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MONITORING THE DRUG-SENSITIVITY OF *PLASMODIUM FALCIPARUM* IN COASTAL TOWNS IN MADAGASCAR BY USE OF *IN VITRO* CHEMOSENSITIVITY AND MUTATION DETECTION TESTS

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Summary:

The dissemination of mutant and resistant strains of *Plasmodium falciparum* makes a considerable contribution to the spread of drug-resistant malaria. Populations around harbours and airports could be particularly exposed to *Plasmodium* isolates introduced with imported cases of malaria. The use of chloroquine as well as the use of and sulfadoxine/pyrimethamine is currently an effective method for treating uncomplicated cases of malaria in Madagascar. As part of a monitoring programme, *in vitro* methods were used to assess the sensitivity of *P. falciparum* isolates in two coastal towns in Madagascar: Mahajanga on the west coast and Toamasina on the east coast. All of the isolates from both sites were sensitive to amodiaquine, quinine, pyrimethamine and cycloguanil. All of the isolates from Mahajanga were sensitive to chloroquine (n = 25; mean IC50 = 22.6 nM, 95 % confidence interval: 16.8-28.7 nM), whereas three of the isolates from Toamasina were resistant to chloroquine (n = 18; mean IC50 = 66.3 nM; 95 % confidence interval: 42.6-90 nM). The frequency of the *Pfcrt* Thr-76 and the *dhfr* Asn-108 mutations was estimated by PCR/RFLP. The 43 *P. falciparum* isolates examined, including the three *in vitro* chloroquine-resistant isolates from Toamasina were all wild-type (Lys-76). Phenotyping and genotyping studies suggested that the prevalence of chloroquine- and pyrimethamine-resistant isolates and of mutant strains of *P. falciparum* is very low. These results showed that *in vitro* test and genotyping of resistance markers approaches could be successfully used to monitor the emergence of drug-resistant malaria and to try to alleviate the lack of medical teams able to carry out *in vivo* test. The possible hazard/risk associated with imported cases of malaria is discussed.

KEY WORDS : *Plasmodium falciparum*, resistance, *Dhfr* Asn-108, *Pfcrt* Thr-76, Indian Ocean sub-region, coastal towns, Madagascar.

Résumé : SURVEILLANCE DE LA SENSIBILITÉ DE *PLASMODIUM FALCIPARUM* DANS DES VILLES CÔTIÈRES À MADAGASCAR PAR LE TEST DE CHIMIOSENSIBILITÉ *IN VITRO* ET LA DÉTECTION DES MUTATIONS

La dissémination des souches de Plasmodium falciparum *mutantes et résistantes contribue à la diffusion de la résistance aux antipaludiques. Les populations des zones portuaires sont particulièrement exposées aux isolats de* Plasmodium *introduits associés à des cas de paludisme d'importation. Le traitement par la chloroquine aussi bien que par l'association sulfadoxine/pyrimethamine demeure une méthode efficace pour la prise en charge des accès palustres simples à Madagascar. Dans le cadre du programme de surveillance du paludisme, des approches in vitro étaient utilisées pour évaluer la sensibilité des isolats de* P. falciparum *dans deux villes côtières et portuaires malgaches : Mahajanga sur la côte Ouest et Toamasina sur la côte Est. Les isolats testés dans ces deux sites étaient tous sensibles à l'amodiaquine, à la quinine, à la pyrimethamine et au cycloguanil. À Mahajanga, les isolats ont été sensibles à la chloroquine (n = 25; moyenne CI50 = 22,6 nM, intervalle de confiance 95 % : 16,8-28,7 nM), alors que trois isolats de Toamasina étaient de phénotype chloroquinorésistant (n = 18; moyenne CI50 = 66,3 nM; intervalle de confiance 95 % : 42,6-90 nM). La fréquence des mutations* Pfcrt *Thr-76 et de* dhfr *Asn-108 était estimée par PCR/RFLP. Les 43 isolats de* P. falciparum *analysés étaient tous de génotype sauvage (*Pfcrt *Lys-76 et* dhfr *Ser-108), classiquement associé à un phénotype sensible. Le phénotypage et le génotypage démontrent que la prévalence de souches de* P. falciparum *résistantes à la chloroquine ou à la pyrimethamine est encore très faible à Madagascar. Il s'avère ainsi que l'approche basée sur le test in vitro et le génotypage des marqueurs de résistance seraient utiles i) pour surveiller l'émergence des plasmodies résistantes aux antipaludiques majeurs à Madagascar, et ii) notamment pour pallier le manque d'équipes médicales compétentes et expérimentées pour la conduite des études in vivo. Le risque associé aux cas importés de paludisme est discuté.*

MOTS CLÉS : Plasmodium falciparum, *résistance*, Dhfr *Asn-108*, Pfcrt *Thr-76*, *Sous région de l'Océan Indien, villes côtières, Madagascar.*

The geographical spread and emergence of drug-resistant *Plasmodium falciparum* have hindered the control of malaria, the most important parasitic disease in the world. Between 1978 and 1988, chloroquine-resistant strains of *P. falciparum* were detected in all tropical African countries (Trape, 2001). Chloroquine remains the first-line antimalarial drug in Madagascar. As shown, unlike in East Africa and South East Asia, the malaria situation is fairly stable in Madagascar, with *P. falciparum* showing low grade resistance (R1 and R2) to chloroquine (Deloron *et al.*, 1985; Lepers *et al.*, 1993; Milijaona *et al.*, 1998) and the prevalence of treatment failure is low (Randrianarivelojosia *et al.*, 2000; Raharimalala *et al.*, 2001). Malaria is endemic in Madagascar and the main vectors are

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Anopheles funestus, *An. arabiensis* and *An. gambiae* (Brutus *et al.*, 2001; Cot *et al.*, 2001; Duchemin *et al.*, 2001). This disease always remains a public health problem (Milijaona *et al.*, 1998; Brutus *et al.*, 2001).

Many infectious diseases have emerged due to factors such as the increased migration of the population, ecological and environmental disasters. Furthermore, the importation of malaria from regions of *Plasmodium* resistance is likely to result in the spread of resistant strains of *P. falciparum* in areas that are free from highly treatment-resistant forms of the disease. Imported cases of malaria are not documented even though international travel and commercial exchange have been increasing dramatically between Madagascar, Africa and Asia over the five last years. Coastal areas are particularly exposed to imported cases. Thus, as part of a Madagascan surveillance programme, we used an *in vitro* antimalarial test to assess the sensitivity of *P. falciparum* in two towns with international harbours: Mahajanga on the west coast and Toamasina on the east coast. We also used genotyping methods to detect two mutations in *pfcrt* and *dhfr* genes, accounting for drug resistance.

MATERIALS AND METHODS

Study sites and collection of *Plasmodium falciparum* isolates

P. falciparum isolates were collected from two coastal towns (Mahajanga and Toamasina), separated by 420 km as the crow flies.

Mahajanga is located on the west coast of Madagascar. Mahajanga port mostly deals with commercial exchange between Madagascar and the Comoro Islands. The transmission of *Plasmodium* is seasonal in this area. Transmission mainly occurs during the rainy season (December to April). *P. falciparum* isolates were collected from patients attending urban primary health centres in Mahajanga between January 2001 and June 2001.

Toamasina is located in the wet area on the east coast. This town is home to the biggest international harbour in Madagascar. The transmission of *Plasmodium* is perennial in this area. *P. falciparum* isolates were collected from patients attending urban primary health centres in Toamasina between April 2001 and September 2001.

The Madagascan Ministry of Health approved the experimental protocols used for the study. Thin and thick blood smears were prepared for each outpatient admitted to the clinics in Toamasina and Mahajanga with suspected malaria. These smears were stained with Giemsa stain and examined under a light microscope to determine whether malaria parasites were present. Mono-specific *P. falciparum* isolates were included in the study if at least 3,000 ring stage parasites were detected per microlitre of blood, and if patients had not recently (< 7 days) taken antimalarial drugs. Blood samples (2 to 5 ml) were collected from consenting patients (or patients whose guardians or parents gave consent) by venopuncture. The samples were collected in citric acid dextrose tubes. They were then transported at + 4° C to the Malaria research Unit at the Institut Pasteur de Madagascar in Antananarivo within 48 hours. Patients presenting signs and symptoms of severe and complicated malaria, as defined by the World Health Organization, were excluded (Warrell *et al.*, 1990).

The medical teams from the local health centres decided how their patients should be treated. There was no follow-up. Thus, the treatment outcomes are not reported.

Test antimalarial drugs

Amodiaquine base, chloroquine diphosphate, pyrimethamine and quinine base were obtained from Sigma Chemicals, cycloguanil was obtained from Zeneca and mefloquine from Roche Products. Sterile stock solutions were prepared in methanol/water and serial dilutions were made in distilled water. Test concentrations ranged from 2.5 to 1,280 nM for amodiaquine; from 12.5 to 1,600 nM for chloroquine; from 0.3 to 32,000 nM for cycloguanil and pyrimethamine; from 2.5 to 400 nM for mefloquine and from 25 to 3,200 nM for quinine. The chloroquine- and pyrimethamine-susceptible *P. falciparum* clone 3D7 and the chloroquine- and pyrimethamine-resistant *P. falciparum* clone FCM29 were used as references to test each batch of plates containing antimalarial molecules.

The susceptibility of isolates to antimalarial molecules was tested by measuring the inhibition of tritium-labelled hypoxanthine incorporation. After arrival in Antananarivo, parasites were tested immediately. A part of the red blood cells pellet reserved for the parasite genotyping was kept at – 20° C until use. *In vitro* chemosensitivity tests were performed according to the isotopic microtest method (Le Bras & Deloron, 1983; Randrianarivelojosia *et al.*, 2001). RPMI 1640 medium was used to test the quinoline-containing antimalarial drugs (amodiaquine, chloroquine, mefloquine and quinine). RPMI medium without *p*-aminobenzoic and without folic acid (Biowhittaker Europe, A Cambrex Company, Belgium) was used to test the antifolates (cycloguanil and pyrimethamine). Both media were supplemented with 10 % (*v/v*) non-immune human type AB-positive serum. [^{3}H]-hypoxanthine was added (1 µCi per well) to the culture plates. The parasites were incubated for 42 hours.

The percentage growth relative to the control one was calculated. The 50 % inhibitory concentration

(IC50) of the test antimalarials was calculated by regression analysis of log-concentration/response probit curves. Isolates were considered to be resistant *in vitro* to chloroquine if the IC50 was greater than 120 nM (Milijaona *et al.*, 1998). Isolates were considered to be resistant when the IC50 was > 80 nM for amodiaquine, > 50 nM for mefloquine, > 800 nM for quinine, > 500 nM for cycloguanil and > 2,000 nM for pyrimethamine (Basco & Le Bras, 1994; Basco *et al.*, 1994; Brasseur *et al.*, 1995; Parzy *et al.*, 1997; Randrianarivelojosia *et al.*, 2001). The results are expressed as the mean IC50s and 95 % confidence intervals (95 % CI). The correlation between the *in vitro* response of *P. falciparum* to two different drugs, based on IC50s, was analysed by Pearson's correlation.

DNA EXTRACTION, PCR AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM

The red blood cells were harvested by centrifugation before the sensitivity test and were kept at – 20° C until use as mentioned above. Parasite DNA was extracted from 200 µl of red blood cells by use of the phenol and chloroform (Ariey *et al.*, 1999). Only *P. falciparum* isolates that could be assessed by the *in vitro* sensitivity tests were analysed.

The *P. falciparum dhfr* gene was amplified by PCR using a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). The reaction mixture contained approximately 200 ng of genomic DNA, 15 pmol of primers 5'-ttctccttttttatgatggaacaagt-3' (sense) and 5'-aaaataaacaaaatcatcttattcttc-3' (anti-sense), buffer (25 mM KCl, 10 mM Tris-HCl, pH 8.8), 2.5 mM $MgCl_2$, 200 µM dNTP and two units of *Taq* polymerase (Amersham Pharmacia Biotech, Piscataway, USA) in a 50-µl volume (Parzy *et al.*, 1997). The cycling conditions were slightly modified as follows: 94° C for five minutes for one cycle, and 40 cycles of 94° C for 30 sec, 58° C for 30 sec and 72° C for one minute. Three characterized clones of *P. falciparum* 3D7 (Ser-108), Palo Alto (Thr-108) and FCM29 (Asn-108) maintained in continuous culture were used as positive controls, and double-distilled water was used as a negative control.

The PCR products were incubated with *Alu*I (New England Biolabs, UK), *Bsr*I (New England Biolabs, UK) or *Scr*FI (New England Biolabs, UK) in a final volume of 25 µl according to the manufacturer recommendations.

The products present/generated in the reaction mixtures (15 µl) were separated in a 1.2 % agarose gel, stained with 0.5 µg/ml ethidium bromide, and visualised under ultraviolet light. The 890 base pair PCR products each contained one restriction site for either *Alu*I, *Bsr*I or *Scr*FI, depending on the amino acid residue at position 108 of the *dhfr* gene. *Alu*I, *Bsr*I and *Scr*FI each cleaved the PCR product into one 339 bp product and one 551 bp product. *Alu*I cut the product when the wild-type codon was present at position 108 (Ser-108), *Bsr*I cut the product when an Asn codon was present at position 108 (Asn-108) and *Scr*FI cut the product when a Thr codon was present at position 108 (Thr-108).

The *pfcrt* gene of *P. falciparum* was amplified by PCR. The reaction mixture contained approximately 200 ng of genomic DNA, 15 pmol of primers 5'-ggtggaggttcttgtcttgg-3' (sense) and 5'-ataaagttgtgagtttcggatg-3' (anti-sense), buffer (25 mM KCl, 10 mM Tris-HCl, pH 8.8), 2.5 mM $MgCl_2$, 200µM dNTP and one unit of *Taq* polymerase (Amersham Pharmacia Biotech, Piscataway, USA) in a 25 µl reaction. The cycling conditions were as follows: 94° C for five minutes for one cycle, and 30 cycles of 94° C for 30 sec, 59° C for 30 sec and 72° C for one minute. The resulting PCR products were digested with *Apo*I according to the manufacturer recommendations. Ten to fifteen microliters of the solution expected to contain or cut or uncut fragments were loaded on a 1.2 % agarose gel, subjected to electrophoresis, stained with ethidium bromide at a final concentration of 0.5 µg/ml, and visualized by ultraviolet transillumination. Only single restriction site for *Apo*I is present in the 194-bp polymerase chain reaction product, depending on the amino acid residue at position 76 of the *Pfcrt* gene. The cleavage of the 194-bp DNA fragment into two fragments of 71-bp and 123-bp indicates the presence of the wild-type codon Lys-76.

RESULTS

IN VITRO ANTIMALARIAL TEST

We tested 32 isolates of *P. falciparum* from Mahajanga and 21 isolates from Toamasina. Table I shows the results of the *in vitro* test.

- Isolates from Mahajanga

This is the first study to measure the *in vitro* sensitivity of *P. falciparum* isolates from Mahajanga in twenty five years. All of the *P. falciparum* isolates that could be assessed were sensitive to chloroquine (n = 25), to pyrimethamine (n = 9) and to the other antimalarials tested (Table Ia). There was a low prevalence of *in vitro* chloroquine-resistant isolates in Mahajanga (95 % confidence interval: 0-13.7 %). Even though we only tested a limited number of isolates, we found a positive correlation between the antimalarial activities of quinoline antimalarials (Table IIa). Significant correlations were recorded between 4-aminoquinoline chloroquine and amodiaquine (n = 24; r = 0.54; p = 0.006), between chloroquine and quinine (n = 25; r = 42; p = 0.03) and between quinine and mefloquine (n = 15; r = 0.83; p = 0.0001).

(Ia): in Mahajanga	**Antimalarial molecules** (IC50 threshold indicating *in vitro*-resistance)					
	Chloroquine (120 nM)	**Amodiaquine** (80 nM)	**Mefloquine** (50 nM)	**Quinine** (800 nM)	**Pyrimethamine** (2000 nM)	**Cycloguanil** (500 nM)
Number of tests done	32	31	20	32	32	22
Assessable tests	25 (78.1 %)[a]	24 (77.4 %)[a]	15 (75 %)[a]	25 (78.1 %)[a]	9 (28.1 %)[a]	7 (31.8 %)[a]
Mean of IC50s (nM)	22.7 (16.8-28.7)[b]	7.9 (5.5-10.4)[b]	11.6 (7.2-15.9)[b]	88.8 (69-108.6)[b]	9.1 (< 19.8)[b]	6.3 (0.4-12.2)[b]
Lowest IC50 (nM)	0.15	0.23	3.8	32.5	0.2	0.02
Highest IC50 (nM)	51.6	24.5	35.5	218.8	51.1	18.9
Resistant isolates	0	0	0	0	0	0

[a]: test success rate; [b]: 95 % confidence interval.

(Ib): in Toamasina	**Antimalarial molecules** (IC50 threshold indicating *in vitro*-resistance)					
	Chloroquine (120 nM)	**Amodiaquine** (80 nM)	**Mefloquine** (50 nM)	**Quinine** (800 nM)	**Pyrimethamine** (2000 nM)	**Cycloguanil** (500 nM)
Number of tests done	21	20	14	19	13	10
Assessable tests	18 (85.7 %)[a]	17 (85 %)[a]	12 (85.7 %)[a]	17 (89.5 %)[a]	12 (92.3 %)[a]	10 (100 %)[a]
Mean of IC50s (nM)	66.3 (42.6-90)[b]	17.8 (10.4-25.3)[b]	12.9 (8.1-17.8)[b]	194.2 (125.6-262.9)[b]	20.4 (7.8-33)[b]	9.8 (2.7-16.8)[b]
Lowest IC50 (nM)	7.5	3.4	3.5	67.7	0.3	0.5
Highest IC50 (nM)	206.6	57.9	29.8	584.2	75.1	35.1
Resistant isolates	3 (16.7 %)[c]	0	0	0	0	0

[a]: test success rate; [b]: 95 % confidence interval; [c]: resistance rate from assessable tests.

Table I – *In vitro* sensitivity of *Plasmodium falciparum* isolates to major antimalarials.

• Isolates from Toamasina

Between 85 to 100 % of the isolates from Toamasina could be assessed (Table Ib).These isolates were tested within the 24 hours following the blood collection. The chloroquine IC50 values were between 7.5 nM and 206.6 nM (n = 18; mean IC50 = 66.3 nM and 95 % CI = 42.6-90 nM). Based on our criterion (IC50 > 120 nM), three (16.7 %) of the 18 isolates of *P. falciparum* from Toamasina were chloroquine–resistant, with chloroquine IC50 values of 123.9, 140 and 206.6 nM. These resistant isolates were sensitive to amodiaquine, quinine, mefloquine and pyrimethamine. Significant correlations were recorded between 4-aminoquinoline chloroquine and amodiaquine (n = 17; r = 0.51; p = 0.03), between chloroquine and quinine (n = 17; r = 0.57; p = 0.01) and between quinine and amodiaquine (n = 17; r = 0.54; p = 0.02).

GENOTYPING

None of the 43 assessable isolates of *P. falciparum* (25 from Mahajanga and 18 from Toamasina) yielded a digestion product after the *dhfr* PCR product was digested with *Bsn*I or *Scr*FI. AluI cut all *dhfr* PCR products related to these 43 isolates tested. This indicates that none of the isolates contained the Asn-108 or Thr-108 mutations. The phenotypic study based on the *in vitro* quantification of pyrimethamine activity against *P. falciparum* and the pyrimethamine resistance genotypic marker analysis based on the determination of mutations at the codon 108 of *dhfr* showed that none of our collection of *P. falciparum* isolates were potentially resistant to antifolate. The digestion of the *Pfcrt* PCR product with *Apo*I indicated that all 43 isolates tested harboured a lysine residue at position 76.

DISCUSSION

All of the tested isolates were sensitive to amodiaquine, quinine, cycloguanil and pyrimethamine. Given that chloroquine is the first line antimalarial drug and that pyrimethamine/sulfadoxine is the second line, we paid particular attention to the activity of chloroquine and pyrimethamine against *P. falciparum* in Madagascar. This is the first study in

(IIa): in Mahajanga

Drug pair		Number of isolates	Spearman R	*p* level
Chloroquine	Amodiaquine	24	0.54	**0.0006**
Chloroquine	Quinine	25	0.42	**0.03**
Quinine	Amodiaquine	24	0.10	NS
Chloroquine	Mefloquine	15	0.48	NS
Mefloquine	Amodiaquine	15	0.41	NS
Quinine	Mefloquine	15	0.83	**0.0001**
Chloroquine	Pyrimethamine	8	0.40	NS
Cycloguanil	Pyrimethamine	7	0.85	**0.01**

Correlation is significant for $p < 0.05$; NS: non significant.

(IIb): in Toamasina

Drug pair		Number of isolates	Spearman R	*p* level
Chloroquine	Amodiaquine	17	0.51	**0.03**
Chloroquine	Quinine	17	0.57	**0.01**
Quinine	Amodiaquine	17	0.54	**0.02**
Chloroquine	Mefloquine	12	0.22	NS
Mefloquine	Amodiaquine	12	0.57	0.05
Quinine	Mefloquine	12	0.49	NS
Chloroquine	Pyrimethamine	11	– 0.05	NS
Cycloguanil	Pyrimethamine	9	0.92	**0.0005**

Correlation is significant for $p < 0.05$; NS: non significant.

Table II. – Correlation of *in vitro* response of *Plasmodium falciparum* to major antimalarial drugs.

twenty five years to assess the *in vitro* susceptibility of *P. falciparum* isolates from Mahajanga. The results of the *in vitro* antimalarial tests showed that none of the 25 *P. falciparum* isolates from Mahajanga were chloroquine-resistant. The highest chloroquine IC50 recorded was 51.6 nM (mean IC50 = 22.7 nM). A study carried out in Mahajanga in 1998 showed that chloroquine treatment fails in 3.8 % of cases (2/52). Only two of the 50 patients who gave accurate clinical responses were parasitaemic between day 16 and day 24 (Razafindrakoto, unpublished). This situation is in contrast with that in the Comoro Islands, where chloroquine treatment fails in 40 % of cases (Ariey *et al.*, 2001). Given the amount of air and sea traffic between Mahajanga and the Comoro Islands, the importation of potentially chloroquine-resistant malaria parasites is a matter of concern.

Three of 18 isolates from Toamasina were chloroquine-resistant *in vitro*. There was no significant difference between the frequency of chloroquine resistant isolates (p = 0.13) and the mean chloroquine IC50s (p = 0.07) in Toamasina and in Mahajanga. An *in vitro* study has already been carried out on isolates from Toamasina. All *P. falciparum* isolates tested in this study were sensitive to chloroquine *in vitro* (Randrianarivelojosia *et al.*, 2000). As the *in vitro* test makes it possible to assess and to quantify the intrinsic activity of the antimalarials, these results imply that the overall sensitivity of *P. falciparum* to chloroquine is stationary in Toamasina.

It is worth to underline that the extreme values of test antimalarials IC50 recorded in Mahajanga were much neatly lower than in Toamasina, except for mefloquine (Table Ia and Ib). Regarding the lowest IC50s, the ratio was 50 for chloroquine (7.5 nM in Toamasina against 0.15 nM in Mahajanga), 25 for cycloguanil (0.5/0.02) and two for quinine (67.7/32.5). Our study demonstrated also that none of the isolates from either site harboured mutations at position 76 of the *Pfcrt* gene (Thr-76) or at position 108 of the *dhfr* gene (Asn-108 or Thr-108). Conversely, our previous study on isolates from the Comoro Islands, showed that the Pfcrt mutation resulting in the substitution of threonine (Thr-76) for lysine at position 76 was present in 67 % of samples obtained before treatment from 49 randomly selected patients (Ariey *et al.*, 20021). No pyrimethamine-resistant isolates have been detected in Madagascar in the three last years, despite the fact that hundreds of tests have been performed (Randrianarivelojosia, personal communication). Even though several different studies concluded that there is not a perfect correlation between the presence of the *pfcrt* codon 76 mutation and the clinical response to chloroquine (Babiker *et al.*, 2001; Dorsey *et al.*, 2001; Djimde *et al.*, 2001; Mayor *et al.*, 2001), the prevalence of chloroquine treatment failure is higher when the prevalence of the *pfcrt* Thr-76 mutation is high. Durand *et al.* (2001) found a clear correlation between *in vitro* chloroquine resistance and the *pfcrt* Thr-76 mutation. Mockenhaupt *et al.* (2001) reported that *pfcrt* and *pfdhfr* mutations are frequent *in P. falciparum* isolates from Ghana. These studies suggest that *in vitro* methods are important for monitoring the sensitivity of *P. falciparum*.

Numerous factors contribute to the introduction, spread and intensification of drug resistance, although their relative contribution is unknown. The dissemination of resistant parasites from a zone of resistance to a zone of sensitivity is a factor leading to the spread of resistance. Transportation, travel and migration all contribute to the emergence of infectious diseases (Morse, 1995). Resistant malaria is a clear example of an emerging disease. The biggest danger may be imported cases. Madagascar is located between Asia and Africa, both of which generally have high prevalence of chloroquine- and pyrimethamine-resistance. International travel and trade between Madagascar and these two continents are becoming more common. This might increase the frequency of imported cases of malaria. Imported cases of resistant malaria that occurred and are documented in malaria-free countries (Durand *et al.*, 2001), might be introduced silently into a country where malaria is endemic like Madagascar. Known that Since *Plasmodium* vectors are present (Brutus *et al.*, 2001; Cot *et al.*, 2001; Duchemin *et al.*, 2001), autochthonous transmis-

sion of imported drug resistant parasite strains would occur tragically. Thus, malaria resistance should be monitored particularly in coastal and port areas.

The lack of scientists and medical teams that can conduct the *in vivo* study encourages the use of these *in vitro*-based techniques for the monitoring of malaria resistance in Madagascar and other developing tropical countries. We are convinced that the *in vitro* test and the genotyping of resistance markers are useful 1) for monitoring resistance, 2) for detecting the emergence of resistant and/or mutant parasites in a region free from prevalent mutants and 3) for identifying potential alternatives to the first line drugs among the major antimalarials recorded and available in the local market. Our preliminary study shows that the *in vitro* technique-based monitoring can be successfully used to assess malaria resistance. We demonstrated that chloroquine-resistant isolates of *P. falciparum* are absent or present in very low numbers in coastal towns in Madagascar. We also demonstrated the very low prevalence of isolates harbouring *pfcrt* and *dhfr* mutations. A larger sample size and a study coupling *in vivo*, *in vitro* and genotyping techniques should be performed to validate the information provided by the *in vitro* approaches and to predict the risk of treatment failure associated with the commonly used antimalarials such as chloroquine and sulfadoxine/pyrimethamine.

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