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Reduced Levels of chloroquine resistant *Plasmodium falciparum* in selected *foci* of the South West Province, Cameroon

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Abstract

Objectives: To evaluate the state of chloroquine resistant malaria in the South West Province of Cameroon. To estimate the prevalence of different species of *Plasmodium* in the area.
Design: A cross sectional study.

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Setting: South West Province of Cameroon.
Subjects: 326 febrile patients.
Main Outcome Measures: Chloroquine resistance studies using the WHO seven-day standard *in vivo* test. Speciation of malaria by polymerase chain reaction. Prevalence of *falciparum* malaria by light microscopy of thick blood smears.
Results: Prevalence of chloroquine resistance ranged from 4.3 to 13.2%. A total of six cases showed RIII resistance, and three cases showed RII resistance.
Plasmodium falciparum was the predominant species, 96.8% of those testing positive for *P. falciparum*, 6.3% of cases showed mixed infections of *P. falciparum* and *P. malariae*. *P. ovale* accounted for 3% of cases.
Conclusion: *P. falciparum* resistance to chloroquine may not be on the increase compared to earlier reports from the South West Province. The results have important implications in treatment of malaria in view of the low cost of chloroquine.

Introduction

The control of malaria relies mainly on chemotherapy, chemoprophylaxis and the use of mosquito bed nets. The emergence and rapid spread of resistance of parasites and vectors to antimalarials and insecticides respectively has exacerbated the problem of control. Chloroquine is the most common and cheapest antimalarial drug available. Unfortunately, resistance to this drug has reached alarming proportions in most countries affected by *Plasmodium falciparum* malaria.

Drug resistance was first reported in the 1950s in South East Asia and South America.[1] The first case of *P. falciparum* chloroquine resistance in Cameroon was reported in Limbe, Fako Division.[2] Subsequent studies confirmed that resistance had spread to different parts of the country.[3,4] From 1989 to 1991 several *in vivo* studies were carried out in different bio-climatic areas in Cameroon. The results showed that the rate of chloroquine resistance varied from 6% in the Northern Savannah region to 42% in industrial zones like Edea where drug pressure was particularly high.[5] Studies have shown that drug pressure facilitates the development of cross-resistance to antimalarial drugs.[6]

Although conventional microscopy has been the major method of identification of malaria parasites, proper reading and interpretation of results requires experience and expertise. The difficulty, which many laboratory technicians encounter in reading thick smears and interpreting the results correctly has resulted in the common practice by medical personnel of treating all febrile cases with antimalarials even when laboratory tests are reported negative. Correct identification of the various plasmodial species using stained smears also demands expertise and for these reasons mixed infections and single infections due to species other than *P. falciparum* are often missed. The superiority of the polymerase chain reaction amplification technique over microscopy was clearly demonstrated in a field study in Thailand where 16% of infected samples were missed, 3% misdiagnosed and 13% mixed infections missed.[7] PCR amplification is sensitive and accurate enough to detect as low as four parasites per microlitre by gel electrophoresis.

The objectives of this study were firstly to evaluate the state of chloroquine resistant malaria in the South West Province 13 years after it was first reported and secondly, to obtain accurate information on the prevalence of the different species of *Plasmodium* in the area using the more sensitive PCR amplification technique.

Materials and Methods

Study Design and Sampling.

A random cluster sampling was used to evaluate chloroquine resistance in the study populations. All malaria patients recruited into the study had to have been born in the study area (in the case of children less than two years old) or must have lived in the study area for at least two years. For inclusion into the study, the patient was not to have received any form of malaria treatment within the previous 14 days as confirmed with the Saker-Solomons urine test.[8] In addition the patient was required to have a parasite count of at least 1 000 per ml of blood. After the first day (DO) of sampling, each patient returned for further sampling and follow up on the second day (Dl), the third day (D2) and the eighth day (D7).

Study Areas.

All three areas from which patients were recruited are located in the South West Province of Cameroon. These areas are mesoendemic for malaria in the dry season and hyperendemic in the rainy season. The semi-urban town of Tiko has a population of 14 000 inhabitants. The area is almost entirely at sea level, with a small region that is 20 to 40 metres above sea level. The majority of inhabitants are plantation workers of the Cameroon Development Corporation (CDC). Limbe is a larger town with a population of 200 000 engaged in varied professions. Many parts of Limbe are at sea level with a few being 30 to 80 metres above sea level. Molyko is a village in the South West provincial capital of Buea and has a population of approximately 10 000 inhabitants of which 75% are students. The village is situated 580 to 630 metres above sea level at the foot of Mount Cameroon. All three areas have a rainy season (March to October) and a dry season (November to February).

Study Population.

A total of 326 febrile participants between the ages of one to 51 years and of both sexes were recruited into the study after individual or parental consent. Of these 113 (one to 41 years old) were recruited at the CDC Central Clinic in Tiko; 150 (one to 51 years old) from the Provincial Hospital in Limbe and 63 (one to 40 years old) from the University of Buea Health Unit and private clinics in Molyko. The study period between January and April 1998 coincided with the dry and rainy seasons. Ethical clearance was obtained from Ethical Review Board of the South West Province.

The Simplified WHO Seven Day Test to Detect Chloroquine Resistance.

The procedure used was as outlined by WHO (1973) and similar to the *in vivo* seven day assay used in 1987 to evaluate chloroquine resistance in Cameroon.[3] Baseline data using 14 days follow up showed a similar trend as obtained using the seven day test. However, a disadvantage was the high rate of withdrawal of patients from the study during 14 days of observation. Essentially, on DO, D2 and D7, thick smears were done on each subject. On DO, Dl and D2 each individual received orally chloroquine tablets containing 100mg chloroquine base (Cinpharm, Cameroon) at doses of 10mg/Kg on DO and DI and 5 mg/kg on D2. The drug was administered each day as a single dose with water and the patient was observed for 15 minutes after intake. When necessary, the patients were visited at home to complete treatment. Patients in whom parasites were still detected on D7 were referred to the doctor for further treatment with alternative drugs. Parasite densities were determined by microscopy after staining thick smears with fields's stain. Parasites were enumerated following the procedure outlined by Earle and Perez.[9] At least 100 microscopic fields were scanned prior to reporting any result as negative.

Parasite DNA Extraction.

About 2 ml of blood samples were each collected into tubes containing sodium citrate. The blood samples were stored at -20°C at the University of Buea research laboratory and later transported on ice to the Biotechnology Centre at the University of Yaounde. At the Centre the blood samples were dispensed into I.5ml eppendorf tubes, centrifuged at 1 000 x g for five minutes and the supernatant discarded. Each volume was made up to 500 ml with Tris Sodium Chlorid EDTA buffer. The samples were stored at –20°C overnight. Parasite DNA was extracted using phenol. The extracted DNA was precipitated by addition of ethanol followed by centrifugation. The pellets were washed, dried and dissolved in Tris-EDTA buffer and stored at –20°C until PCR amplification.

PCR Amplification.

All PCR reactions were carried out in a total volume of 20 ml containing 2mM MgCL2, 50mM KCL, 10mM Tris-HCL(PH 8.3), 0.1 mg/ml gelatin, 125 micromolar of each of the deoxyriboncleotide triphosphates (dNTp) and 0.4 units of amplitag polymerase. One microlitre of the purified template DNA extracted from the blood was used for the SPE-Nest I (SNI) reaction in which the fragment spanned by the primers rPLU5(CCTGTTGTTGCCTTAAACTTC) and rPLU(TTAAAATTGTTGTTGCAGTTAAAAGC) was amplified (these primers were validated by Snounou *et al.*[7] and are highly sensitive). This was to detect the presence of the genus *Plasmodium*. One microlitre of the product of SNI reaction was employed as DNA template in the SPE-Nest 2(SN2) reaction in which the species-specific oligonucleotide primer pairs were used.[7] The negative controls were FMO (F = *P. falciparum*; M = *P. malariae*; O = *P. ovale*) both obtained from Georgetown University, Maryland, USA (Courtesy of Dr Diane Taylor) and the Biotechnology Centre, Yaoundé. The amplification assays were performed in a DNA thermal cycler at the biotechnology Centre. The amplified products were run on agarose gels, visualized under a UV transilluminator and photographed.

Statistical Analysis.

The Chi square test with $p<0.05$ was used to determine the level of significance.

Results

Prevalence of Malaria.

Of the 326 individuals recruited into the study 247 (75.8%) were positive. The prevalence of malaria as determined by micoscopy was 82.3% and 69.8% for Tiko, Limbe and Buea respectively (Table I). Parasite counts ranged from1 040 to 120 000 parasites per µI of blood.

Table I: The prevalance of malaria per locality.

Locality	No. of positive patients	No. of negative patients	Total	Prevalance
Tiko	93	20	113	82.3%
Limbe	110	40	150	73.3%
Buea	44	19	63	69.8%

WHO *in vivo* Seven Day Test.

Of the 247 patients with a positive diagnosis, only 122 agreed to be treated with chloroquine and followed up thereafter for detection of chloroquine resistance. Resistant parasite strains were detected in 10 patients of whom two, seven and one were from Tiko, Limbe and Buea respectively. There were a total of 4 R11 and 6 RIII type resistance detected (Table II). No statistically significant difference ($p>0.05$) was observed in the pattern of sensitivity and resistance between the three areas.

Parasite Counts at the Various Levels of Resistance.

Classification into the different levels of resistant types was based on parasite counts on DO, D2 and D7. The mean parasite count in cases of R11 type resistance dropped appreciably from DO to D2 but by D7, a fourfold increase was recorded (Table III). In cases with RIII type resistant parasite strains, parasite counts continued to increase steadily from DO to D7.

Table II: Prevalence of chloroquine sensitivity in the three areas studied.

Locality	Sensitive to chloroquine	Chloroquine resistance R11	R111	No. tested	Prevalence of resistance (%) ±Confidence* Interval*
Tiko	45	1	1	47	4.3± 5.79
Limbe	46	2	5	53	13.2 ± 9.11
Buea	21	1	0	22	4.5 ± 8.66
Total	112	4	6	112	812± 4.86

Chi-square=3,131.** ***5% signficant.
Degrees of freedom=2.** **p>0.05.

*Table III: Evolution of parasitaemia in patients with resistant **P.falciparum.***

Resistance level	No. of cases	DO	D2	D7
RII	4	21 400 (±10 374.97)	26 530 (± 14 233.52)	26 500 (±12 257.65)
RIII	6	18 403.33 (± 10 770.33)	23 826.67 (± 1 136.51)	25.333.33 (± 10.982.21)

Plasmodium Species Detected by PCR.

A total of 96 positive blood samples as detected using microscopy were analysed for speciation by PCR. An equal number of 32 samples each were randomly selected from samples collected in the three study areas. The genus specific primers strongly hybridized with RNA from blood with all four species of malaria parasites. No significant signals were detected with human DNA or in the absence of DNA isolates (negative controls). Visible bands were detected on all four positive controls (FMO was used as positive control for *P. falciparum*, *P. malariae* and *P. ovale* respectively while a separate control was used for *P. vivax*). Of the 96 samples analysed by PCR, 93 (96.8%) were positive for *P. falciparum* and three for *P. ovale* (3%). Of those positive for *P. falciparum* six (6.3%) had a mixed infection with *P. malariae* (Table IV). No *P. vivax* infection was detected.

*Table IV: Prevalence of the different **Plasmodium** species in the study areas.*

Species	Tiko n*=32	Limbe n=32	Buea n=32	Total n=126
P. falciparum	29 (90.6%)	28 (87.5%)	30 (93.8%)	87 (90.6%)
P. ovale	1 (3.1%)	2 (6.3%)	0	3 (3.1%)
P. falciparum & *P. malariae*	2 (6.3%)	2 (6.3%)	2 (6.3%)	6 (6.3%)

** n= number of samples.*

Discussion

The results from this study indicated that resistance to chloroquine in the South West of Cameroon has dropped since it was first reported. The status of drug resistant *P. falciparum* in the Southern part of Cameroon as determined from 1987 to 1988 clearly showed R11and R111 levels of resistance in 18 and 62% respectively of the 389 cases studied.[10] The overall prevalence for resistance was 85.08% (CI = 1.96). While there are no previous figures on the level of chloroquine resistance in Buea, the prevalence of resistance from our study was 4.3%, 13.2% and 4.5% for Tiko, Limbe and Molyko (Buea) respectively with an overall prevalence of 8.2% (CI = 4.86). An earlier study had reported a resistance level of 20.6% in Tiko and 55% in Limbe.[11]

Since the first reports of chloroquine drug resistance in the mid 1980's the use of this drug as the first line drug for treatment of uncomplicated *P. falciparum* malaria in the study areas was discouraged. As a result, many physicians stopped the use of chloroquine as the first line drug. Results of a questionnaire that was administered in the study areas on the types of antimalarial used in self-medication showed a frequency rate of 45.5%, 35.9% and 16.3% for sulfadoxine pyrimethamine, quinine sulfate and chloroquine respectively (unpublished data). This further indicated that chloroquine was no longer used frequently. The itching effect of chloroquine which makes it unattractive to many patients has also contributed to the infrequent use of this drug. It was noticed from the present study, however, that of 13 individuals with malaria who claimed to be allergic to chloroquine, only one of these experienced pruritis following oral chloroquine intake. The others were able to complete the dose for treatment without further complains. Furthermore none of the patients vomited during the course of the study. All of the above factors seem to have contributed to a significant reduction in chloroquine drug pressure.

The standard seven day test was used in this study because it is appropriate in detecting R11 and R111 type resistance[12] which had previously been reported to be the common types in the study areas. The parasitaemia reported in the study ranged from 1 040 to 120 000 parasites/ml of blood. Although parasitaemia was generally not high because of the period of study, a relatively true prevalence of resistance was obtained. Although the primers used in our study have been validated elsewhere, this is the first study in South West Cameroon in which parasite speciation has been done using such a sensitive technique. The findings are therefore significant. *P. falciparum* was found to be the predominant species in all three areas accounting for 96.8% of all positive cases. This study has provided additional information with the finding that *P. malariae* occurred in 6.3% of positive cases as mixed infections with *P. falciparum* while *P. ovale* accounted for single infections in 3% of positive cases. The superiority of the PCR technique over slide microscopy is evident from the fact that two of the three cases of *P. ovale* infections and four mixed infections were missed by microscopy. Information on proper speciation of' parasites is essential for the institution of effective treatment of malaria.

Our results indicated that there was no significant influence of altitude on the prevalence of malaria in the

three areas studied (p>0.05). It is expected that with Molyko situated at a higher altitude than Tiko and Limbe a significant difference in prevalence would have been observed. It is worth mentioning that between 1993 when the University of Buea was opened and the present date, the population of Molyko has just about doubled and this could have brought about a significant change in the epidemiology of malaria in this area. Unfortunately there are no previous data on prevalence studies in Molyko for comparison. Further investigations at much higher altitudes in Buea are necessary to be more conclusive.

In conclusion, the data from our study showed that *P. falciparum* resistance to chloroquine may no longer be such an alarming problem as reported earlier in some areas of the South West Province. The results presented here have important implications in the treatment of malaria considering that chloroquine remains the cheapest antimalarial in Cameroon. The drug is generally well tolerated. We suspect that the decline in chloroquine resistance may well reflect a decrease in drug pressure over the years. The superiority of the PCR technique with reference to slide microscopy was also evident.

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