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A point mutation in codon 76 of *pfcrt* of *P. falciparum* is positively selected for by Chloroquine treatment in Tanzania

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Abstract

This study was undertaken to validate the relevance of Chloroquine (CQ) resistance markers *pfcrt*^{76} and *pfmdr1*^{86} in an endemic area in Tanzania. After treatment with CQ, recrudescence was distinguished from new infection by *msp2* genotyping, and the number of concurrent infections was also determined. The rate of children with recrudescent parasites at day 7 and/or day 14 amounted to a parasitological failure rate of 22.4% using PCR. The mean multiplicity of infection at day 0 was 3.2 ($n = 71$). The allelic frequencies of the mutated *pfcrt*^{76} and *pfmdr1*^{86} were estimated to be 92 and 77%, respectively. Both values exceeded by far the observed frequency of 14% of recrudescent parasites as calculated on the whole analysed parasite population taking multiple infections into account. Although neither mutant allele is of predictive value for parasitological resistance, there is evidence for a role of *pfcrt*^{76} in CQ resistance in the natural parasite population. All wild-type *pfcrt*^{76} alleles were eliminated before day 3, after the onset of CQ treatment and no recrudescent parasite with the wild-type allele was observed at later time points. The discrepancy between the rate of resistant parasites (14%) and the frequency of the mutant *pfcrt*^{76} allele (92%), however, indicates that other polymorphisms and other factors must be involved in CQ resistance. No selective elimination of the *pfmdr1*^{86} wild-type allele was observed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Drug resistance; Chloroquine; Multiplicity of infection; *P. falciparum*; Malaria tropica; Genotyping; *pfcrt*; *pfmdr*; *msp2*

1. Introduction

Chloroquine (CQ) has long been used as first line drug for the treatment of uncomplicated malaria tropica in Africa for the last decades due to its affordability, efficacy and safety. The rapid spread of CQ resistant parasites, however, has limited its use for malaria treatment (White, 1992) and there is an urgent need for alternatives.

The mechanism of CQ action and resistance is still a matter of investigation. CQ accumulates in the digestive vacuole of the parasite (Yayon et al., 1984) where it is supposed to interfere with haem detoxification (Slater and Cerami, 1992), or modifying aspartic and cysteine protease activity (Goldberg et al., 1991) or intravesicular pH (Krogstad et al., 1985). Resistant parasites are characterised by a reduced amount of CQ accumulated in the food vacuole as compared to sensitive parasites (Fitch, 1970). Two genes (*pfmdr1* and *pfmdr2*) homologous to the mammalian multidrug resistance gene (*mdr*) have been identified in *P. falciparum* (Foote and Kemp, 1989). *mdr* codes for an ATP driven P–glycoprotein pump which confers drug resistance in mammalian cancer cell lines (Roninson, 1987). Point mutations leading to amino acid exchanges in *pfmdr1* have been related to drug resistance in *P. falciparum* (Ponnudurai et al., 1981; Foote et al., 1990; Frean et al., 1992; Cox-Singh et al., 1995; von Seidlein et al., 1997). Several field studies, however, failed to show an association between *pfmdr1* polymorphism and drug resistance (Haruki et al., 1994; Bhattacharya et al., 1997; Bhattacharya and Pillai, 1999). Recently, allelic replacement experiments have shown that polymorphisms in *pfmdr1* modify the sensitivity to CQ in parasites already resistant to CQ (Reed et al., 2000). These findings indicate that other genes might be crucial for CQ resistance. Genetic cross experiments mapped CQ resistance to a 36 kb region in chromosome 7 (Su et al., 1997). In this region, the genes *cg2* and *cg1* displayed codon exchanges which associated well with CQ resistance in several strains (Su et al., 1997). Allelic replacement with *cg2* and *cg1* sequences from CQ sensitive parasites, however, did not modify the degree of resistance in a CQ resistant strain (Fidock et al., 2000a).

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Recent evidence points to an essential role of *pfcrt* for CQ resistance, another gene within this 36 kb region in chromosome 7 (Fidock et al., 2000b). This gene codes for a transmembrane protein which is located in the digestive vacuole of the parasite. A mutation in codon 76 leading to a change from lysin to threonine has been found exclusively in CQ resistant strains of *P. falciparum*. This mutation is associated with an increased acidification of the digestive vacuole, the compartment of CQ action (Fidock et al., 2000b). Further evidence was also given in a epidemiological study in Mali by Djimdé et al. (2001), where it was shown that the *pfcrt* mutation was selected after CQ treatment.

The objective of this study was to assess the association of the *pfcrt*^{76} and the *pfmdr1*^{86} polymorphisms with CQ resistance in a field trial in Tanzania. The samples derived from a standard in vivo test series carried out in Masasi, one of the sentinel sites for monitoring drug resistance in Tanzania. To distinguish between recrudescence and new infection, all samples were PCR amplified for the locus of the merozoite surface protein 2 (*msp2*) (Snounou and Beck, 1998).

2. Materials and methods

2.1. Study site and study population

The study was conducted at Nagaga Health Centre in the Masasi District of Tanzania between January and March of 1999. This district is located in the southernmost area bordering Mozambique. The area under study is uniform comprising coastal plains with sandy soils and occasional rocky outcrops with hilly ridges. There are numerous marshy areas which may be associated with rivers or water courses where rice is cultivated during the long rains (March–June). Malaria transmission is intense and perennial. Nagaga Health Centre was randomly selected from a list of all health centres in the district. The WHO protocol for assessment of therapeutic efficacy of antimalarial drugs for uncomplicated *P. falciparum* malaria in areas with intense transmission was followed (World Health Organization, 1996). Children attending the outpatient clinic between the ages of 6 and 60 months were recruited and clinically examined to rule out other causes of fever. Axillary temperatures, weights and blood slides for parasitaemia were taken and packed cell volumes (PCV) were also determined. Those children meeting all inclusion criteria set by World Health Organization (1996) were enrolled and standard oral CQ treatment was administered under supervision. All children were seen at the hospital again on days 1 and 2 for clinical examination and CQ administration. They were again seen on days 3 and 7 for blood slides and those with treatment failure, were given a single dose of pyrimethamine–sulfadoxine (Fansidar (Hoffmann-La Roche, Basel, Switzerland)). They were again seen on day 14, when in addition to the clinical examination, a blood slide was made and PCV was determined.

All children were treated with CQ tablets (150 mg base of CQ phosphate tablets, Helm Pharmaceuticals GMBH Hamburg) with a dose of 10 mg/kg body weight on days 0 and 1 and 5 mg/kg body weight on day 2. Fansidar was given in case of treatment failure as a single dose (1.25 mg pyrimethamine/kg body weight). In addition, Paracetamol tablets were also given for the first 2 days and during the follow-up if fever was documented.

2.2. Laboratory examination

Thick and thin blood smears were made on the same slide and stained with 5% Giemsa stain at pH 7.2 for 20 min. Parasite density was assessed by counting the number of asexual parasites per 200 leucocytes in the thick smear. Parasite numbers were converted to a count per microlitre by assuming a standard leucocyte count of 8000/μl. Species confirmation was done by examining the thin film.

2.3. Classification of therapeutic and parasitological responses

Using the clinical and parasitological criteria set by WHO the therapeutic responses to CQ treatment were classified into early treatment failure (ETF), late treatment failure (LTF) and adequate clinical response (ACR) (World Health Organization, 1996). The sum of the rate of ETF and LTF was referred to as the clinical treatment failure rate. Parasites present at day 7 and/or day 14 after CQ treatment were classified as recrudescent if their *msp2* genotype had been previously detected. Genotypes which were not seen before day 7 were classified as new infections. The parasitological resistance rate was defined as the percentage of children harbouring true recrudescent parasites, a more stringent indicator for drug resistance than the clinical treatment failure rate.

2.4. Molecular analysis

For PCR analysis blood was collected at days 0, 3, 7 and 14 on Whatman 3 MM filter paper and DNA was extracted using the Chelex method. A piece of filter paper of about 10 mm^2 was soaked overnight in 1 ml 0.5% saponin in PBS and subsequently washed in 1 ml PBS for 30 min at 4 °C. It was transferred to 200 μl of a preheated (100 °C) 5% Chelex-100 resin in sterile water, vortexed for 30 s and boiled for 10 min at 100 °C. The reaction tube was centrifuged twice at 10,000 *g* for 2 min and the supernatant was transferred to a new tube after each centrifugation step.

msp2 analysis was carried out as described previously (Felger et al., 1999) with 5 μl of the above DNA preparation. The baseline multiplicity of infection was determined as the number of *msp2* genotypes, which were detected at day 0. For the analysis of *pfmdr1* codon 86 polymorphism 2 μl of DNA were amplified in a volume of 50 μl using a PTC-100 thermocycler (MJ Research). PCR reactions were done with 1 μM of each primer (Frean et al., 1992),

1.5 mM $MgCl_2$, 100 μM dNTPs, 0.75 units Taq-Polymerase (Gibco). The thermal profile was as follows: 94 °C for 30 s (4 min 30 s at cycle 1), 48 °C for 1 min with an increment of +0.2 °C per cycle, and 72 °C for 2 min (7 min at cycle 40) for 40 cycles. Aliquots of 10 μl from each PCR product were cleaved by 1 unit *Apo* I (which cleaves the wild-type) and 1 unit *Afl* III (which cleaves the mutant). For *pfcrt* amplification (Djimdé et al., 2001), a primary PCR was done with 1 μM of primer CCGTTAATAATAAA TACACGCAG (CRTP1) and CGGATGTTACAAAAC-TAGTTACC (CRTP2), 2.5 mM $MgCl_2$, 200 μM dNTPs and 0.75 units Taq-Polymerase (Gibco). The thermal profile was 94 °C for 30 s (3 min at cycle 1), 56 °C for 30 s, and 60 °C for 1 min (3 min at cycle 45) for 45 cycles. A volume 2 μl of primary PCR product were used in a 50 μl nested PCR reaction with 1 μM of primer TGTGCTCAT-GTGTTTAAACTT (CRTD1) and CAAAACTATAGTTA CCAATTTTG (CRTD2), 2.5 mM $MgCl_2$, 200 μM dNTPs and 0.75 units Taq-Polymerase (Gibco). The thermal profile was 92 °C for 30 s (3 min at cycle 1), 48 °C for 30 s, and 65 °C for 30 s (3 min at cycle 30) for 30 cycles yielding a 145 bp PCR product. Aliquots were digested with 1 unit *Apo* I, which cleaves the wild-type into 111 and 34 bp fragments. Genomic DNA from strain 3D7 was amplified and digested in the same way serving as control for complete digestion. Restriction digests of both *pfmdr1* and *pfcrt* were analysed on 10% polyacrylamide gels.

2.5. Statistical analysis

To allow for the effects of varying multiplicity in estimates of resistance rates, we used a non-linear statistical model to estimate the allele frequencies of resistance markers prior to treatment. Assuming resistant parasite clones to be transmitted independently of sensitive clones, the likelihood for a sample containing no resistant clones is $(1-p)^n$, where p is the allele frequency for the resistance marker and n is the multiplicity of infection of the sample. Similarly, the likelihood for a sample with no wild-type clones is p^n and for a mixture of wild-type and mutant clones is $1-p^n-(1-p)^n$. The likelihood over the whole data set for p is computed as the product of these likelihood over all samples, using values of n derived from the *msp2* typing.

A Markov Chain Monte Carlo algorithm (Program Winbugs 1.3) was used to obtain estimates and credible intervals (Bayesian confidence intervals (CI)) for p, making use of this likelihood, and assuming a uniform (0.1) prior distribution for p.

3. Results

Among 79 children enrolled, one was lost during follow-up. According to the clinical and parasitological criteria set by WHO, 54 children (69.2%) had an adequate clinical response (ACR), 17 (21.8%) and 7 children (9.0%) showed early and late treatment failures (ETF, LTF), respectively. Thus, the clinical treatment failure rate in this study was 31%. Parasites on day 7 post-treatment were detected in 20 children by microscopy, amounting to a parasitological resistance rate in the host of 25.6% (Table 1). Among them, three had asymptomatic parasitaemia at day 7 and were classified as ACR, 10 as ETF and 7 as LTF (Table 1).

Table 1
Multiplicity of infection, day 0 *pfcrt*^{76} genotype and clinical and parasitological outcome in patients with parasitological resistance analysed by light microscopy[a]

Patient	Multiplicity of infection				D0 *pfcrt*^{76}	Clinical response	Parasite response by *msp2*
	D0	D0 + 3	Rec.				
9	3	6	4	1	Mut	ETF	Rec. + new
45	4	6	1	–	Mut	ETF	Rec.
55	4	4	2	–	Mix	ETF	Rec.
61	1	1	–	1	Mut	ACR	New
65	1	2	1	–	Wild	ACR	Rec.
67	9	10	10		Mix	ACR	Rec.
71	1	3	1	1	Mut	LTF	Rec. + new
75	1	1	1	–	Mut	LTF	Rec.
81	4	4	1	–	Mut	LTF	Rec.
83	6	6	3	–	Mut	LTF	Rec.
87	2	2	1	–	Mix	LTF	Rec.
107	3	4	–	1	Mut	ETF	New
121	3	3	3	–	Mut	ETF	Rec.
127	3	5	4	–	Mut	ETF	Rec.
135	3	3	1	–	Mut	ETF	Rec.
147	4	4	2	–	Mut	ETF	Rec.
117	ND	ND	ND	ND	ND	ETF	ND
131	ND	ND	ND	ND	ND	LTF	ND
133	ND	ND	ND	ND	ND	LTF	ND
137	ND	ND	ND	ND	ND	ETF	ND

[a] D0: day 0; D0+3: day 0 and 3; Rec.: recrudescent; new: new at day 7 and/or day 14; ND: not determined.

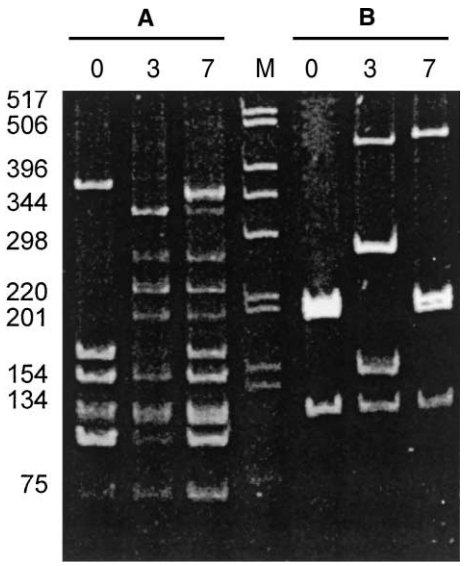

Fig. 1. Tracing of parasites by PCR–RFLP of *msp2* alleles in two patients (A and B) at days 0, 3 and 7. Restriction fragments after *Hin*f I digest were analysed by polyacrylamide electrophoresis (10%) and ethidium bromide staining. Patient A harboured several recrudescent and one new genotype at day 7, patient B harboured one recrudescent and one new genotype.

msp2 analysis revealed that 12 children harboured recrudescent infections, two only new infections and two a mixture of recrudescent and new infections (Fig. 1, Table 1). In four children, parasite genotypes at day 7 and/or day 14 could not be analysed. Thus, the parasitological resistance rate in the host population after the subtraction of new infections determined by *msp2* genotyping was 22.4% ($14/16 \times 25.6\%$).

The mean multiplicity of infections per child prior to CQ treatment was 3.2 with a total number of 229 infections in 71 children. The mean multiplicity amounted to 3.5, if genotypes newly identified at day 3 post-treatment were included in the baseline, resulting in a total number of 248 individual infections on day 0 and/or day 3. Among those, 35 parasite infections were found to be recrudescent on day 7 and/or day 14 by *msp2* genotyping (Table 1), amounting to a resistance rate of 14% within the parasite population.

Baseline frequencies of both resistance markers *pfcrt*^{76} (Fig. 2) and *pfmdr1*^{86} (Fig. 3) were analysed. About 79% of children carried only infections with the *pfcrt*^{76} mutation, 18% had mixed infections, and only 3% had the wild-type lysin genotype (Table 2). Both children with

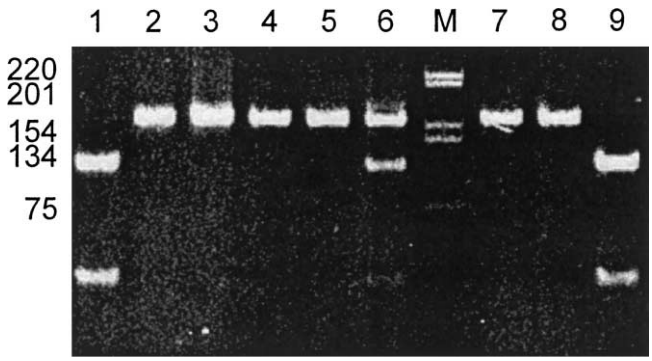

Fig. 2. Representative polyacrylamide gel of *Apo* I restriction digests of amplified regions of *pfcrt* containing the codon 76 polymorphism. The wild-type codon is cleaved into a 111 and a 34 bp fragment.

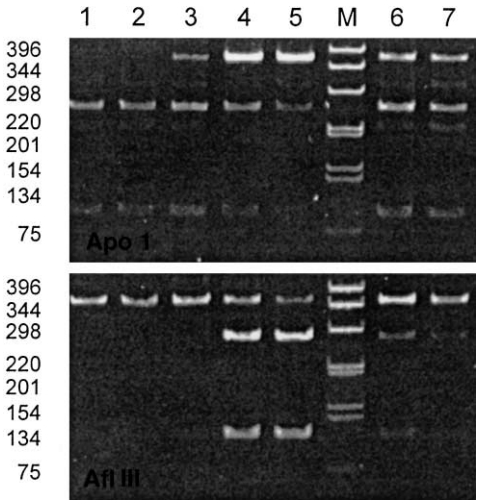

Fig. 3. Representative polyacrylamide gel of amplified regions from *pfmdr1* containing the codon 86 polymorphism. Each PCR product was analysed by *Apo* I which cleaves the wild-type, and *Afl* III which cleaves the mutant.

wild-type *pfcrt*^{76} genotype infections at day 0 cleared their parasitaemia by day 3, one had a recrudescence on day 7 after CQ treatment, which was identified as a mutant *pfcrt*^{76} infection already present on day 3. At days 0, 13 children (45%) harboured parasites with only the Tyrosine *pfmdr1*^{86} mutation, 12 children (41%) carried both mutant and wild-type infections, and 4 children (14%) carried only asparagine wild-type infections (Table 2).

Table 2
Day 0 frequencies of patients carrying wild-type, mutant and mixed *pfcrt*^{76} and *pfmdr1*^{86} genotypes

	pfcrt^{76}	*pfmdr1*^{86}
No. of patients	71	29
Mutation	78.9% (56)	44.8% (13)
Mutation and wild-type	18.3% (13)	41.4% (12)
Wild-type	2.8% (2)	13.8% (4)
Estimated allelic frequency	0.918 (95% CI: 0.876; 0.951)	0.766 (95% CI: 0665; 0.850)

By using the multiplicity of infection the allelic frequencies of *pfcrt*^{76} and *pfmdr1*^{86} mutation within the parasite population were estimated to be 92% (CI_{95}: 88, 95%) and 77% (CI_{95}: 67, 85%), respectively. Both values exceeded by far the observed frequency of resistant parasites which was 14%. No difference in multiplicity of infection was found between children with sensitive and those with resistant parasites. There was also no increased frequency of either mutant allele on day 0 in the population of children with recrudescent parasites as compared to children who cleared their parasitaemia before day 7. Nonetheless, there was evidence for a role of *pfcrt*^{76} in CQ resistance. If the mutant allele was not positively selected for its frequency after CQ treatment should not increase as compared to day 0. At a wild-type *pfcrt*^{76} frequency of 8% (CI_{95}: 5, 12%) we would expect between 3.5 and 8.6 *pfcrt*^{76} wild-types among the 70 parasite clones found at day 3, but we detected none. Also, at days 7 and 14 no *pfcrt*^{76} wild-type infection was detected. This indicates that the parasites with *pfcrt*^{76} wild-type are selectively eliminated by CQ treatment and treatment selects for mutant parasites. In contrast *pfmdr1*^{86} wild-types were still observed in at least three children bearing resistant parasites at day 7.

4. Discussion

In our study site in Tanzania, allele frequencies of mutated *pfcrt*^{76} and *pfmdr1*^{86} within the parasite population were very high before the onset of drug therapy with 92 and 77%, respectively. This corresponds to a parasitological failure rate of 22.4% as determined by PCR. The identification of new infections emphasises the value of tracing single infections using genetic markers. The rate of parasitological resistant patients of 22.4% determined by PCR would have been overestimated by 14% using light microscopy only.

The complete elimination of the *pfcrt*^{76} wild-type before day 3 after the onset of CQ treatment indicates that this mutation is under selective pressure and is involved in CQ resistance in vivo. It is not unexpected to find the mutant *pfcrt*^{76} allele with a frequency of over 90% in patients prior to CQ treatment. The widespread use of CQ in the Tanzanian population might have highly selected for CQ resistant mutants in the parasite population. Indeed high perennial parasite transmission rates in combination with a high proportion of hosts treated with drugs are key factors both for the survival of a newly arisen drug resistance mutant (Mackinnon, 1997) and its rapid spread once the frequency has reached a threshold level (Mackinnon and Hastings, 1998). Epidemiological modelling predicts that with continuous drug pressure the frequency of a mutant drug resistance gene can increase from 10 to 90% within 2–5 years in areas of intense transmission. (Mackinnon and Hastings, 1998). Since our samples were taken only at admission from patients with malaria, the true allelic frequency in the parasite population could not be determined. This sample set is highly selected, and we would expect a much lower allelic frequency within the circulating parasite population.

The relevance of the *pfcrt*^{76} point mutation for CQ resistance is also supported by a field study in Mali from an area with seasonal transmission where the mutation was found in 40% of patients at day 0, and was selected for in all patients with resistant parasites at day 14 post-treatment (Djimdé et al., 2001). The high allelic frequency of 92% for the mutant *pfcrt*^{76} allele and the comparatively low frequency of resistant parasite clones of 14% indicates that additional mutations identified in *pfcrt* (or in yet unidentified genes) might also account for CQ resistance (Fidock et al., 2000b; Djimdé et al., 2001).

We failed to find an association between the *pfmdr1*^{86} mutation and CQ resistance. This is in line with other field studies in which CQ resistance was not associated with mutations in this gene (Haruki et al., 1994; Bhattacharya et al., 1997; Bhattacharya and Pillai, 1999). Our results confirm the hypothesis that *pfmdr1* is at most a modulator of CQ resistance (Reed et al., 2000).

In conclusion, the complete elimination of the *pfcrt*^{76} wild-type after CQ treatment gives strong evidence for the relevance of this gene in CQ resistance in a natural parasite population.

In our study, the mean baseline multiplicity of infection prior to treatment was 3.2 which is in the range of other regions in Tanzania known for their high endemicity of malaria (Smith et al., 1999). Many studies have shown that multiplicity varies with the season, between closely related areas and also by age (Smith et al., 1999). But the effect of multiple infections on resistance rates has not generally been considered. With increasing multiplicity of infections the proportion of patients bearing either pure mutant or pure wild-type infections decreases and the proportion of patients bearing mixed infections increases assuming allelic frequencies to be independent of multiplicity (Fig. 4). The predicted trends of an increasing multiplicity are supported by our experimental data, however, they are less prominent (Fig. 4). At a given gene frequency a parasite population in individuals harbouring many parasite clones is more likely to contain resistant clones than in individuals with only one or a few concurrent infections (Fig. 4). in vivo tests of resistance will thus lead to apparently higher resistance rates in highly endemic areas, even if there is no difference in the parasite populations. In this study, a parasitological resistance rate of 22.4% within the host population was determined, whereas the frequency of resistant parasites was only 14%. In order to make resistance rates of studies with different multiplicities of parasites fully comparable allelic frequencies within the parasite population have to be determined for each mutation of a given resistance marker.

Hence, it is necessary to establish a genetic resistance index (GRI), which would link the observed frequency of

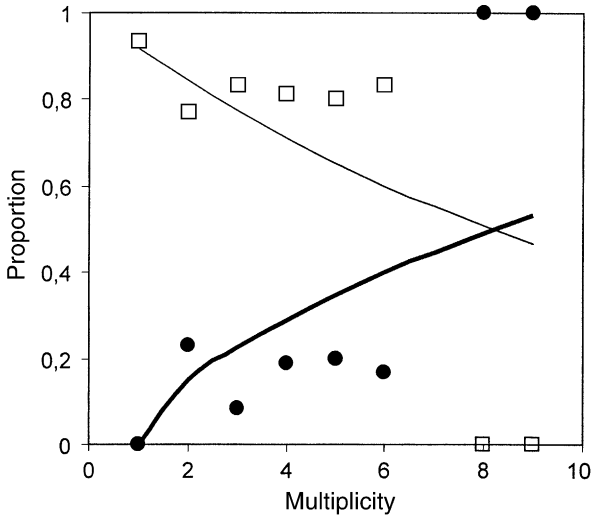

Fig. 4. Modelling the proportion of mutant and mixed *pfcrt*^{76} alleles for different multiplicities of infections based on the calculated mutant allele frequency of 92%. Symbols: mutant expected (—); mixture expected (▬); mutant observed (□); mixture observed (●).

point mutations at the community level with the observed clinical or parasitological failure rate at the health facility level. Such GRI would allow quantification of resistance and would provide support in decisions concerning drug policies.

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