ELSEVIER

International Journal for Parasitology 33 (2003) 1051–1058

www.parasitology-online.com

Intensity of transmission and spread of gene mutations linked to chloroquine and sulphadoxine-pyrimethamine resistance in falciparum malaria

Ambrose O. Talisuna[a,e], Peter Langi[a], Theonest K. Mutabingwa[b,c], Eric Van Marck[d], Niko Speybroeck[e], Thomas G. Egwang[f], William W. Watkins[g], Ian M. Hastings[h], Umberto D'Alessandro[e,*]

[a]*Ministry of Health, P.O. Box 7272, Kampala, Uganda*
[b]*National Institute for Medical Research, Amani, Tanzania*
[c]*London School of Hygiene and Tropical medicine, Gates Malaria Partnership, London, UK*
[d]*University of Antwerp, Antwerp, Belgium*
[e]*Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium*
[f]*Department of Parasitology, Med Biotech Laboratories, Kampala, Uganda*
[g]*Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX, UK*
[h]*Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK*

Received 20 March 2003; received in revised form 27 May 2003; accepted 4 June 2003

Abstract

The number of malaria parasite clones per infection-multiplicity of parasite clones-is affected by the transmission intensity, multiplicity increases with increasing transmission. This affects the frequency of parasites' sexual recombination and, if several mutations in different genes are involved, can break down drug resistant genotypes. Therefore, the effects of malaria transmission intensity on the spread of drug resistance could vary depending on the number of genes involved. Here we show that, compared to low transmission, intermediate-high transmission is associated with a 20–100-fold lower risk for the mutations linked to chloroquine resistance and a 6–17 times higher risk for those linked to sulphadoxine-pyrimethamine resistance. This is consistent with the hypothesis of a multigenic basis for chloroquine resistance and a monogenic basis for that of sulphadoxine-pyrimethamine. Reducing transmission intensity could slow the spread of resistance. However, a reduction below a critical threshold (e.g. when parasite prevalence in children 2–9 years old is around 60–80%) could, paradoxically, accelerate the spread of resistance to chloroquine and possibly to other drug combinations whose basis is multigenic. Our findings have important implications for malaria control because increasing drug resistance has a substantial impact on mortality.
© 2003 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

Keywords: Malaria transmission intensity; *Plasmodium falciparum*; Drug resistance; Gene mutation; Chloroquine; Sulphadoxine-pyrimethamine

1. Introduction

Plasmodium falciparum resistance to chloroquine (CQ) has been linked to mutations in the *P. falciparum* multidrug resistance (*pfmdr1*) gene and the *P. falciparum* chloroquine related transporter (*pfcrt*) gene (Foote et al., 1990; Fidock et al., 2000; Reed et al., 2000; Sidhu et al., 2002). Mutations at codon 76 in the *pfcrt* gene (K76T) are probably the last to occur in the long process leading to CQ resistance (Djimde et al., 2001a; Hastings et al., 2002). Resistance to sulphadoxine-pyrimethamine (SP) has been linked to progressive accumulation of mutations in the dihydrofolate reductase (*dhfr*) gene (Peterson et al., 1990), though mutations in the dihydropteroate synthase (*dhps*) gene could also increase the tolerance to the drug (Sibley et al., 2001). The role of malaria transmission intensity in the spread of drug resistance is still a subject for scientific debate (White, 1999; Hastings and D'Alessandro, 2000). Theory is not consistent on this point and depends amongst other things on the underlying assumptions on falciparum population biology (Hastings and D'Alessandro, 2000).

* Corresponding author. Tel.: +32-3-247-6354; fax: +32-3-247-6359.
E-mail address: udalessandro@itg.be (U. D'Alessandro).

0020-7519/03/$30.00 © 2003 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/S0020-7519(03)00156-5

If intra-host competition occurs and two or more genes are required to encode resistance, resistance may spread faster in areas of high and low transmission with the slower in places with intermediate transmission. If a single gene encodes resistance and intra-host competition occurs, it may spread faster in areas where transmission is the highest or, if no intra-host competition occurs, is unaffected by the intensity of transmission. Furthermore, the early stages of increasing drug tolerance may be unaffected by transmission intensity. Epidemiological studies on the relationship between transmission intensity and spread of resistance are needed as their results could have important consequences on the implementation of control measures aimed at decreasing transmission. In a previous study (Talisuna et al., 2002a) we reported on the relationship between malaria transmission intensity and clinical treatment failure to CQ and SP. However, treatment failure might be affected by pharmacokinetic factors and some normally susceptible parasites may survive. Conversely, resistant parasites might be cleared by host immunity and appear as susceptible. These factors make clinical efficacy a less-than-ideal tool for tracking the evolution of drug resistance. We have circumvented this problem by directly tracking the genes responsible for encoding resistance to SP and CQ (*dhfr* and *pfcrt*) and investigated their relationship with transmission intensity, number of parasite clones per infection-multiplicity of parasite clones (MPC), and drug usage in six non-contiguous human populations in Uganda.

2. Materials and methods

2.1. Study populations

The East Africa Network for Monitoring Antimalarial Treatment (EANMAT) and the Uganda National Malaria Control Programme (UNMCP) jointly chose eight non-contiguous populations for the surveillance of antimalarial drug efficacy and we conducted the study in six of these populations. The study populations were selected according to the following criteria: transmission intensity (high, medium, low), moderate/high population density, easy accessibility/practicability, broad geographical representation, health unit with a defined catchment population (hospitals were excluded) and the presence of a stable population with limited in and out migration. Five of the study populations (Arua, Apac, Tororo, Kyenjojo and Rukungiri) were rural and one (Jinja) is peri-urban. The sites lie between 945–1219 m and above sea level and have a similar rainfall pattern with two peaks per year. The first peak is from March to May and the second is from August to October. The Uganda National Council for Science and Technology (UNCST) and the research ethics committee at the Prince Leopold Institute of Tropical Medicine in Antwerp, Belgium, reviewed and approved the study. Informed consent was obtained from all survey participants or their parents or guardians. In 1999, a population-based cross-sectional survey was conducted in each of the study populations.

2.2. Explanatory and dependent variables

Malaria transmission intensity and drug selection pressure were the primary and secondary explanatory variables, respectively, while our dependent variables were: the frequency of the *pfcrt* codon 76 (K76T) or the *dhfr* codon 59 (C59R) mutant allele in the entire *P. falciparum* parasite population (mutant and wild) at each site. A prerequisite for the derivation of the mutant allele frequency in the parasite population is the number of asexual parasite clones per infection in the human host. We therefore took into account the number of parasite clones per infection to determine the frequency of the *pfcrt* K76T and the *dhfr* C59R allele. We then assessed whether the mutant allele frequency varied significantly according to different levels of exposure to falciparum infection. Secondly, we used the risk of having *P. falciparum* infections with the pure (no wild type present) *pfcrt* K76T or three *dhfr* (Asn-108, Ile-51 and Arg-59) gene mutations among human hosts as another dependent variable. We further assessed, using multivariate analysis, whether the risk of falciparum infections having the pure *pfcrt* T76 gene mutation or having three *dhfr* gene mutations varied significantly with malaria transmission intensity

2.3. Estimation of malaria transmission intensity and drug selection pressure

For each population, we estimated malaria transmission intensity and drug selection pressure using the parasite prevalence (PR) in children 2–9 years old and the proportion of the population with detectable drug metabolites in their urine, respectively. Details of the survey have been described elsewhere (Talisuna et al., 2002a). Briefly, a census of the population (maximum 10,000 individuals) at each sentinel site was carried out and a sample of 250 people 1–45 years old was randomly selected from the census database. Duplicate thick and thin blood films for parasitologic examination were collected. The presence of *P. falciparum* in the peripheral blood was determined by microscopy examination of 100 high-power magnification oil immersion fields. A urine sample was also collected from each individual into a universal container. Drug pressure was estimated as the percentage of selected individuals with detectable levels of CQ or SP metabolites in the urine by means of an enzyme-linked immunosorbent assay blocking test (Schwick et al., 1998). Furthermore, demographic data, information on bed net use and self-reported antimalarial drug use were collected from all surveyed individuals. In addition a blood sample was

collected from each individual onto 3MM Whatman filter paper and stored at room temperature in an individual zip-lock dry polythene bag. The latter were subsequently used for molecular analysis in the laboratory.

2.4. Determination of number of parasite clones per infection and identification of pfcrt and dhfr mutant alleles

To determine the multiplicity of parasite clones (MPC) per host infection, the polymorphic repetitive regions of block 2 of the merozoite surface protein *(MSP) 1* and block 3 of *MSP2* were amplified by a nested PCR to identify different parasite clones in all microscopy positive samples as previously described (Ranford-Cartwright et al., 1997). A conservative estimate of the minimum number of asexual parasite clones per infected individual for each population was determined as described previously (Farnert et al., 1999). The frequency of polyclonal infections in each population was computed as the percentage of infections with more than one clone among all the successfully genotyped samples. To identify mutant alleles in the *dhfr* and *pfcrt* gene we used nested mutation-specific PCR (MS-PCR) performed according to the techniques already described (Plowe et al., 1997; Djimde et al., 2001b). Briefly, amplified *DHFR* domain from the primary PCR, which used the primer pair AMP1 (Sense), and AMP2 (antisense) was used in a secondary PCR reaction to identify DHFR genotypes at codons 108, 51 and 59. In successful mutant and wild-type PCR amplification reactions, the relevant band was seen under ultraviolet transillumination, after electrophoresis on 2% agarose gels stained with ethidium bromide. For the *pfcrt* gene flanking primers TCRP1 and TCRP2, amplifying a 537 bp region encompassing the K-76T mutation were employed in the first PCR run. 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. In successful mutant and wild-type amplicons, a band of 366 bp was observed. Both Polaroid and digital photographs were taken.

2.5. Statistical analysis

Statistical analysis was done using Stata (Stata Corp., 2001). Differences amongst sites in MPC (a non-negative discrete count variable) were compared by Poisson regression, while those for categorical variables were compared using the Pearson chi-square test and the test for linear trend.

We used maximum likelihood estimation (MLE) to derive the mutant allele frequency at codon 59 (C59R) in the *dhfr* gene and at codon 76 in the *pfcrt* (K76T) gene. The K76T and the C59R mutations were selected because they are believed to occur last among the *pfcrt* and *dhfr* triple mutations and are more likely to be closely associated with CQ and SP resistance. The method analogous to that described by Schneider et al. (2002) was used except that our 95% confidence intervals were obtained at 2 natural logs below the (ln) maximum likelihood. We then assessed whether the mutant allele frequency varied significantly according to different levels of exposure to falciparum infection. The responses, namely K76T allele frequency and C59R allele frequency, being proportions were transformed by means of an arcsine transformation, as recommended by Zar (1984) for data originating from a binomial distribution (active versus nonactive). We then checked whether a linear term was sufficient to describe the relation between these responses. This was done by means of the likelihood ratio test statistic, which allows to investigate if higher terms than a linear term were necessary. We further assessed, using multivariate analysis, whether the risk of falciparum infections having the pure *pfcrt* T76 (no wild genotype present) mutation or with three *dhfr* (Asn-108, Ile-51 and Arg-59) mutations varied significantly with malaria transmission intensity.

3. Results

We observed a significantly higher *pfcrt* K76T allele frequency in populations with either low ($<30\%$) or very high ($>90\%$) as compared to those with intermediate (30–60%) or high (61–90%) PR (Table 1). A unique feature of our data is the 'valley phenomenon' in the relationship between the *pfcrt* K76T allele frequency and PR (Fig. 1a). Conversely the *dhfr* C59R allele frequency was lower in populations with low PR and higher in those with intermediate or high PR (Table 1). The likelihood ratio test statistic indicated that a linear model was sufficient ($P = 0.0777$) to describe the relation between the C59R allele frequency and the PR ages 2–9 years while for the K76T allele frequency (at least) a cubic term was necessary ($P = 0.0097$). Fig. 1 shows the fits of a cubic model and a linear model for respectively the K76T allele frequency (Fig. 1a) and C59R allele frequency (Fig. 1b) data. The frequency of polyclonal (more than one clone) infections increased significantly with increasing PR (P for trend < 0.001) (Table 1). Furthermore, the mean MPC for the reference population (lowest PR) was significantly lower compared to that of two of four populations with intermediate or high PR ($P < 0.03$) (Table 1). Compared to the reference population, intermediate or high PR was significantly associated with a 20–100-fold lower risk for falciparum infections having the pure *pfcrt* K76T gene mutations. Conversely, intermediate or high PR was associated with a 6–17-fold higher risk for infections with three *dhfr* gene mutations (Table 2 and Fig. 2). Such an opposite trend between the *pfcrt* and *dhfr* gene mutations

Table 1
Parasite prevalence, drug usage, multiplicity of infections, and the *pfcrt* K76T and *dhfr* C59R allele frequency

Site	PR (ages 2–9)	PR (all ages)	CQ use (all ages)	SP use (all ages)	Frequency of polyclonal infections (all ages)	Mean MPC (SD)	K76T allele frequency (95% CI)	C59R allele frequency (95% CI)
Jinja	13 (6–25)	10 (6–15)	82 (75–87)	3.4 (1.2–7.2)	52 (31–73)	2.1 (1.2)	0.96 (0.82–1.0)	0.28 (0.14–0.47)
Rukungiri	39 (27–53)	35 (26–39)	68 (61–75)	4.6 (2.0–8.9)	78 (66–87)	2.5 (1.2)	0.89 (0.82–0.94)	0.42 (0.32–0.5)
Kyenjojo	67 (55–78)	46 (39–53)	43 (36–51)	0.5 (0.0–2.7)	75 (65–83)	2.1 (0.9)	0.67 (0.59–0.77)	0.54 (0.45–0.64)
Apac	79 (68–88)	53 (46–59)	32 (26–38)	0.4 (0.0–2.3)	93 (86–97)	3.5 (1.3)	0.48 (0.39–0.57)	0.45 (0.35–0.56)
Arua	83 (71–91)	55 (48–62)	33 (26–40)	1.7 (0.3–4.8)	76 (66–84)	2.5 (1.2)	0.55 (0.47–0.62)	0.53 (0.43–0.61)
Tororo	91 (84–95)	71 (65–76)	41 (34–47)	0.4 (0.0–2.3)	90 (83–96)	2.9 (1.0)	0.86 (0.78–0.91)	0.53 (0.42–0.63)

Unless stated all values are percentages and numbers in parentheses are the 95% confidence intervals (CI). PR, parasite prevalence (proportion of individuals with patent parasitaemia); MPC, multiplicity of parasite clones in infected individuals. Polyclonal infection: more than one parasite clone per infection.

was unexpected because increasing PR was significantly associated with decreasing SP ($P = 0.002$) and CQ ($P < 0.001$) use (Fig. 3), a crucial factor for the selection of resistant parasites.

4. Discussion

Our observations suggest that, besides drug pressure, the intensity of malaria transmission plays an important and direct role in the spread of drug resistance within populations. In low transmission areas falciparum infections are more likely to evolve into clinical disease requiring treatment as a consequence of the population's lower acquired immunity. The use of drugs with a long half-life at therapeutic concentrations would increase the proportion of infected individuals with residual drug concentrations and consequently the selective drug pressure (Watkins and Mosobo, 1993) resulting into a high frequency of gene mutations encoding resistance. Moreover, the mean MPC increases with increasing malaria transmission (Babiker and

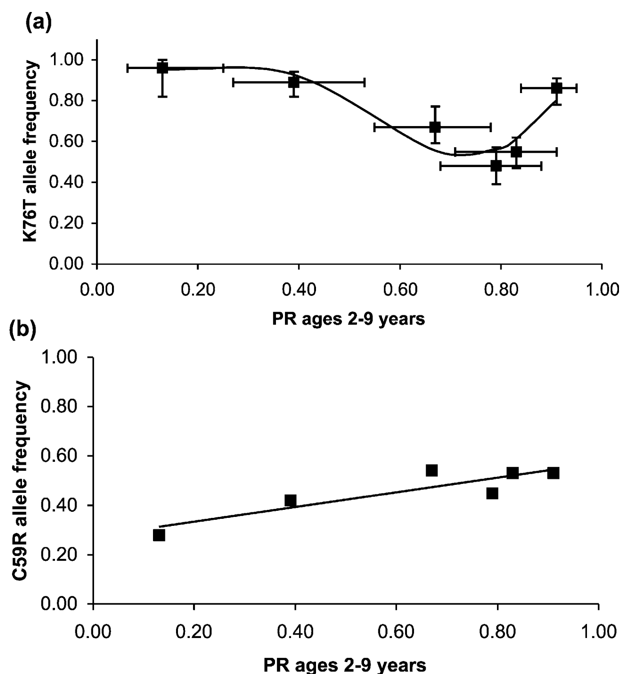

Fig. 1. Malaria transmission intensity and the *pfcrt* K76T or *dhfr* C59R allele frequency. We used the parasite prevalence in children 2–9 years old to estimate malaria transmission intensity and maximum likelihood estimation to derive the K76T and C59R allele frequency. In panel (a), the 95% confidence limits have been included for both parasite prevalence and K76T allele frequency.

Table 2
Malaria transmission intensity and the risk of falciparum infections having the pure *pfcrt T76* or three *dhfr* mutations

Site	Pure *pfcrt* T76 mutations (no wild type present)			
	PR	%CQ use	Odds ratio (OR)	95% CI
Jinja	<30	>60	Reference	Reference
Rukungiri	30–60	>60	0.3	0.034–2.6
Kyenjojo	61–90	30–60	0.05	0.005–0.39*
Apac	61–90	30–60	0.01	0.001–0.12*
Arua	61–90	30–60	0.02	0.002–0.19*
Tororo	>90	30–60	0.13	0.02–1.34
	Three *dhfr* mutations (Asn-108, Ile-51 and Arg-59)			
	PR	%SP use	Odds ratio (OR)	95% CI
Jinja	<30	>3	Reference	Reference
Rukungiri	30–60	>3	2.9	0.73–11.8
Kyenjojo	61–90	<1	6.0	1.48–24.4*
Apac	61–90	<1	17.3	3.30–90.2*
Arua	61–90	1–3	1.62	0.44–5.95
Tororo	>90	<1	8.78	1.97–39.1*

*Statistically different. PR, parasite prevalence in children 2–9 years old; SP, sulphadoxine-pyrimethamine; CQ, chloroquine. PR and drug use are divided into equal and ordered subgroups of thirds (Terciles).

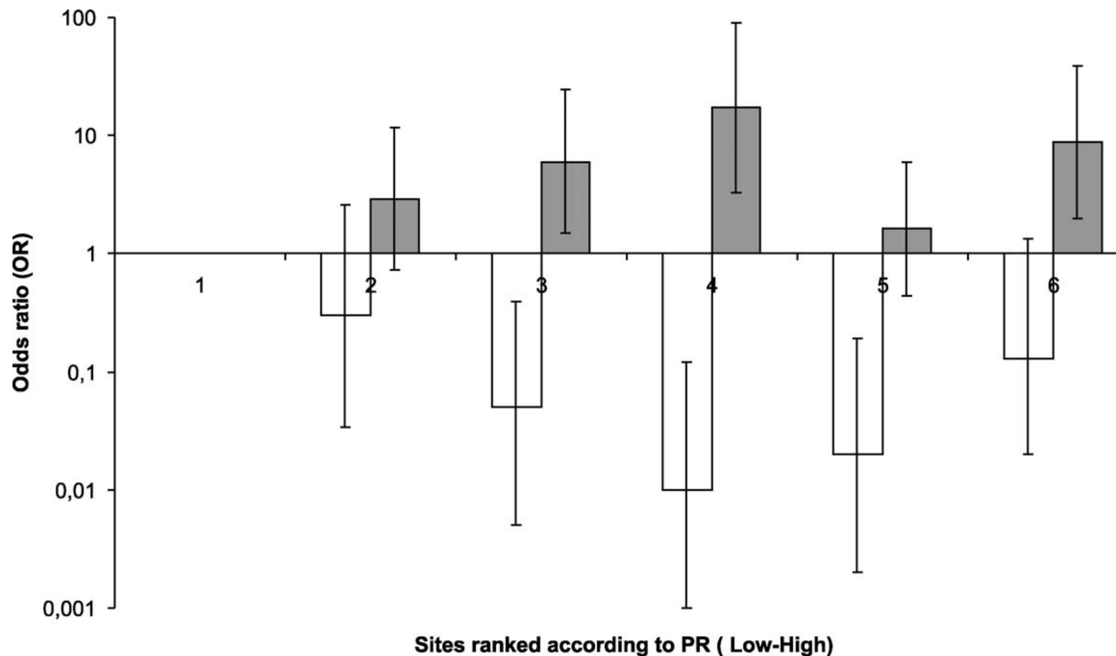

Fig. 2. Malaria transmission intensity and risk of falciparum infections with the pure *pfcrt* T76 or three *dhfr* mutations. A logarithmic scale has been used and a site with the lowest parasite prevalence (PR) is the reference. Shaded and clear bars represent the odds ratios (ORs) and their 95% confidence intervals for the three *dhfr* mutations (Asn-108, Ile-51 and Arg-59) and the pure K76T mutations, respectively. 1. = Jinja – reference (PR = 13%); 2. = Rukungiri (PR = 39%); 3. = Kyenjojo (PR = 67%); 4. = Apac (PR = 79%); 5. = Arua (PR = 83%); 6. = Tororo (PR = 91%).

Walliker, 1997; Arnot, 1998). A lower MPC or a lower prevalence of polyclonal infections would increase the probability of self-fertilisation in the mosquito vector by gametocytes from the same parasite clone (Hill et al., 1995; Paul et al., 1995) and of transmitting mutant gene combinations to the next generation. A high CQ use and the lower MPC or prevalence of polyclonal infections can therefore explain the higher frequency of the *pfcrt* K76T allele in populations with low PR. In high transmission areas the opposite phenomenon should occur: a high frequency of asymptomatic infections due to a high partial immunity, a lower proportion of infections treated hence a lower fraction of the parasite population exposed to drug selective pressure and a higher MPC or frequency of polyclonal infections. The frequency of gene mutations linked to drug resistance should be lower in these settings as compared to areas with a lower transmission. However, we have observed a higher frequency of mutations linked to SP and CQ resistance in areas with very high transmission intensity, an observation that suggests that malaria transmission intensity has other

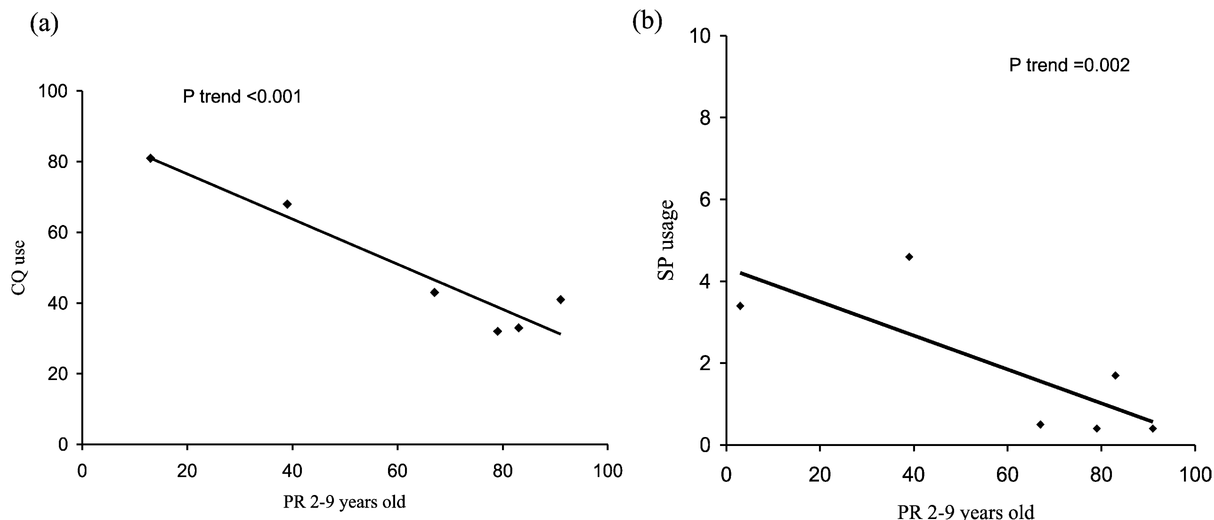

Fig. 3. Malaria transmission intensity and drug use. Drug (chloroquine (CQ) and sulphadoxine-pyrimethamine (SP)) pressure in the population was estimated as the percentage of surveyed individuals with detectable levels of CQ and SP metabolites in the urine by means of a dipstick. Panels (a) and (b) show the relationship between the PR and CQ and SP use, respectively. Regression lines based on ordinary least square have been superimposed.

effects antagonistic to those linked to sexual recombination. Theory suggests that the density dependent constraints on parasite survival or establishment, a feature of the population biology of helminth species, apply also to *P. falciparum* population biology. If intra-host competition exists then susceptible parasite populations that are eliminated following chemotherapy are replaced by mutant parasite populations that have the ability to survive or recolonise. This phenomenon is boosted if the number of parasite clones per infection is high or the frequency of polyclonal infections is high (Hastings, 1997). Our empirical data provide strong evidence for this theory.

Mathematical models have previously predicted that the relationship between malaria transmission intensity and the spread of mutant alleles would exhibit the 'valley' phenomenon if two or more genes encode resistance. The latter is thought to be a composite of two effects. (1) The increased loss of resistant genotypes through sexual recombination during meiosis and natural selection would generate a concave curve with the highest frequency of resistant alleles in low transmission. (2) Interaction between drug selection and intra-host competition would generate a linear increasing function (Hastings, 1997). Intuitively, if the genetic basis for resistance is monogenic only the linear increasing function is expected because sexual recombination does not apply. Our epidemiological observations support the hypothesis of a multigenic basis of resistance for CQ and a monogenic one for SP. Indeed the frequency of mutations linked to CQ resistance is consistent with the existence of the 'valley phenomenon' (Fig. 1a), although this observation should be taken with caution as its specific pattern is based on the high frequency in the site with the highest PR. Nevertheless, the exclusion of this site from the analysis would also support the hypothesis of a multigenic basis for CQ resistance as the frequency of the mutations would decrease with increasing transmission. The genetic basis for CQ resistance is currently a subject for scientific debate. DNA transfection studies strongly suggest that the *pfcrt* gene mutations are responsible for CQ resistance (Fidock et al., 2000; Sidhu et al., 2002). However, mutations in other genes such as *pfmdr1* could be involved in its modulation (Foote et al., 1990; Reed et al., 2000; Wellems and Plowe, 2001) and could confer the ability for the parasite to survive drug action and the hostile host immunity. Although we studied one gene (*pfcrt*), its mutations particularly the K76T are thought to be the last to occur in the long process for the development of CQ resistance (Djimde et al., 2001a; Hastings et al., 2002). The *pfcrt* K76T allele frequency would therefore measure the sub population of resistant (multiple mutant) parasites (with ability to survive) that are circulating within a given parasite population. Our choice of the *pfcrt* K76T mutation is supported by studies that have found a strong positive correlation of the prevalence of host infections with this mutation to in vivo CQ treatment failure (Djimde et al., 2001b; Talisuna et al., 2002b). However, the prevalence of host infections having the mutant allele and not the frequency of the mutant allele in the entire parasite population was used in such studies and a stronger correlation is likely if the latter is used. The frequency of the mutations linked to SP resistance follows a liner trend (Fig. 1b). The genetic basis for SP resistance has been studied extensively and is thought to be predominantly due to progressive accumulation of mutations in the *dhfr* gene (Peterson et al., 1990) though mutations in the *dhps* gene increase tolerance to the drug. Furthermore, the *dhfr* codon 51 or 59 mutations have been hardly observed in nature in the absence of the *dhfr* codon 108 mutation. Therefore, the *dhfr* C59R allele frequency derived by MLE would measure the subpopulation of parasites with ability to go through the 'drug-selective filter' (White and Pongtavornpinyo, 2003). Indeed, several studies have demonstrated a strong association of either the triple *dhfr* mutations or the *dhfr* Arg-59 mutation with in vivo SP treatment failure (Nzila et al., 2000; Kublin et al., 2002; Mberu et al., 2002) and support our choice to use the *dhfr* C59R allele or the three *dhfr* mutations.

There are some limitations to our data and interpretation and the main one is that we used the PR as a proxy for the intensity of transmission. The PR in children less than 10 years has previously been proposed as a good index to estimate parasite challenge (Snow et al., 1997). In the past this index has been widely used to estimate exposure to infection (Metselaar and Van Thiel, 1959) because PR surveys are relatively easier to conduct and can cover larger geographical areas than entomological surveys. However, a major limitation is that PR saturates and reaches a plateau when transmission is high. The annual entomological inoculation rate (AEIR), the product of the human biting rate and the sporozoite rate (the proportion of vectors with sporozoites in their salivary glands), would have been preferable although it has its own limitations as well. It is subject to the individual collectors (Snow and Marsh, 2002) and its value might vary with the method used. Furthermore, the mosquito sampling is normally conducted in a few households, usually three to six, and the AEIR derived is then extrapolated to whole villages or communities. However, parasite exposure may vary considerably in small geographical areas or even households (Cattani et al., 1985; Mbogo et al., 1995). The relationship between AEIR and PR has been recently reviewed (Beier et al., 1999); the 25th–75th percentile for the PR is about 25–50% when the AEIR is 1–10, 51–74% when the AEIR 11–100 and above 74% when the AEIR is above 100. Such distribution is similar to the classification we have used.

Our field data suggest that the net effects of transmission intensity depend on the genetic basis of resistance. If two or more genes encode resistance, the interaction between sexual recombination and intra-host dynamics could lead to faster spread of resistance in very high and low transmission areas with a minimum in those of intermediate to high transmission. However, the spread of resistance could be

faster in areas of intense transmission if the genetic basis of resistance is monogenic. These findings have important implications for malaria control interventions. Rational drug use and reduction of malaria transmission intensity could slow the rate of spread of antimalarial drug resistance. However, further reduction of transmission below a critical threshold could have the paradoxical and negative effect of increasing the rate of spread of resistance to CQ and possibly to new drug combinations with a multigenic basis for resistance. Such a threshold could be at relatively high transmission intensity, where the PR in children 2–9 years old is around 60–80% (Fig. 1a). This implies that non-selective control measures in areas with less intense transmission could facilitate a faster spread of parasite resistance to drugs with a multigenic basis. Our results have important consequences for malaria control programmes because increasing *P. falciparum* drug resistance has a substantial impact on mortality.

Acknowledgements

We thank the study communities, sentinel site staff, the survey team and laboratory staff at the Med Biotech Laboratories (MBL), namely, Jackie Kyosiimire-Lugemwa, Constance Agwang, Betty Magambo, Godfrey Mujuzi and Alex Ogwal. This study was conducted under the auspices of the East Africa Network for Monitoring Antimalarial Treatment (EANMAT) and was financed by the Belgian Development Co-operation (BEADC-ITM) in collaboration with the Institute of Tropical Medicine in Antwerp and the Fonds voor Wetenschappeljk Onderzoek-Vlanderen (FWO). A.O.T is supported by the Belgian Development Co-operation (BEADC-ITM) in collaboration with the Institute of Tropical Medicine in Antwerp.

References

Arnot, D., 1998. Unstable malaria in Sudan: the influence of the dry season. Clone multiplicity of Plasmodium falciparum infections in individuals exposed to variable levels of disease transmission. Trans. R. Soc. Trop. Med. Hyg. 92, 580–585.

Babiker, A., Walliker, D., 1997. Current views on the population structure of *Plasmodium falciparum*: implications for control. Parasitol. Today 13, 262–267.

Beier, J.C., Killeen, G.F., Githure, J.I., 1999. Short report: entomological inoculation rates and *Plasmodium falciparum* malaria prevalence in Africa. Am. J. Trop. Med. Hyg. 61, 109–113.

Cattani, J.A., Moir, J.S., Gibson, F.D., Ginny, M., Paino, J., Davidson, W., Alpers, M.P., 1985. Small area variations in the epidemiology of malaria in Madang Province. Papua New Guinea Med. J. 29, 11–17.

Djimde, A., Doumbo, O.K., Cortese, J.F., Kayentao, K., Doumbo, S., Diourte, Y., Dicko, A., Su, X.Z., Nomura, T., Fidock, D.A., Wellems, T.E., Plowe, C.V., Coulibaly, D., 2001a. A molecular marker for chloroquine-resistant falciparum malaria. N. Engl. J. Med. 344, 257–263.

Djimde, A., Doumbo, O.K., Steketee, R.W., Plowe, C.V., 2001b. Application of a molecular marker for surveillance of chloroquine-resistant falciparum malaria. Lancet 358, 890–891.

Farnert, A., Rooth, I., Svensson, A., Snounou, G., Bjorkman, A., 1999. Complexity of *Plasmodium falciparum* infections is consistent over time and protects against clinical disease in Tanzanian children. J. Infect. Dis. 179, 989–995.

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M., Sidhu, A.B., Naude, B., Deitsch, K.W., Su, X.Z., Wootton, J.C., Roepe, P.D., Wellems, T.E., 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol. Cell 6, 861–871.

Foote, S.J., Kyle, D.E., Martin, R.K., Oduola, A.M., Forsyth, K., Kemp, D.J., Cowman, A.F., 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. Nature 345, 255–258.

Hastings, I.M., 1997. A model for the origins and spread of drug-resistant malaria. Parasitology 115, 133–141.

Hastings, I.M., D'Alessandro, U., 2000. Modelling a predictable disaster: the rise and spread of drug- resistant malaria. Parasitol. Today 16, 340–347.

Hastings, I.M., Bray, P.G., Ward, S.A., 2002. Parasitology. A requiem for chloroquine. Science 298, 74–75.

Hill, W.G., Babiker, H.A., Ranford-Cartwright, L.C., Walliker, D., 1995. Estimation of inbreeding coefficients from genotypic data on multiple alleles, and application to estimation of clonality in malaria parasites. Genet. Res. 65, 53–61.

Kublin, J.G., Dzinjalamala, F.K., Kamwendo, D.D., Malkin, E.M., Cortese, J.F., Martino, L.M., Mukadam, R.A., Rogerson, S.J., Lescano, A.G., Molyneux, M.E., Winstanley, P.A., Chimpeni, P., Taylor, T.E., Plowe, C.V., 2002. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. J. Infect. Dis. 185, 380–388.

Mberu, E.K., Nzila, A.M., Nduati, E., Ross, A., Monks, S.M., Kokwaro, G.O., Watkins, W.M., Hopkins-Sibley, C., 2002. *Plasmodium falciparum*: in vitro activity of sulfadoxine and dapsone in field isolates from Kenya: point mutations in dihydropteroate synthase may not be the only determinants in sulfa resistance. Exp. Parasitol. 101, 90–96.

Mbogo, C.N.M., Snow, R.W., Khamala, C.P.M., Kabiru, E.W., Ouma, J.H., Githure, J.I., Marsh, K., Beier, J.C., 1995. Relationships between *Plasmodium falciparum* transmission by vector populations and the incidence of severe disease at nine sites on the Kenyan coast. Am. J. Trop. Med. Hyg. 52, 201–206.

Metselaar, D., Van Thiel, P.H., 1959. Classification of malaria. Trop. Geog. Med. 11, 157–161.

Nzila, A.M., Mberu, E.K., Sulo, J., Dayo, H., Winstanley, P.A., Sibley, C.H., Watkins, W.M., 2000. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. Antimicrob. Agents Chemother. 44, 991–996.

Paul, R.E., Packer, M.J., Walmsley, M., Lagog, M., Ranford-Cartwright, L.C., Paru, R., Day, K.P., 1995. Mating patterns in malaria parasite populations of Papua New Guinea. Science 269, 1709–1711.

Peterson, D.S., Milhous, W.K., Wellems, T.E., 1990. Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. Proc. Natl. Acad. Sci. USA 87, 3018–3022.

Plowe, C.V., Cortese, J.F., Djimde, A., Nwanyanwu, O.C., Watkins, W.M., Winstanley, P.A., Estrada-Franco, J.G., Mollinedo, R.E., Avila, J.C., Cespedes, J.L., Carter, D., Doumbo, O.K., 1997. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J. Infect. Dis. 176, 1590–1596.

Ranford-Cartwright, L.C., Taylor, J., Umasunthar, T., Taylor, L.H., Babiker, H.A., Lell, B., Schmidt-Ott, J.R., Lehman, L.G., Walliker, D., Kremsner, P.G., 1997. Molecular analysis of recrudescent parasites in a *Plasmodium falciparum* drug efficacy trial in Gabon. Trans. R. Soc. Trop. Med. Hyg. 91, 719–724.

Reed, M.B., Saliba, K.J., Caruana, S.R., Kirk, K., Cowman, A.F., 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 403, 906–909.

Schneider, G., Premji, Z., Felger, I., Smith, T., Abdulla, S., Beck, H.P., Mshinda, H., 2002. A point mutation in codon 76 of *pfcrt* is positively selected for by chloroquine treatment in Tanzania. Infect. Genet. Evol. 1, 183–189.

Schwick, P., Eggelte, T.A., Hess, F., Tueumuna, T.T., Payne, D., Nothdurft, H.D., von Sonnenburg, F., Loscher, T., 1998. Sensitive ELISA dipstick test for the detection of chloroquine in urine under field conditions. Trop. Med. Int. Health 3, 828–832.

Sibley, C.H., Hyde, J.E., Sims, P.F., Plowe, C.V., Kublin, J.G., Mberu, E.K., Cowman, A.F., Winstanley, P.A., Watkins, W.M., Nzila, A.M., 2001. Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? Trends Parasitol. 17, 582–588.

Sidhu, A.B., Verdier-Pinard, D., Fidock, D.A., 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Science 298, 210–213.

Snow, R.W., Marsh, K., 2002. The consequences of reducing transmission of *Plasmodium falciparum* in Africa. Adv. Parasitol. 52, 235–264.

Snow, R.W., Omumbo, J.A., Lowe, B., Molyneux, C.S., Obiero, J.O., Palmer, A., Weber, M.W., Pinder, M., Nahlen, B., Obonyo, C., Newbold, C., Gupta, S., Marsh, K., 1997. Relation between severe malaria morbidity in children and level of *Plasmodium falciparum* transmission in Africa. Lancet 349, 1650–1654.

Talisuna, A.O., Langi, P., Bakyaita, N., Egwang, T., Mutabingwa, T.K., Watkins, W., Van Marck, E., D'Alessandro, U., 2002a. Intensity of malaria transmission, antimalarial-drug use and resistance in Uganda: what is the relationship between these three factors? Trans. R. Soc. Trop. Med. Hyg. 96, 310–317.

Talisuna, A.O., Kyosiimire-Lugemwa, J., Langi, P., Mutabingwa, T.K., Watkins, W., Van Marck, E., Egwang, T., D'Alessandro, U., 2002b. 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. Trans. R. Soc. Trop. Med. Hyg. 96, 551–556.

Watkins, W.M., Mosobo, M., 1993. Treatment of *Plasmodium falciparum* malaria with pyrimethamine- sulfadoxine: selective pressure for resistance is a function of long elimination half-life. Trans. R. Soc. Trop. Med. Hyg. 87, 75–78.

Wellems, T.E., Plowe, C.V., 2001. Chloroquine-resistant malaria. J. Infect. Dis. 184, 770–776.

White, N., 1999. Antimalarial drug resistance and combination chemotherapy. Philos. Trans. R. Soc. Lond. B Biol. Sci. 354, 739–749.

White, N.J., Pongtavornpinyo, W., 2003. The de novo selection of drug resistant malaria parasites. Proc. R. Soc. Lond. B Biol. Sci. 270, 545–554.

Zar, J.H., 1984. Biostatistical Analysis, 2nd Edition, Prentice-Hall International, Englewood Cliffs, NJ.