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Role of the *pfcrt* codon 76 mutation as a molecular marker for population-based surveillance of chloroquine (CQ)-resistant *Plasmodium falciparum* malaria in Ugandan sentinel sites with high CQ resistance

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Abstract

The mutant genotype at codon 76 of the *pfcrt* gene (T76) has been proposed as a molecular marker for surveillance of chloroquine (CQ)-resistant *Plasmodium falciparum* malaria but this proposal has not been validated by population-based surveys. In 1998–99, in 6 Ugandan sentinel sites, the prevalence of *P. falciparum* infections with the T76 genotype and the level of CQ use were measured by community surveys, and CQ resistance was determined by in-vivo tests on 6–59-month-old children with clinical malaria. The prevalence of T76 was not related to the overall clinical (early and late treatment failure: ETF+LTF; $r = 0{\cdot}14$, $P = 0{\cdot}78$) or parasitological (RI+RII+RIII; $r = 0{\cdot}17$, $P = 0{\cdot}73$) CQ resistance. However, the percentage of individuals carrying only infections with the T76 genotype (T76 alone) increased with increasing ETF ($r = 0{\cdot}76$, $P = 0{\cdot}07$) and type RIII parasitological failure ($r = 0{\cdot}69$, $P = 0{\cdot}12$). Similarly, the ratio between T76 and K76 (the wild type) prevalences (T76/K76) was strongly and positively correlated with ETF ($r = 0{\cdot}85$, $P = 0{\cdot}03$) and RIII ($r = 0{\cdot}82$, $P = 0{\cdot}04$). Moreover, T76 alone ($r = 0{\cdot}90$, $P = 0{\cdot}01$) as well as T76/K76 ($r = 0{\cdot}90$, $P = 0{\cdot}01$) significantly increased with increasing community CQ use. T76 alone and T76/K76 can be useful markers to estimate the ETF and RIII prevalence as well as the amount of CQ use in the community.

Keywords: malaria, *Plasmodium falciparum*, gene mutation, genes, *pfcrt*, drug resistance, chloroquine, Uganda

Introduction

Resistance of *Plasmodium falciparum* to chloroquine (CQ) emerged in the late 1950s in south-east Asia and South America (WERNSDORFER, 1991) and was first reported on the African continent among the non-immunes in the late 1970s (FOGH *et al.*, 1979). Parasite resistance among semi-immune individuals is already high in some sub-Saharan countries and this has prompted National Malaria Control Programmes in East and Central Africa to change their treatment policies from a first-line CQ-based regimen to a sulfadoxine–pyrimethamine (SP) regimen either as mono- or combination therapy.

The role of mutations linked to parasite resistance to CQ is not clear yet. Parasite resistance to CQ has been linked to polymorphism in the *pfmdr1* gene, although multiple mutations in different genes in the 36-kb segment of chromosome 7 are probably required for clinical resistance (FOOTE *et al.*, 1990). A single candidate gene, *cg2*, a polymorphic gene encoding a 330 kDa protein, was proposed as the one responsible for CQ resistance (SU *et al.*, 1997). However, the observed association is probably due to linkage disequilibrium (LD) on chromosome 7 with the *pfcrt* gene, which encodes an integral transmembrane protein, the *P. falciparum* CQ resistance related transporter (PfCRT) protein, recently linked to CQ resistance (FIDOCK *et al.*, 2000; DJIMDÉ *et al.*, 2001a). Several substitutions have been reported at various codons in the *pfcrt* gene. These include the substitution of threonine for lysine at position 76 (K-76T), serine for alanine at position 220 (A220S), glutamic acid for glutamine at position 271 (Q271E), serine for asparagine at position 326 (N326S) and isoleucine for arginine at position 371(R371I). However, the mutation with the most significant association to in-vivo failure in a clinical setting in Mali was the K-76T (DJIMDÉ *et al.*, 2001a).

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Molecular markers have been suggested as potential tools for surveillance of malaria parasite resistance (PLOWE *et al.*, 1997; DJIMDÉ *et al.*, 2001b). Validated molecular markers would allow the mapping of parasite resistance over large areas. However, the use of molecular markers in general and more specifically the use of polymorphism in the *pfcrt* gene at codon 76 for predicting the level of in-vivo parasite resistance to CQ within communities has not yet been validated with extensive field data. Previous studies measured the prevalence of the T76 mutation in samples collected from patients with clinical malaria attending health clinics (BABIKER *et al.*, 2001; DJIMDÉ *et al.*, 2001a; DORSEY *et al.*, 2001). Considering the widespread self-medication in most African countries (FOSTER, 1995; MCCOMBIE, 1996), parasites from patients recruited for in-vivo tests are likely to have been exposed to antimalarial drugs prior to presentation. In this study on the K-76T polymorphism in the *pfcrt* gene, a random sample of healthy individuals from the general population, rather than patients attending health centres, were investigated. More specifically, we studied the CQ clinical and parasitological resistance and the population-based prevalence of the T76 and K76 genotypes of the *pfcrt* gene at 6 Ugandan sentinel sites with different patterns of malaria transmission and drug use. Our aim was to determine the relationship between CQ resistance and the prevalence of the T76 genotype in these communities.

Materials and Methods

Study population and study design

The Uganda National Council for Science and Technology (UNCST), and the Ethical Review Board (ERB) of the Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium, approved the ethical aspects of the study. The study was conducted in 6 sentinel sites jointly chosen by the East Africa Network for Monitoring Antimalarial Treatment (EANMAT) and the Uganda National Malaria Control Programme (UNMCP) for the surveillance of antimalarial drug efficacy. The study sites were selected according to the following criteria: transmission intensity (high, medium, low), moderate/high population density, easy

accessibility/practicability, inclusion of at least a country border area, geographical representation, health unit with a defined catchment population (hospitals excluded) and the presence of a stable population with limited in and out migration. Prior to the study, profiles were compiled for each of the study sites, which included: location of the sites, climate and vegetation, ethnography and life-style, drug outlets and common antimalarial drugs found in the study sites. Five of the study populations (Arua, Apac, Tororo, Kabarole and Rukungiri) are rural and one (Jinja) is peri-urban. The sites lie between 945 m and 1219 m above sea level. They have similar rainfall pattern with 2 yearly peaks, the first peak from March to May and the second from August to October. The main occupation of the population around the sentinel sites is subsistence agriculture. Population-based cross-sectional surveys and in-vivo efficacy tests were conducted at all the 6 sites.

In vivo efficacy tests in patients with uncomplicated P. falciparum *malaria*

Over the period 1998–99, drug efficacy tests were conducted *in vivo* at the 6 sentinel sites using a standard protocol developed by the World Health Organization (WHO, 1996) and a modified field manual developed by EANMAT on patients attending the local health centre. Children aged 6–59 months with fever (body temperature $\geq$37·5°C) or history of fever in the past 24 h, with a *P. falciparum* mono-infection and a parasite density between 2000 and 100 000/μL of blood were recruited, treated and followed-up to day 14 after treatment. Children with general danger signs or severe malaria, other causes of fever, severe malnutrition and history of allergic reactions to sulpha drugs were excluded. CQ, 150-mg base, (quality assured) was administered orally under supervision and according to bodyweight (25 mg/kg given in divided doses of 10 mg/kg on day 0, and day 1 and 5 mg/kg on day 2). Patients were followed-up on days 1, 2, 3, 7 and 14 and on any other day if they developed symptoms. Children who took drugs from other sources or those who developed concomitant infections were excluded from the study. Patients were actively followed-up when they did not arrive for a scheduled visit. Clinical outcomes were classified into 3 groups: early treatment failure (ETF), late treatment failure (LTF) and adequate clinical response (ACR) as defined previously (WHO, 1996). Total treatment failure (TTF) is defined as the sum of ETF and LTF. Parasitological response was classified according to 4 categories: sensitive (clearance of parasites after treatment without subsequent recrudescence), RI (initial clearance followed by recrudescence), RII (reduction of parasitaemia by day 3 to <25% of initial parasitaemia but no clearance) and RIII (no reduction of parasitaemia or reduction to a level $\geq$25% of the initial parasitaemia). Sample size estimations were performed using the lot quality assurance sampling (LQAS) method (LEMESHOW & TABER, 1991). The upper threshold level of clinical failures beyond which replacement of CQ is deemed necessary as a national policy was taken as 25% and the lower threshold level of clinical failures below which it would be acceptable to continue the utilization of CQ was taken as 10%. Based on these assumptions, approximately 42 children were required per site to derive valid estimates with a confidence level of 95% and 80% power for detecting that the in-vivo CQ resistance at each site was significantly less or greater than 25%.

Population-based cross-sectional surveys

Prior to each cross-sectional survey, a census of the population (maximum 10 000 individuals) was carried out at each study site in order to have a sampling frame from which to select individuals to be included in the cross-sectional surveys. A sample of 250 people aged 1–45 years was selected randomly from the census file using a computer-generated sample list (EpiInfo, CDC, Atlanta, GA, USA) for each sentinel site. During the survey, carried out between September and December 1999, selected individuals were assessed clinically (splenomegaly) and the axillary temperature was determined with an electronic thermometer. Two thick and 2 thin blood films for parasitological examination were collected. Blood samples for polymerase chain reaction (PCR) analysis were collected on to 3M Whatman filter-paper and stored in individual zip-lock bags at room temperature. In addition a urine sample was collected from each individual into a universal sterile container, stored at 4–8°C, and was subsequently used for determination of CQ metabolites.

Estimation of CQ drug pressure within the community

CQ drug pressure in the community was estimated as the percentage of selected individuals with detectable levels of CQ metabolites in the urine as determined by a dipstick. The dipstick test is a qualitative test based on an enzyme-linked immunosorbent assay (ELISA) blocking test where an immobilized antibody is first reacted with the test sample, then with a drug–peroxidase-enzyme conjugate and finally with the peroxidase-enzyme substrate (EGGELTE, 1990; EGGELTE *et al.*, 1992; SCHWICK *et al.*, 1998).

Mutation-specific polymerase chain reaction

DNA was extracted from the corresponding filter-paper samples using the Chelex-100 resin method as described previously (PLOWE *et al.*, 1995). Nested mutation-specific PCR (MS-PCR) was performed on samples randomly selected among those microscopy positive. Parasite DNA was used as template to amplify the relevant portion of the *pfcrt* gene using nested PCR as described previously (details are found at http://medschool.umaryland.edu/CVD/plowe.html). In brief, flanking primers TCRP1 and TCRP2, amplifying a 537-bp region encompassing the K-76T mutation were employed in the first PCR run, in a reaction mixture containing 2·5 units *Taq* polymerase, 2·5 mM magnesium chloride, 200 μM dNTPs and 1 μM concentration of TCRP1 and TCRP2. The cycling parameters consisted of an initial denaturation step at 94°C for 3 min and 45 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 60°C for 1 min; in the last cycle, there was a final extension step at 60°C for 3 min. In addition to DNA from field samples, DNA from the CQ-sensitive strain 3D7 and the CQ-resistant strain FCB1 were used as positive controls for the wild-type and mutant codons respectively and water served as a negative control. The success of the PCR was assessed by running 15 μL of the PCR products on an agarose gel, which was stained with ethidium bromide, and examined under ultraviolet transillumination. For the nested PCR, 1 μL of the outer product was amplified using a common primer (TCRP3) and either a mutant-specific primer (TCRP4m) for the mutant codon or wild-type specific primer (TCRP4w) for the wild-type codon. The cycling conditions were as above except that the annealing and extension temperatures were 47°C and 64°C, respectively. The positive control for the mutant and wild-type codon was FCB1 and 3D7, respectively; again water served as the negative control. In successful mutant and wild-type amplicons, a band of 366 bp was observed. Twenty percent of the samples from each site were subjected to quality control analysis by having 2 different technicians genotype the same samples; the degree of disagreement between 2 independent experimenters was <3%.

Statistical analysis

Data were double entered in EpiInfo and range and consistency checks were done before statistical analysis with Stata Version 6.0 (Stata Corporation, Texas,

USA). The frequency of wild and mutant genotypes at codon 76 of *pfcrt* among infected individuals at the different sites was compared using the χ^2 test. Because the sum of the prevalence of wild (K76) and mutant (T76) genotypes was higher than 100%, as a consequence of the high number of mixed infections, the percentage of individuals carrying only infections with the T76 genotype (T76 alone) and the ratio between T76 and K76 (the wild type) prevalences (T76/K76) were used. The relationship between these 2 markers and the prevalence of resistance (clinical and parasitological) and community CQ use was determined by linear regression where T76 alone or T76/K76 were the dependent variables. In a univariate analysis in which CQ use was a covariate, the Mantel–Haenszel (MANTEL & HAENSZEL, 1959) technique was used to compare the occurrence of the T76 point mutation in isolates from infected people aged <10 years to that from people aged ⩾10 years. Age was used as a proxy for host immunity.

For CQ resistance, we categorized the sites according to the stages for making a systematic change in the antimalarial drug policy based on TTF of first-line therapy into: grace period (0–5%), alert period (6–14%), action period (15–24%) and policy change (⩾25%) (KITUA, 2000).

Results

Parasite prevalence and community CQ use

Overall, 1314 people were surveyed, of whom 1300 (99%) provided a blood sample for *P. falciparum* parasitaemia determination: 591 (45·5%) of the 1300 had parasitaemia. The prevalence of malaria infection within the community varied significantly according to site ($P = 0·001$, Table 1). Parasite prevalence (PR) in the age-group 2–9 years is normally used as an index for malaria endemicity. Malaria is classified as hypoendemic when the PR is <10%, mesoendemic when the PR is 11–50%, hyperendemic when the PR is >50% and holoendemic when the PR is constantly >75% (METSELAAR & THIEL, 1959). Using this classification, 3 sites (Arua, Apac and Tororo) can be described as holoendemic, one site (Kabarole) as hyperendemic and the other 2 sites (Jinja and Rukungiri) as mesoendemic (Table 1). The median parasite prevalence for all ages was 49·3% (range 10–71%); however, the median parasite prevalence among children aged 2–9 years was 73·2% (range 13·3–88·3%). With respect to CQ community use, 93% (1219 of 1313) of the surveyed population provided a urine sample for CQ biochemical tests and of these 48·2% (587 of 1219) were positive for CQ metabolites. CQ use (all ages) varied significantly according to endemicity ($P < 0·001$, Table 1). Higher CQ use was observed in the mesoendemic sites (Jinja and Rukungiri) as compared to the holo- or hyperendemic ones (Kabarole, Apac, Arua and Tororo) (Table 1) and was inversely correlated to parasite prevalence ($r = -0·88$, $P = 0·01$).

In-vivo CQ treatment outcome

The in-vivo outcomes for 221 children aged 6–59 months who were followed-up for 14 days or until they reached an outcome are reported in Table 2. The loss at follow-up for each site was <8%, which is less than the 10% upper cut-off acceptable for in-vivo efficacy studies. The prevalence of CQ treatment fail-

Table 1. Mean age, *Plasmodium falciparum* parasite prevalence and chloroquine (CQ) use by Ugandan site established by cross-sectional surveys in 1998–99

Site	Classification according to malaria endemicity	Children aged 1–9 years % (*n*/*N*)	Mean age in years (SD)	Parasite prevalence (all ages) (*n*/*N*)	Parasite prevalence (age 2–9) % (*n*/*N*)	CQ use (biochemical test)[a] % (*n*/*N*)
Jinja	Mesoendemic	32·1 (68/212)	19·3 (14·2)	10·0 (21/210)	13·3 (8/60)	81·6 (146/179)
Rukungiri	Mesoendemic	31·8 (62/195)	18·5 (12·8)	32·3 (63/195)	39·3 (22/56)	68·4 (119/174)
Tororo	Holoendemic	48·0 (118/246)	14·4 (12·4)	70·7 (174/246)	88·3 (83/94)	40·5 (98/242)
Kabarole	Hyperendemic	42·0 (89/212)	15·4 (12·3)	45·7 (96/210)	67·1 (49/73)	43·3 (88/203)
Arua	Holoendemic	38·6 (78/202)	19·5 (16·6)	55·3 (110/199)	82·5 (52/63)	32·6 (58/178)
Apac	Holoendemic	37·4 (92/246)	15·7 (12·2)	52·9 (127/240)	79·4 (54/68)	32·1 (78/243)
Total		38·6 (507/1313)	16·9 (13·5)	45·5 (591/1300)	64·7 (268/414)	48·2 (587/1219)

[a]Proportion of population with positive urine test for CQ.

Table 2. Clinical and parasitological failures for malaria treatment with chloroquine, by site in Uganda for children aged 6–59 months

			Parasitological failure				Clinical failure	
Site	Year of study	Stages towards antimalarial policy change	RI % (*n*/*N*)	RII % (*n*/*N*)	RIII % (*n*/*N*)	RI+RII+RIII % (*n*/*N*)	TTF (ETF+LTF) % (*n*/*N*)	ETF % (*n*/*N*)
Jinja	1998	Change period	0 (0/24)	4·2 (1/24)	29·2 (7/24)	33·3 (8/24)	28·0 (7/25)	28·0 (7/25)
Rukungiri	1999	Alert period	0 (0/49)	6·1 (3/49)	4·1 (2/49)	10·2 (5/49)	10·0 (5/50)	10·0 (5/50)
Tororo	1999	Change period	0 (0/57)	70·2 (40/57)	17·5 (10/57)	87·7 (50/57)	42·4 (25/59)	20·3 (12/59)
Kabarole	1999	Change period	31·3 (5/16)	37·5 (6/16)	12·5 (2/16)	81·3 (13/16)	43·8 (7/16)	12·5 (2/16)
Arua	1998	Action period	14·8 (8/54)	22·2 (12/54)	5·6 (3/54)	42·6 (23/54)	21·1 (12/57)	8·8 (5/57)
Apac	1999	Alert period	23·5 (12/51)	15·7 (8/51)	1·96 (1/51)	41·2 (21/51)	14·8 (8/54)	7·4 (4/54)

Numbers in parentheses are actual figures observed.
TTF, total treatment failure; ETF, early treatment failure; LTF, late treatment failure.
See the text for definitions of the levels of parasitological resistance (RI, RII and RIII).

ure for 2 sites (Rukungiri and Apac) is in the stage categorized as alert period, one site (Arua) is in the action period and 3 sites (Jinja, Tororo, and Kabarole) are in the change policy period (Table 2).

T76 and K76 genotype frequency, CQ treatment outcome and CQ use

The molecular analysis of the *pfcrt* mutation at codon 76 was carried out on 302 blood samples and the presence of the K76 or T76 genotypes was determined in 296 samples. T76 frequency was extremely high (median 93·6%, range 86·3–100%); 120 infections (41%) had T76 alone, 16 (5·4%) K76 alone and 159 (54%) carried both genotypes. Their prevalence varied significantly between sites ($P < 0·001$) (Table 3). There was no correlation between the T76 genotype (alone or in combination with K76) and the clinical (ETF+LTF) ($r = 0·14$, $P = 0·78$) or parasitological (RI+RII+RIII) ($r = 0·17$, $P = 0·73$) resistance. However, T76 alone was positively correlated with ETF ($r = 0·76$, $P = 0·07$), with RIII ($r = 0·69$, $P = 0·12$) and CQ use ($r = 0·90$, $P = 0·01$) (Fig. 1). Similarly, T76/K76 significantly increased with increasing ETF ($r = 0·85$, $P = 0·03$) (Fig. 2), RIII ($r = 0·82$, $P = 0·04$) and CQ use ($r = 0·90$, $P = 0·01$) (Fig. 3).

T76 genotype frequency and age

Host immunity can affect treatment outcome in malaria endemic regions. In order to ascertain whether the prevalence of the T76 genotype varies with immunity, we used age as a proxy for host immunity. The frequency of the T76 genotype was significantly lower, after stratifying by site, in individuals aged ≥10 years as compared to those aged <10 years (Mantel–Haenszel odds ratio = 0·26; 95% confidence interval 0·10–0·63).

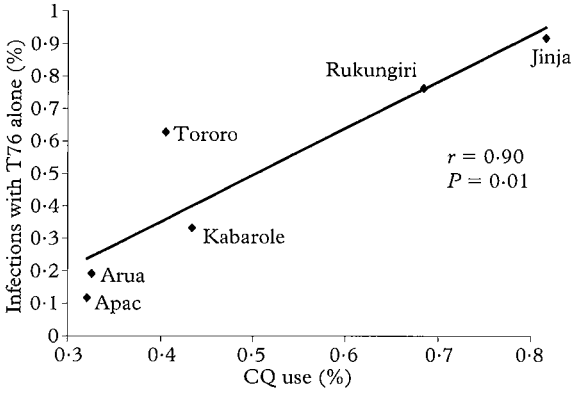

Fig. 1. The correlation between the prevalence of the *Plasmodium falciparum* T76 genotype alone and the level of chloroquine (CQ) use in the Ugandan community (1998–99), with the corresponding regression line.

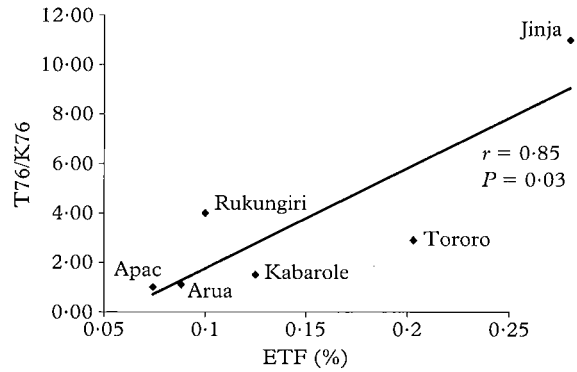

Fig. 2. The correlation between the *Plasmodium falciparum* T76/K76 ratio and the prevalence of early treatment failure (ETF) for chloroquine in the Ugandan community (1998–99), with the corresponding regression line.

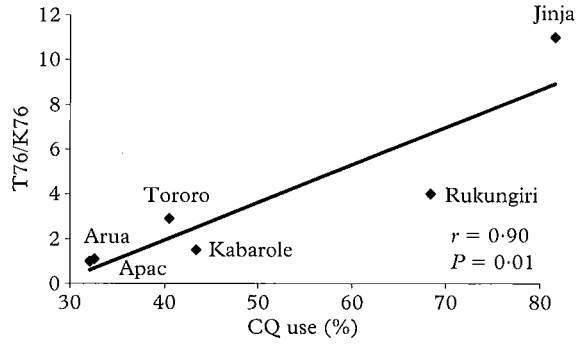

Fig. 3. The correlation between the *Plasmodium falciparum* T76/K76 ratio and the level of chloroquine (CQ) use in the Ugandan community (1998–99), with the corresponding regression line.

Discussion

Our study of the K-76T mutation in the *pfcrt* gene in *P. falciparum* isolates in Uganda is unique in several important aspects. First, the study was carried out at sentinel sites in various parts of the country, representing different intensities of malaria transmission. This is probably an important element for the rate of spread of antimalarial drug resistance (HASTINGS & D'ALESSANDRO, 2000) and might therefore have an impact on the selection of mutant genotypes linked to drug resistance. Secondly, we took into consideration the level of CQ

Table 3. Prevalence of wild-type (K76) and mutant (T76) genotypes at position 76 of *pfcrt* and ratio K76/T76 by Ugandan site

Site	*n*	Prevalence of the wild (K76) genotype (alone + mixed infections) % (95% CI)	Prevalence of the mutant (T76) genotype (alone + mixed infections) % (95% CI)	Ratio of T76:K76	Prevalence of the T76 genotype alone % (95% CI)
Jinja	12	9·1 (0·2–41·3)	100 (71·5–100)	11·0	91·7 (76·0–100)
Rukungiri	59	23·3 (13·4–36·0)	93·6 (78·6–99·2)	4·0	76·3 (65·4–87·1)
Tororo	43	32·3 (16·7–51·4)	93·6 (78·6–99·2)	2·9	62·8 (48·3–77·2)
Kabarole	48	65·3 (50·4–78·3)	98·0 (89·9–99·9)	1·5	33·3 (20·0–46·7)
Arua	73	80·8 (69·9–89·1)	86·3 (76·2–93·2)	1·1	19·2 (10·1–28·2)
Apac	61	88·2 (77·8–95·3)	90·2 (79·8–96·3)	1·0	11·7 (3·5–19·7)

95% CI, 95% confidence interval.

use in each site. This approach has provided an invaluable insight about the level of drug pressure and the prevalence of the mutant genotype at codon 76 of the *pfcrt* gene, which has been linked to CQ resistance. This is the first report, as far as we are aware, of a population-based investigation on the *pfcrt* T76 mutation and its role as a genetic marker for the surveillance of CQ resistance at community level. Indeed, we determined the prevalence of the *pfcrt* T76 mutation in a randomly selected sample of the population. Our approach differs from that used by DJIMDÉ *et al.* (2001b) in Mali where the prevalence of the point mutation was determined in blood samples collected before treatment in individuals with uncomplicated falciparum malaria. The prevalence of the T76 mutant genotype was extremely high, in most sites above 90%, and did not vary significantly between them. However, the prevalence of the K76 wild genotype, as well as that of individuals carrying infections either with both genotypes or with the T76 genotype alone, varied significantly between sites. A high prevalence of infections with the T76 genotype in pretreatment isolates has been reported from Sudan and Uganda (BABIKER *et al.*, 2001; DORSEY *et al.*, 2001) and was not predictive of in-vivo failure at individual level. However, pretreatment isolates are normally taken from patients seeking medical care, a selected group more likely to have taken antimalarial drugs as compared to the general population (FOSTER, 1995; MCCOMBIE, 1996). Although we tried to avoid such selection bias by randomly sampling individuals from the general population, we were unable to link the overall T76 prevalence to that of CQ resistance, mainly because the mutant genotype was present in most of the malaria infections we identified. More promising markers to estimate CQ resistance seem to be the percentage of individuals carrying infections with only the T76 mutated genotype and the ratio between T76 and K76 prevalence. Although these were weakly correlated to the overall clinical (ETF+LTF) and parasitological (RI+RII+RIII) CQ resistance, a positive and strong relationship with ETF and RIII was found. Such correlation was stronger for the T76/K76 ratio, suggesting that the K76 prevalence and its relationship with the T76 prevalence is more important than their individual values taken alone. It is important to notice that these markers correlate well only with the last stages (ETF and RIII) of drug resistance. The mutation at codon 76 in the *pfcrt* gene is probably necessary but not sufficient for the development of CQ resistance. In this work we did not examine mutations at other *pfcrt* codons nor in the other genes such as *pfmdr1*, because *pfcrt* 76 has been previously suggested as a molecular marker for surveillance of CQ-resistant *P. falciparum* malaria (DJIMDÉ *et al.*, 2001b). However, according to our results, the prevalence of infections carrying the K76 wild genotype is more closely related to CQ resistance than that of the T76 mutated genotype. Therefore, the disappearance of infections with the wild genotype may be one of the last stages of the long process resulting in high CQ resistance. Drug pressure must be an important factor in this process. Increasing CQ drug pressure would probably select the T76 mutation and would consequently decrease the prevalence of the wild-type (K76) genotype (KYOSIIMIRE-LUGEMWA *et al.*, 2002). In our study the prevalence of the T76 genotype alone as well as the ratio T76/K76 significantly increased with increasing CQ use, mainly because of the disappearance of the K76 wild genotype. This observation supports the idea that increasingly important drug pressure selects mutations involved in CQ resistance.

Can molecular markers such as the T76/K76 ratio be deployed for the surveillance of CQ resistance? We tried to validate them in sites where CQ resistance was already relatively high, none of the sites had a TTF lower than 10%. A T76/K76 value higher than 1·5 was a marker of high ETF, the only exception being Rukungiri where a T76/K76 value of 4 corresponded to 10% ETF and 0% LTF, suggesting the presence of highly resistant parasites. Moreover, T76/K76 could also be used to estimate CQ use at community level not only in countries where CQ is still the first-line drug, such as in West Africa, but also in Eastern and Central Africa, where a change of treatment policy from CQ as first-line drug has already occurred. There is evidence in Malawi that CQ sensitivity probably increases after prolonged withdrawal (TAKECHI *et al.*, 2001). In these situations the T76/K76 ratio could be used to monitor the amount of CQ circulating within the community.

In areas with intense transmission, premunition against malaria develops as a result of repeated infections and this immunity has been observed as a potent factor in the efficacy of antimalarial therapy. Our observation of a higher prevalence of the T76 genotype in isolates from people aged <10 years compared to that in isolates from people $\geqslant 10$ years indicates the role of host immunity in the clearance of mutant malaria parasite strains.

We believe that decisions on antimalarial drug policy will never be entirely based on molecular data alone. However, molecular markers could have an important role in roughly estimating the prevalence of antimalarial resistance over a region or a country. Subsequently, in-vivo tests could be carried out in sites selected on the basis of molecular data. Although our findings should be confirmed in other settings, the relationship between ETF and T76/K76 ratio is sufficiently strong to consider it as a valid molecular marker for CQ resistance; values over 1·5 are probably indicators of high resistance.

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