Inoculum effect leads to overestimation of *in vitro* resistance for artemisinin derivatives and standard antimalarials: a Gambian field study

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SUMMARY

Artemisinin (QHS) and its derivatives are new antimalarials which are effective against *Plasmodium falciparum* parasites resistant to chloroquine (CQ). As these drugs are introduced it is imperative that resistance is monitored. In this paper we demonstrate that the inoculum size used in *in vitro* testing influences the measured *in vitro* susceptibility to QHS and its derivative dihydroartemisinin (DHA) and to mefloquine (MEF) and CQ over the range of parasitaemias routinely used in testing with the WHO *in vitro* microtest. An increase in parasitaemia and/or haematocrit was accompanied by a decrease in the measured sensitivity of 2 laboratory lines. In the context of a field study testing *in vitro* susceptibility of parasite isolates from patients with uncomplicated malaria in Fajara, The Gambia we demonstrate that failure to control for inoculum size significantly overestimates the level of resistance to QHS and DHA as well as MEF, halofantrine (HAL) and quinine (QUIN). When controlling for the inoculum effect, cross-resistance was observed between QHS, MEF and HAL suggesting the presence of a multidrug resistance-like mechanism. These studies underline the importance of inoculum size in *in vitro* susceptibility testing.

Key words: *Plasmodium falciparum*, artemisinin, *in vitro*, susceptibility, The Gambia, inoculum effect.

INTRODUCTION

A major problem in the control of malaria is the increase in resistance to chloroquine (CQ) and pyrimethamine/sulphadoxine in *Plasmodium falciparum* worldwide, the causative agent of approximately 400 million clinical cases and between 2 and 3 million deaths each year. Even more worrying has been the emergence of resistance to the newer antimalarials mefloquine (MEF) and halofantrine (HAL) in S.E. Asia (Nosten *et al*. 1991; ter Kuile *et al*. 1994). Artemisinin (QHS) derivatives have recently been introduced and have been demonstrated to be highly efficacious in areas of multiple drug resistance (Hien & White, 1993). Differences in *in vitro* sensitivity of individual parasite isolates has been noted with the QHSs, suggesting the genetic potential for the emergence of *in vivo* resistance (Basco & Le Bras, 1993; Gay *et al*. 1997; Pradines *et al*. 1998). It is essential that the levels of resistance to these newer antimalarials are monitored.

In vitro resistance data are useful because they can provide an indication of the relative sensitivity to a drug in a given area (Wernsdorfer & Payne, 1988). Moreover cross-resistance information is important as it may reveal the mechanism of drug-resistance, particularly with the identification of putative molecular determinants of resistance. Cross-resistance between MEF and HAL in Thailand appears to be an mdr-like phenomenon (Wilson *et al*. 1993). In Africa several reports have indicated cross-resistance between the QHSs and MEF and HAL and either an inverse or no relationship with CQ (Basco & Le Bras, 1993; Gay *et al*. 1997; Pradines *et al*. 1998). In contrast other reports have demonstrated either no cross-resistance with MEF and HAL, or cross-resistance with CQ (Alin *et al*. 1995; Alin 1997; von Seidlein *et al*. 1997). The basis of these discrepancies remains unexplained.

A major confounder of *in vitro* susceptibility tests with CQ is the inoculum effect (Ponnudurai, Leewenburg & Meuwissen, 1982). This phenomenon refers to the need for an increased concentration of drug to inhibit growth when greater numbers of microorganisms per volume are inoculated. The basis of this effect was attributed to accumulation of drug in the parasitized cells leading to its depletion from the test wells at higher inoculum sizes (Gluzman, Schlesinger & Krogstad, 1987). This has also been observed when susceptibility testing with other antimalarials such as amodiaquine derivatives

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(Bray, Hawley & Ward, 1996) and MEF and HAL (Ritchie *et al*. 1996). In this paper we demonstrate a significant inoculum effect with the antimalarials QHS and dihydroartemisinin (DHA) as well as other antimalarials over a range of inoculum size routinely used in susceptibility testing with the WHO *in vitro* microtest. Further, by means of varying the inoculum size during *in vitro* susceptibility testing of field isolates obtained in The Gambia we show that levels of resistance to QHS and DHA as well as to quinine (QUIN), MEF and HAL are overestimated when inoculum effect is not considered. The occurrence of cross-resistance between these antimalarials was also falsely determined in some cases. Cross-resistance between QHS, DHA, MEF and HAL and between CQ and QUIN was seen when the inoculum size was controlled.

MATERIALS AND METHODS

Parasite cultivation

The *P. falciparum* laboratory isolates used in these studies were K1 (CQ-resistant and MEF-sensitive) and T9/96 (CQ-sensitive and MEF-resistant). Parasites were cultivated at 37 °C in human erythrocytes (A+) in 25 ml narrow-neck, flat-bottomed culture flasks (Corning) containing medium (RPMI 1640/25 mM Hepes/23 mM $NaHCO_3$/0·2% glucose/50 μg/ml gentamycin/pH 7·3) and 10% AB serum. The same pooled serum was used in all experiments. Cultures were maintained in a 4% O_2/3% CO_2/93% N_2 atmosphere. Parasitaemia was calculated by counting at least 1000 erythrocytes using a 3×3 graticule and red cell count was read using a Neubauer haemocytometer (5×10^6 erythrocytes/μl was taken to equal 50% haematocrit).

In vitro *susceptibility testing*

A standard [^{3}H]hypoxanthine incorporation-based assay, a modification of the method developed by Desjardins *et al*. (1979), was used to measure the antimalarial sensitivity of the parasites. Briefly, serial dilutions of drug were made in 96-well plastic microtitre plates (Sigma) with 50 μl culture medium in each well. Cell suspensions of a certain parasitaemia or haematocrit were added to the plates to a final volume of 100 μl and these were placed in modular incubators flushed with the gas mixture described above at 37 °C. After 24 h 0·2 μCi [^{3}H]-hypoxanthine (Amersham) was added to each well, and after 19 h further incubation the contents of the well were harvested onto glassfibre filter mats using a Skatron harvester and dried. Radioactivity incorporated into parasite nucleic acid was determined by liquid scintillation counting. 'IC_{50}' values refer to the drug concentrations which result in a 50% reduction in incorporation of [^{3}H]hypoxanthine relative to untreated controls (O'Neill *et al*. 1985). These were calculated by using the XLFIT add-on of EXCEL and a sigmoidal log dose–response fit (equation 205). Stock solutions of CQ diphosphate (Sigma) and QUIN hydrochloride (Sigma) were prepared in sterile distilled water and of MEF hydrochloride (gift from Dr R. Ridley, Hoffman–La Roche), HAL hydrochloride (gift of Dr J. Horton, SKB), QHS (Sigma) and DHA (gift of Dr P. Olliaro, WHO) were prepared in methanol.

Laboratory experiments

Ring-stage parasites were obtained by several rounds of sorbitol-lysis (Lambros & Vanderburg, 1979). Inoculum size has previously been defined as the product of the haematocrit and the parasitaemia (Geary *et al*. 1990). The WHO *in vitro* microtest uses 1000–80000 parasites/μl of whole blood diluted 1 in 10 in medium (equivalent to a parasitaemia between 0·02 and 1·6%) as one of its criteria for inclusion. Correspondingly we varied the inoculum size by altering both the haematocrit and the parasitaemia in the experiments with laboratory isolates. Parasitaemia was initially varied at 4/2/1/0·5/0·2 and 0·125% at a fixed HC of 2·5%. In subsequent experiments HC was set at 5 or 1·25% at parasitaemias of 0·5 and 1·0%. All tests were carried out in duplicate and repeated 3 times.

Field study

This study was carried out at the Medical Research Council Laboratories (MRC), Fajara, The Gambia (Sept.–Oct. 1996). It was approved by the MRC Laboratories Scientific Co-ordinating Committee and the Gambian Government/MRC Ethical Committee. Venous blood samples (1–5 ml) were obtained in heparinized tubes following consent from patients with uncomplicated malaria recruited at the Outpatients Clinic. Samples were prepared for the tests by the removal of buffy coat and at least 3 washes in RPMI. Samples were resuspended at a haematocrit of 5%. The parasitaemia was estimated from a thin film. If greater than 2% it was adjusted to approximately 2% with 5% haematocrit uninfected erythrocytes (from the same 2 O+donors throughout the field studies). The samples were then diluted 4 times in serial 2-fold dilutions with 5% haematocrit uninfected erythrocytes to give parasitaemias ranging from the highest parasitaemia, with 2-, 4- and 8-fold dilutions. Subsequently the highest parasitaemia of the sample was accurately measured by counting at least 1000 erythrocytes on a thin film using a 3×3 graticule. Therefore each parasite sample was prepared as 4 different inoculum sizes.

Hypoxanthine assays were carried out with each field isolate at the 4 different inoculum sizes with QHS, DHA, CQ, QUIN, MEF and HAL. The

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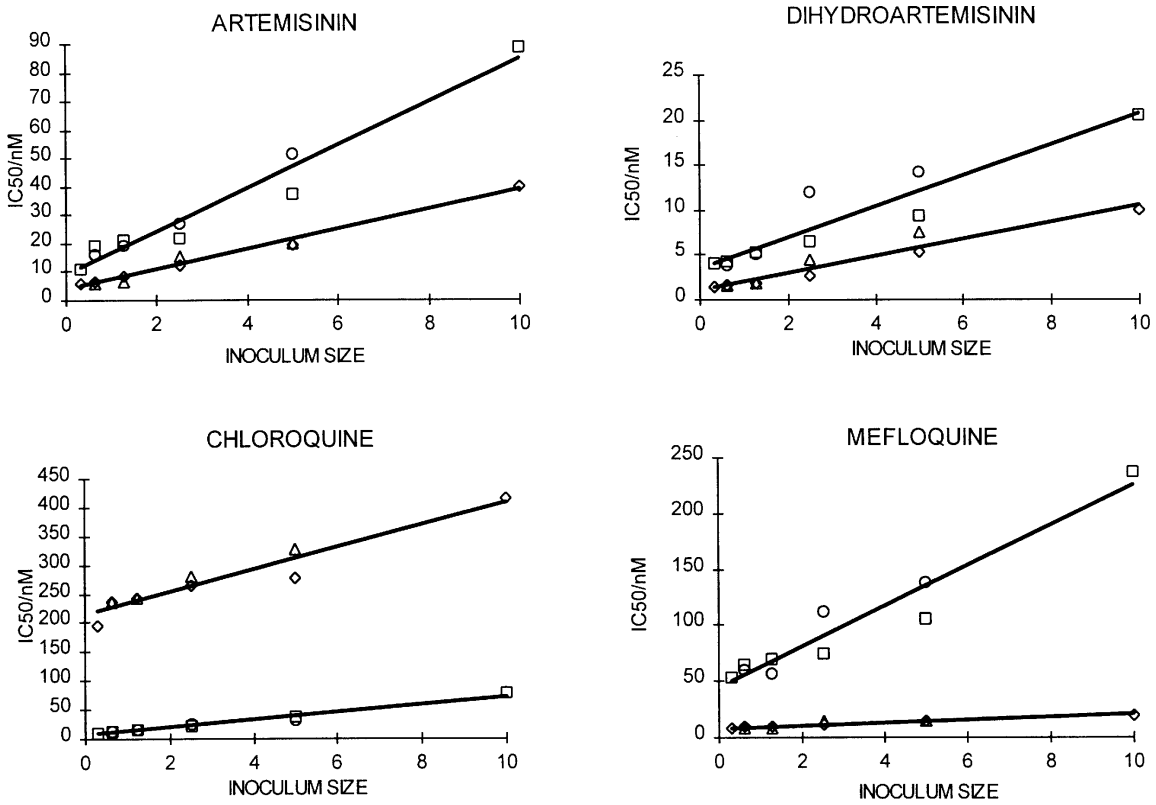

Fig. 1. Graphs illustrating the inoculum effect in T9/96 and K1 with the drugs QHS, DHA, CQ and MEF. Linear trend-lines are all highly significant. (□, ◇) Values at inoculum sizes obtained by varying parasitaemia. (○, △) Values of inoculum sizes, obtained by varying haematocrit. (△, ◇) Values derived for K1; (□, ○) values for T9/96. Each value is the mean of 3 separate determinations in duplicate.

same pooled AB serum was used in all tests. Hypoxanthine was added at 0·2–1·0 μCi per well. A test was deemed successful if counts were greater than 5 times that of uninfected erythrocytes. IC_{50} values were determined by XLFIT as above. IC_{50} values were plotted against inoculum size and linear trend-lines were drawn to estimate the IC_{50} at 0·5 % parasitaemia/2·5 % haematocrit (IC_{50}(cont)). We have defined the IC_{50} at the uncontrolled top parasitaemia as IC_{50}(top). Correlations between drug sensitivities were measured by the Spearman correlation co-efficient using the SPSS package.

RESULTS

Sensitivity testing with T9/96 and K1

Results of these experiments when parasitaemia was varied (Fig. 1) demonstrate clear inoculum effects with all the drugs tested for both laboratory lines K1 and T9/96. IC_{50} values increase linearly with inoculum size over the range tested with QHS and DHA, and as shown before with CQ (Geary *et al.* 1990) and MEF (Ritchie *et al.* 1996).

Increasing haematocrit to increase inoculum size also increased IC_{50} values for all the drugs tested (Fig. 1). The values obtained at the same inoculum size by varying haematocrit were not significantly different from those obtained when the parasitaemia was varied, implying that the uninfected cell component is not important in affecting the IC_{50} value over the range of parasitaemia and haematocrits tested.

Linear trend-lines fitted to the data were highly significant in all cases ($P<0·001$). The gradients of the lines were different for the 2 laboratory lines, indicating that the magnitude of the inoculum effect is dependent on intrinsic characteristics of each line. Modelling has previously indicated that the gradient is a function of the IC_{50} at an inoculum size of zero and the cellular accumulation ratio of the drug (Geary *et al.* 1990).

Sensitivity testing in the field

IC_{50} values were obtained for 26 samples with CQ/MEF/QHS and 27 with QUIN, DHA and HAL. Parasitaemias of the samples ranged between 0·55 and 15·04 % with a mean value of 3·64 %. Parasitaemias higher than 4 % were diluted to approximately 4 % at a haematocrit of 2.5 % (approximately the accepted upper limit for the WHO *in vitro* microtest) with uninfected erythrocytes. When IC_{50} values were plotted against inoculum size a positive relationship was observed in all cases. Linear trend-lines were fited to these and most were significant ($P<0·05$). Predicted IC_{50} values at a controlled

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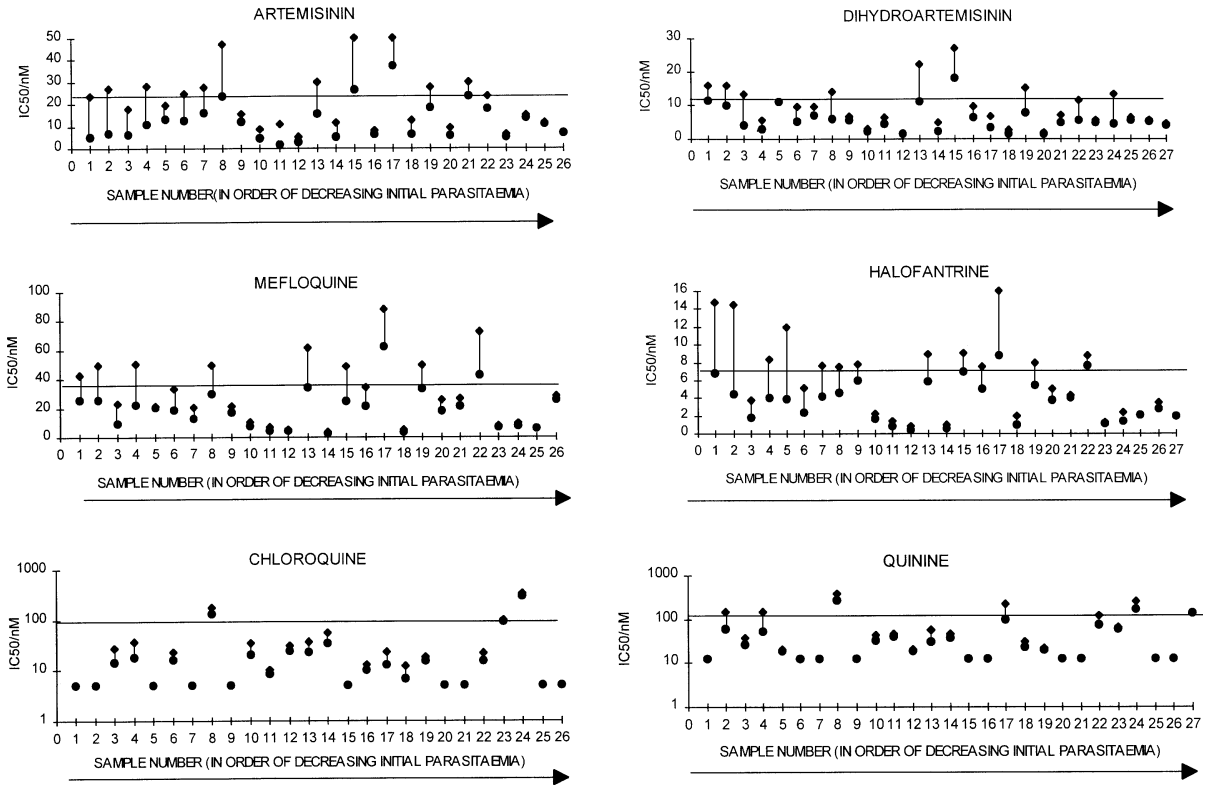

Fig. 2. Graph of the sensitivities of the Gambian field isolates at both the uncontrolled parasitaemia, IC_{50}(top) (◆), and the controlled parasitaemia, IC_{50}(cont) (●) for the drugs QHS, DHA, MEF, HAL, CQ and QUIN. Thin lines connecting the diamonds and the circles reflect the magnitude of the discrepancy. Horizontal lines are the 90th percentile values.

Table 1. Correlation of *in vitro* responses between artemisinin, dihydroartemisinin and the other antimalarials in the Gambian field study. Correlation of IC_{50}(top) values are also shown

Drug 1	Drug 2	*n*	*r*(cont)	*P*(cont)	*r*(top)	*P*(top)
Artemisinin	Mefloquine	26	0·74	<0·001	0·75	<0·001
	Halofantrine	26	0·68	<0·001	0·65	<0·001
	Dihydroartemisinin	26	0·33	0·1	0·62	0·001
	Chloroquine	26	0·008	n.s.	0·04	n.s.
	Quinine	26	0·35	n.s.	0·48	0·01
Dihydroartemisinin	Mefloquine	26	0·33	0·1	0·52	0·007
	Halofantrine	27	0·55	0·003	0·53	0·004
Mefloquine	Halofantrine	26	0·88	<0·001	0·79	<0·001
	Quinine	26	0·24	n.s.	0·38	0·05
Chloroquine	Quinine	26	0·72	<0·001	0·71	<0·001

n, number of paired results; *r*, correlation coefficient; *P*, significance of the test; n.s., not significant.

parasitaemia of 0·5 % (IC_{50}(cont)) and 2·5 % haematocrit were derived from these trend-lines. The IC_{50} at the uncontrolled highest inoculum size (IC_{50}(top)) and that at IC_{50}(cont) are summarized in Fig. 2 to demonstrate the discrepancy in IC_{50} due to failure to control for the inoculum effect. It is noted that there was a high degree of sensitivity to CQ and QUIN in this study.

When the inoculum size is not controlled the degree of resistance is overestimated in every case. In some cases this is so great that an isolate designated sensitive at the controlled inoculum size (IC_{50}(cont)) is resistant at the uncontrolled parasitaemia (IC_{50}(top)). To quantify this we have used a criterion for resistance of an IC_{50} value higher than the 90th percentile of IC_{50}(cont) values (Gay *et al.* 1997). In total 7/26 (28 %) isolates for QHS, 7/27 (26 %) isolates for DHA, 7/26 (28 %) isolates for MEF, 11/27 (41 %) isolates for HAL, 3/27 (11 %) for QUIN, but none for CQ, were erroneously noted as resistant, indicating how the level of resistance can be overestimated in a sensitivity trial.

Cross-resistance was observed between QHS versus MEF versus HAL, and between DHA versus HAL at IC_{50}(cont) (Table 1). Interestingly when the uncontrolled IC_{50}(top) values were used it was found

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that QHS *vs*. DHA, QHS *vs*. QUIN, DHA *vs*. MEF and MEF *vs*. QUIN become significantly correlated from only being weakly correlated when the controlled IC_{50}(cont) values are used. This suggests that a proportion of the cross-resistance observed is due to the inoculum effect.

DISCUSSION

In the present study we make explicit the extent to which resistance to QHS and other standard antimalarials can be overestimated due to the inoculum effect. Parasitaemia and haematocrit were varied over the range of inoculum sizes that are routinely used in the WHO *in vitro* microtest and both are found to be important, the sensitivities varying between 2- and 10-fold over a 16-fold range of inoculum sizes depending on the drug and the parasite line.

In real clinical settings, when parasite isolates are obtained directly from a patient for drug susceptibility testing there is a large degree of variation in the level of both parasitaemia and haematocrit. It is not sufficient therefore to use the criteria of the WHO *in vitro* microtest, i.e. 1000–80000 parasites/μl which is an 80-fold range in inoculum size. Previously this has not been a big problem in sensitivity testing for CQ as the differences in IC_{50} values between resistant and sensitive parasites are often greater than 10-fold, while the discrepancy in IC_{50} values over the permissible range of inoculum sizes is less. Note that the CQ IC_{50} value of the sensitive T9/96 line at its highest parasitaemia does not approach that of K1 even at its lowest inoculum size. With the QHS derivatives and arylaminoalcohols the difference between sensitive and resistant parasites is between 2- and 4-fold (Wilson *et al*. 1993; Gay *et al*. 1994) which is similar to the discrepancy in IC_{50} values due to the inoculum effect. Therefore in contrast to CQ, there can be an overlap between the sensitivities of the resistant and sensitive strain at different inoculum sizes with QHS, DHA and MEF.

The uncontrolled inoculum sizes of the samples used in the Gambian field study *in vitro* tests in this study were in the range routinely used in the WHO *in vitro* microtest. Our results clearly indicate how the IC_{50} values can be greatly overestimated even within this range. Furthermore, they raise the question about the appropriateness of 'cut-off' values for resistance. These like the IC_{50} values will be dependent on the inoculum size used in a particular study. Further, it will not be easy to compare resistance levels between studies which use uncontrolled or controlled but different inoculum sizes.

While controlling for the inoculum effect we find that QHS is indeed cross-resistant with MEF and HAL, but not with CQ. This has been observed previously for QHS and its derivative artemether (Basco & Le Bras, 1993; Gay *et al*. 1997) and both these studies controlled for inoculum size. This is in sharp contrast to the cross-resistance between CQ and artemether observed in a study in The Gambia in the previous year which did not control for inoculum size (von Seidlein *et al*. 1997). In a Tanzanian study which did not control for the inoculum effect CQ was found to be cross-resistant with QHS and not with MEF while in another there was no cross-resistance between QHS, CQ, MEF or HAL (Alin *et al*. 1995, 1997). It is nevertheless still possible that these latter results reflect true geographical differences in the basis of resistance.

The cross-resistance between the structurally different drugs QHS and MEF suggests that there is a true multidrug resistance(mdr)-like mechanism. Selection for mefloquine-resistance *in vitro* has been associated with a concomitant increase in resistance to both HAL and QHS (Peel *et al*. 1994). The molecular basis of mdr in *P. falciparum* may involve the *pfmdr1* gene (Barnes *et al*. 1993; Peel *et al*. 1994). Alternatively, the drugs may share the same target which is altered to confer resistance. Haemin, a product of haemoglobin degradation within the parasite food vacuole may be involved in the mechanism of action of both QHS and MEF (Meshnick *et al*. 1991).

As the molecular basis of resistance is being elucidated for a number of different antimalarials and new drugs are being developed and tested in the field, we suggest that there is an urgent need for a standardization of methodologies for susceptibility testing throughout the world.

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