FC27-E	520	0 (0)	1 (11)	1 (11)
FC27-F	540	0 (0)	1 (11)	1 (11)
Total		**5 (55.6)**	**4 (44.4)**	**9 (100)**

Table 5
Allele distribution for Glurp

Allele	Fragment size (bp)	Ankazomborona n (%)	Matanga n (%)	Total n (%)
Glurp-A	600 pb	2 (10)	2 (10)	4 (20)
Glurp-B	650 pb	1 (5)	1 (5)	2 (10)
Glurp-C	700 pb	1 (5)	3 (15)	4 (20)
Glurp-D	750 pb	3 (15)	2 (10)	5 (25)
Glurp-E	800 pb	1 (5)	1 (5)	2 (10)
Glurp-F	850 pb	1 (5)	0 (0)	1 (5)
Glurp-G	900 pb	1 (5)	0 (0)	1 (5)
Glurp-H	950 pb	0 (0)	1 (5)	1 (5)
Total		**10 (50)**	**10 (50)**	**20 (100)**

For *glurp* gene, out of the 38 *P. falciparum* positive samples, 20 (52.6%; 95%IC: 36.0 – 68.7%) were successfully amplified. As for *msp1* and *msp2* genes, a high proportion of different alleles (N=8) was observed (Figure 4A and 4B, Table 4).

The mean value of MOI was 1.79 ± 0.74, similar in both sites (1.79 ± 0.80 in Ankazomborona and 1.79 ± 0.70 in Matanga). No significant difference in MOI was also observed between *msp1* (1.33 ± 0.48), *msp2* (1.0±0) and *glurp* (1.0±0) genes.

Discussion

Compared methods for DNA extraction showed that simple water elution method gave higher successful result. Thus, this method was adopted in the study. One hundred seventy used mRDT samples were analyzed. The *Plasmodium* positivity rate was 23.5% (95%CI: 17.5 – 30.8%) including 92.2% *P. falciparum* positive, 5% *P. vivax* positive and 2.5% *P. falciparum/P. vivax* mixed positive samples. Although this low positivity rate, this study highlighted the opportunity using used mRDT for malaria diagnosis and molecular analysis [4]. The main advantage is that no other consumable, nor reagent for blood sampling are needed and mRDT are widely available in public health facilities since 2008 in Madagascar [24]. However, data presented here suggest that DNA extraction method should be improved for increasing the yield of parasite DNA [5]. Here, the laboratory analysis were carried out 8 months after the collection of used mRDT that were stored two months in field. We suspect that the storage of used mRDT in the field conditions has likely affected the DNA integrity. Conducting a study with large samples, about the impact of storage duration and conditions of used mRDT on the quality of the DNA extract is necessary.

Out of 38 confirmed *P. falciparum* samples, 21 were successfully amplified for *msp1* (55.3%, 95%CI: 38.5 – 71.0%). The most frequent allelic family was MAD20 (52.4%), followed by RO33 (47.6%) and K1 (28.6%), similar to those found in Equatorial Guinea, Myanmar and Senegal [22, 25, 26], but in contrast to those observed in Congo DR and Yemen where the K1 allelic family predominate about 40 to 60% [27, 28].

For the *msp2*, only 9 of 38 confirmed samples were successfully amplified and belonged to the FC27 allelic family, concordant with data observed by Rakotomanjaka in the same locality (Matanga) in 2016 [29]. This is not the case in all localities in Madagascar, since several studies reported higher frequency of 3D7 allelic (up to 48%) such as in Andapa, Mahasolo and Saharevo [30, 31].

Analysis based on the PCR product fragments showed 4 distinct alleles for MAD 20 allelic family (11 samples), 1 allele for RO33 (10 samples) and 6 alleles for K1 (6 samples). The K1 allelic family was found at low frequency (28.6%) compared to MAD20 and RO33, but this allelic family was more polymorphic (6 distinct alleles for 6 samples). For FC27 allelic family of *msp2*, out of the 9 positive samples, 6 distinct alleles were detected. Regarding to *glurp* gene, 8 distinct alleles were observed in the 20 positive samples. Polymorphism analysis showed high genetic diversity of *P. falciparum* populations in Ankazomborona and Matanga. Majority of alleles detected during in this study were single with a high polymorphism level. No significant difference of polymorphism level was detected in two study sites. Parasite genetic diversity is one of key elements to consider for strategies implementation to fight against malaria [28]. It has an impact on parasite transmission and control strategies, and it varies according to transmission in endemic areas [7-10]. According to the results, it seems, transmission intensity is similar in Ankazomborona and in Matanga. Nevertheless, a study on large samples is crucial before giving final conclusion.

This study showed 28.6% multiple infection for *P. falciparum msp1* gene not for *msp2* and *glurp*. These results indicate that polyclonal infection frequency is low compared to those found in other countries [32, 33]. The MOI average value was 1.79 ± 0.74, a lower value than in other northwestern Ethiopia (2.6), in

Republic of Congo (2.64), and in Equatorial Guinea (5.5) [23, 25, 28]. MOI for Ankazomborona and Matanga did not differ significantly. Thus, hypothesis concerning the link between the high level of MOI and malaria severity, particularly in areas with high transmission rate [11], is not verified during this study due to the limited number of samples. Despite this, we can deduce that *P. falciparum* populations circulating in Madagascar showed a high level of genetic polymorphism. That high polymorphism reflects transmission intensity. Consequently, it would be interesting to increase samples number in order to continue genetic diversity study over time and space for carrying out a cartographic study about *Plasmodium* allelic families' distribution in Madagascar.

Conclusion

The present study highlighted the possibility of performing malaria diagnosis and molecular genetic diversity analysis of *Plasmodium* using blood samples collected on mRDT as DNA source. Preliminary data from this study showed high genetic diversity of *P. falciparum* populations in Ankazomborona and in Matanga but multiple infection rate was low in the both study sites.

This study provides fundamental information on *P. falciparum* genetic diversity and allow to update available data. The results of this study can be used as a baseline information for future studies on parasite transmission dynamics and to evaluate effectiveness malaria prevention and control strategies.

Declarations

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Authors' contributions: All authors contributed equally to preparing the final version of the manuscript. VA and ER were in charge of analysis and parasite genotyping. AR is guarantor of the paper.

Availability of data and materials

The data are available from the National Malaria Control Programme of Madagascar.

Ethics approval and consent to participate

The study was part of malaria surveillance approved by the Ethics Committee of the Ministry of Health of Madagascar (No. 083/MSANP/CE/11-2012).

Consent for publication

All the authors have agreed to the submission of this manuscript for publication.

Competing interests

The authors declare that they have no other conflict of interest.

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Figures

Figure 1

Geographical situation of Ankazomborona and Matanga (Source: BD 500 and FTM)

Figure 2

Fragment sampling of RDT strip

A: Distal part, B: Central part, C: Proximal part, D: All parts

Figure 3

PCR product from DNA extacted by the two methods: Instagena matrix (1 to 8) and simple elution in water (9 to 16)

Expected size 206 bp for P. falciparum (A) and 120 bp for P. vivax (B),

PM: 100bp DNA ladder marker, 1 - 16: samples

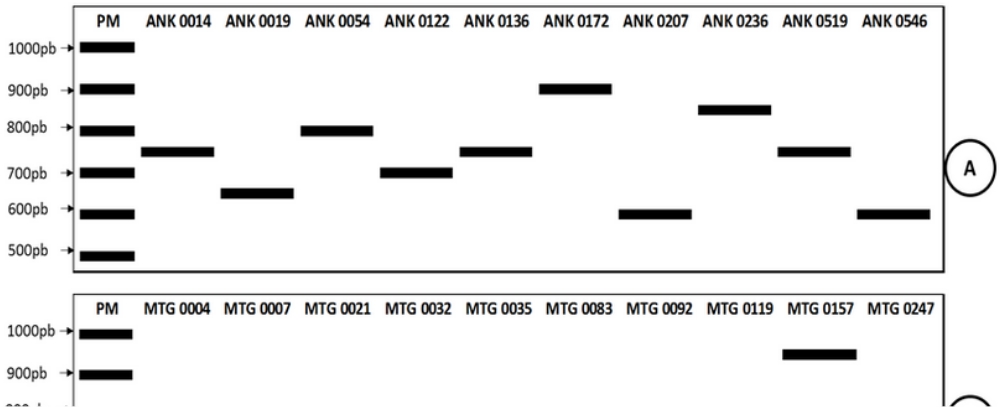

Figure 4

Polymorphism of P. falciparum glurp gene