Multiplicity of *Plasmodium falciparum* infection predicts antimalarial treatment outcome in Ugandan Children

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Abstract

Background: In areas with intense malaria transmission, individuals are often simultaneously infected with multiple parasite strains. This study assessed the effect of multiple infections on treatment response in Ugandan children with uncomplicated malaria.

Methods: Four hundred and seventy six blood specimens were analysed for parasite genetic diversity. The *P.falciparum* merozoite surface protein-2 (*msp-2*) was analysed to establish multiplicity of infection for pre and post treatment specimens.

Results: There were 32 different *msp-2* alleles, 15 corresponding to the IC/3D7 and 17 to the FC27 allelic family. The majority of the isolates (343, 72 %) were multiple infections resulting into an overall mean multiplicity of infection of 2.15 (SD±1.02). Children infected with multiple strains had nearly a 3-fold increase in treatment failure (Hazard Ratio = 2.8, 95 % CI: 1.5-5.3) compared to their age mates infected with a single strain.

Conclusion: Multiple-strain infection reduced response to antimalarial therapy. Strategies that reduce multiple-strain infections (intermitted presumptive treatment, indoor residual spraying, insecticide treated nets and efficacious drugs) are likely to improve antimalarial drug efficacy and reduce rate of spread of drug resistance.

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Background

In Uganda, like in most of sub-Saharan Africa, malaria is among the leading causes of morbidity and mortality [1]. Efforts to eradicate or control malaria have had limited success due to the spread of drug resistant *Plasmodium falciparum* malaria, for almost all available drugs[2]. The emergence and spread of chloroquine resistance has led to increased incidence of disease, progression to severe disease, and increased malaria specific mortality especially in children[3, 4].

Molecular studies have demonstrated that one individual can be simultaneously infected with multiple *P. falciparum* strains that are genetically distinct[4-6]. Such multiple-strain infections are believed to play an important role in the development of strain-specific immunity. Individuals may develop immunity to infection from some but not all strains to which they are exposed [7]. A lower multiplicity of infection in severe compared to uncomplicated malaria has been reported and the parasite genetic diversity in both groups was very large [8]. However, the relationship between the number of strains in a single malaria infection and antimalarial treatment failure has not been conclusively established. To assess the effect of multiple infections on treatment response in Ugandan children with uncomplicated malaria, we analysed 476 blood specimens for parasite genetic diversity using merozoite surface protein - 2[9].

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Materials and methods

Clinical trial and study site

The clinical data were collected during a randomised clinical trial performed between January and June 2004 in Arua, Uganda as part of the Uganda malaria surveillance project[10]. Treatment efficacy of combination therapies of chloroquine (CQ) plus sulfadoxine-pyrimethamine (SP), amodiaquine (AQ) plus SP, and AQ plus artesunate (AS) were compared. Patients were given directly observed therapy and actively followed for 28 days. The outcome was classified according to WHO guidelines for areas of intense transmission as; adequate clinical and parasitological response (ACPR) or early treatment failure (ETF), late clinical failure (LCF) or late parasitological failure (LPF). For purposes of analysis, treatment outcome was grouped into treatment success (ACRP) or treatment failure (ETF, LCF and LPF)[11].

Molecular genotyping and diversity determination

Blood spots were collected onto filter paper (Whatman No.3, Whatman Inc., Clifton, NJ) on the day of enrolment and on the day treatment failure occurred. Merozoite surface protein-2 (*msp-2*) genotyping was performed on all day zero samples for patients with adequate clinical response. For all patients experiencing late clinical or parasitological failures during follow-up (days 4 to 28), genotyping was undertaken on samples for day zero and the day of failure (paired genotyping). Parasite DNA was extracted with Chelex 100 Resin (Bio-Rad Laboratories, Hercules, CA) as previously described [12]. Paired samples from the same patient were run on adjacent lanes. Gel images were digitized and molecular weights assigned to the bands. Each band assigned a molecular weight was considered an individual strain. Molecular weight standards were used to estimate the size of PCR products (50bp DNA ladder) using GelCompar software (Applied Maths).

Data management and statistical analysis

All data were double entered and verified using Epi-Info 6.04 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and analyzed using SPSS version 12.0. Treatment outcomes were assessed by comparing genotyping patterns for specimens collected at treatment failure (day of failure) with corresponding patterns for specimens obtained at day of enrolment (day 0). Strains were considered the same if molecular weights were within 20 base pairs. Recrudescence was defined as all early treatment failure and late treatment failure where all strains present on day of failure were present on day of enrolment. Definitive new infections were defined as any recurrent parasitaemia where none of the strains present on day of recurrent parasitaemia were present on the day of enrolment. Mixed results were defined as late treatment failures where the day of failure sample contained 50 % or more strains present on the day of enrolment otherwise defined as new infections[6].

Multiplicity of infection was derived as the maximum number of PCR fragments visualized for each blood isolate. The treatment outcome was adjusted for genotyping and categorized into treatment success and treatment failure. The cumulative risk of treatment failure over the 28day follow-up period was estimated using the Kaplan-Meier product limit formula (survival analysis) with censoring of mutually exclusive events for each outcome group. Survival functions were compared using the log-rank test.

Multiplicity was categorized into single strain and multiple strains. Association between multiplicity of infection and the rate of treatment failure was determined using the Cox proportional hazard models controlling for patient age, pre-treatment parasite density, gametocyte prevalence, and pre-treatment hemoglobin concentration. Analysis was stratified into two treatment arms of chloroquine (CQ+ SP) and amodiaquine (AQ + SP or AQ + AS) combinations to cater for efficacy differences in medicines administered.

Results

Clinical treatment outcomes

Of the 485 children eligible for the study, 476 (98 %) who had complete genotyping data were included in this analysis. One hundred and eighty eight (188/476, 40 %) of the children had parasitological treatment failure during 28 days of follow up. The risk of recrudescence (adjusted for genotyping) for CQ + SP was 46 %, while that for AQ + SP was 14 % and that for AQ + AS was 9 % during 28 days of follow up. All baseline characteristics were similar for children with multiple strain infections and those with a single strain infection. The mean (±SD) temperature at presentation was 37.2 ± 0.9°C and was significantly higher in children with multiple-strain infections compared to single-strain infections (p = 0.003).

Genetic diversity of infection of Plasmodium falciparum

A total of 476 isolates analysed yielded 767 *msp*-2 PCR fragments of which 393 (51 %) and 374 (49 %) belonged to IC/3D7 and FC27 respectively. Thirty two *Plasmodium falciparum* alleles were detected in Arua, 15 of IC/3D7 and 17 of FC27 allelic families of *msp*-2. Of the 476 isolates analysed, the majority (290/476, 61%) were a mixture of both IC/3D7 and FC27 alleles. The remaining isolates (186/476, 39 %) were either IC3D7 or FC27 allelic families only. Pure IC/3D7-type allelic family isolates were103 (22 %) and were comprised of 75 single-strain infections and 28 multiple-strain infections. Pure FC27-type allelic family isolates were 83 (17%) and were comprised of 62 single-strain infections and 21 multiple-strain infections

Multiplicity of Plasmodium falciparum infection

Seventy one percent (343/476) of the isolates contained multiple *msp-2* alleles. The mean multiplicity of infection (MOI) (±SD) was 2.15 ± 1.02 with a range of 1-6 strains. Multiple-strain infections were a mixture of both IC/3D7 and FC27 allelic families. Multiplicity of infection was not associated with parasite density (OR= 0.8, 95 % CI: 0.56-1.24), age (OR = 0.7, 95 %

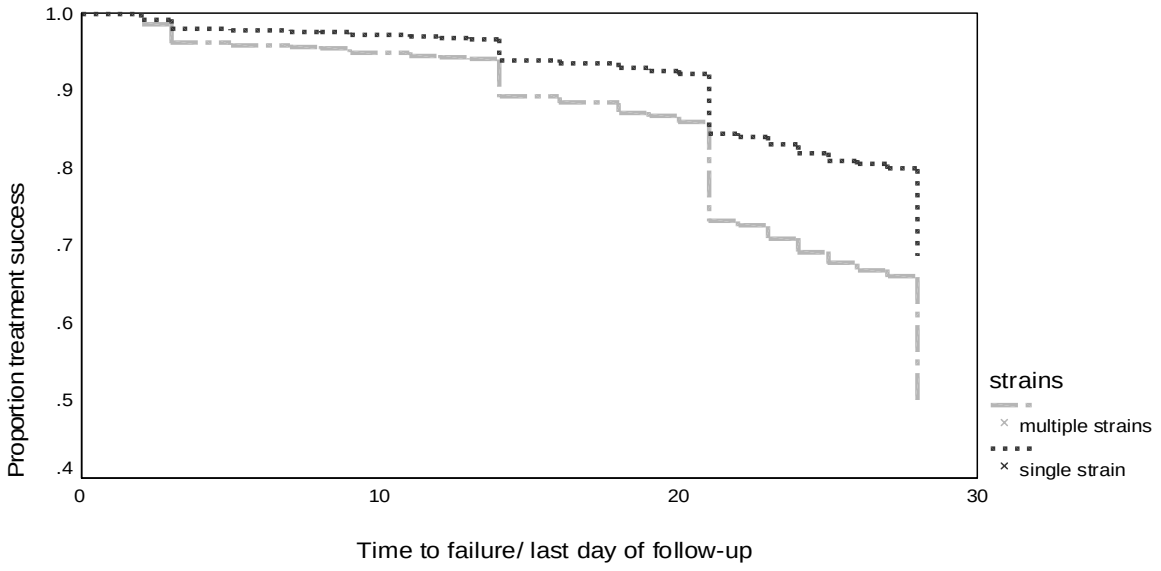
CI: 0.42-1.03), hemoglobin concentration (OR = 1.4, 95 % CI: 0.94-2.10), temperature (OR = 0.7, 95 % CI: 0.47-1.03) or presence of gametocytes (OR = 1.0, 95 % CI: 0.65-1.45). Thirty percent (50/171) of the samples on the day of failure were single strain infections.

Multiplicity of infection and treatment failure

To evaluate the association between multiplicity of infection and treatment failure, Kaplan-Meier survival analysis (Figure 1) and a Cox proportional hazard model were performed controlling for age, pre-treatment parasite density, hemoglobin concentration and temperature. Analysis was stratified for treatment groups.

In the AQ+SP arm, children infected with multiple strains had 3 times the rate of treatment failure compared to children with a single strain (HR = 2.8, 95%CI 1.5-5.4). In the CQ+SP arm, children infected with multiple strains had twice the rate of treatment failure compared to children with single strain infection (HR = 2.0, 95%CI 1.4-2.8) (Table 1). For patients who failed therapy, the mean multiplicity of infection at day of failure was 2.3 (SD 1.1), significantly lower than the mean multiplicity of infection on day zero of 2.5 (SD 1.0) for the same patients (n=188, t =-2.05, P= 0.042), suggesting that chemotherapy decreases multiplicity of infections.

Figure 1 Kaplan Meier survival curve of multiplicity of infection for treatment failure of 476 children in Arua, Uganda, 2004



Pre-treatment "multiplicity of infection" and the risk of recrudescence (true treatment failure) after 28 days of follow-up. Estimates of risk were based on survival analysis techniques with events defined as recrudescence and data censored for re-infections and the end of observation. The curves compare single infection to multiple infections (2 or more strains) in 476 children in Arua, Uganda, 2005.

Discussion

This study has investigated *Plasmodium falciparum* polymorphism and assessed the association between multiplicity of infection and treatment failure in 476 symptomatic Ugandan children in Arua district. The parasite population structure was analysed by allele specific DNA amplification of *msp-2,* which is a suitable means of characterising multiple parasite populations based on our previous analysis[9]. The *P. falciparum* population in Arua was genetically extensive with 32 different alleles of *msp*-2. The highest frequency of a single strain was 16 %, suggesting large parasite diversity in circulation in Arua.

The isolates in our analysis were obtained from children aged less than 5 years old, who are less likely to resist parasite infection due to low immunity and therefore likely to carry a majority of the local strains in circulation before treatment. The level of diversity observed in this study is consistent with what other investigators have previously found in endemic parts of Africa. Several studies have demonstrated an increased diversity of malaria infections in areas of high transmission similar to our observations in Arua, annual entomological inoculation rates (AEIR) = 369 infective bites/person/year, suggesting an association between

Table 1:Bivariate analysis of multiplicity of plasmodium infection in 476 children, Arua, Uganda 2004

Variable	Multiple Infections (%) (n=339)	Single Infection (%) (n=137)	MOR[1] (95%CI)	P-value
Gender				
Male	174(51)	77(56)	0.82(0.55-0.23)	0.335
Age groups				
2yrs and below	228(67)	104(76)	0.65(0.42-1.03)	0.063
Temperature				
>37.2 °C	131(39)	65(47)	0.68(0.47-1.03)	0.077
Parasite density				
>25700 /µl	165(49)	73(53)	0.83(0.56-1.24)	0.362
Gametocytes				
Yes	105(31)	44(31)	0.95(0.62-1.45)	0.808
Hemoglobin				
Hb d"9 g/dl	177(52)	60(44)	1.40(0.94-2.09)	0.096
Medication				
CQ+SP	111(33)	50(36)	0.85(0.56-1.28)	0.433
AQ+SP	112(33)	46(34)	0.98(0.64-1.49)	0.910
AQ+AS	113(34)	41(30	1.22(0.79-1.87)	0.367
Treatment outcome[2]				
Failure	152(45)	36(26)	2.3(1.47-3.53)	<0.001

[1]Mantel-Haenszel odds ratio

[2]28 day parasitological outcome adjusted for genotyping

parasite genetic diversity and malaria transmission intensity[13, 14].

Previous reports have observed variations in parasite genetic diversity. A study in Western Uganda found 8 alleles of *Plasmodium falciparum* using *msp-2* [15], while another in the same region in Uganda reported 24 different alleles of *msp-2* in 40 asymptomatic children aged less than 5 years old[5]. A study done in Kampala, Uganda[9] found 52 alleles of *msp-2* in 394 isolates from children with clinical malaria followed up over a period of one year.

More than two thirds of the malaria episodes investigated in Arua, Uganda, carried multiple *Plasmodium falciparum* strains. Molecular genotyping revealed an average of 2 strains per infection in the 476 isolates. Multiplicity of infection (MOI) has been demonstrated to increase with increasing levels of transmission, until it plateaus at very intense transmission[16]. The majority of multiple-strain infections showed a mixture of both IC/3D7 and FC27 allelic families. The mean MOI observed in Arua, an area with intense transmission was comparable to that observed in Kampala, Uganda, a meso-endemic region but surprisingly lower than that observed in Tororo, Eastern Uganda, an area with holo-endemic malaria[6].

There are conflicting reports about the relationships between multiplicity of infection, age, and parasite density. Some studies have demonstrated that multiplicity varies with age and parasite density[5, 17], and also with transmission intensity[18], but others did not find such relationships[19, 20]. In Kenya, multiplicity of infection increased with decreasing age and the change was more marked below 2 years[21]. In this study, multiplicity of infection did not vary with parasite density or age. However, it was restricted to children under five years of age and therefore can not rule out differences in older children (5-15 years old).

This study confirms an earlier observation that high multiplicity of infection is associated with increased risk of treatment failure[22]. Children infected with multiple *P.falciparum* strains had 3 times the rate of treatment failure compared to those with a single strain, independent of parasite density, hemoglobin concentration, and age and gametocyte prevalence. The rate of treatment failure increased with increasing number of infecting strains. When the number of infecting strains was analysed as a continuous variable, the association with treatment failure was even stronger. Multiple strains increase the probability of carrying a

strain with resistance-conferring mutations, an important factor in determining response to antimalarial response. Further, it is likely that a higher number of infecting strains increases the probability that some strains could evade the immune system and persists in the host. A significant reduction in the number of parasite strains detected on day of failure compared to day zero was observed. This reduction in the mean number of persisting strains suggests that the chemotherapy eliminated some susceptible strains while selecting for the drug-resistant surviving parasites, an observation that could have important implications for the spread of drug resistance in areas with intense transmission. It has been proposed that competition between different parasite strains in the same host (intra-host competition) may lead to evolution and selection of more virulent forms of parasites[23] or to a boosting in the multiplication of the resistance strains after chemotherapy has eliminated the susceptible strains[24]. These observations further support the theories that a high multiplicity has an important role in the evolution and spread of drug resistance within *Plasmodium falciparum* populations.

Children aged two years or less had almost twice the risk of treatment failure compared to older children. This observation is not surprising because age is a surrogate marker for acquired immunity[25]. Higher immunity (older age groups) facilitates drug therapy to kill infecting malaria parasites.

Conclusions

The present study has described *Plasmodium falciparum* genetic diversity and demonstrated a clear association between strain multiplicity of infection and treatment failure in Arua. The majority of clinical episodes of malaria were a result of multiple strain infections with *Plasmodium falciparum* and therefore had increased risk of treatment failure. These findings suggest that diversity and strain multiplicity contribute to drug resistance and add new insight to our understanding of the epidemiology of antimalarial drug resistance and possible interventions to improve treatment outcomes.

Effort towards decreasing the multiplicity of infection (ITNs, IPT, IRS, Chemotherapy and a malaria vaccine when one becomes available) could improve antimalarial treatment response, independent of other parasite and host factors and are likely to slow the spread of drug resistance[16]. More effective therapy in children with symptomatic disease would be expected to reduce the number of parasite strains surviving treatment, reducing the strain multiplicity in the parasite population. These data therefore support the use of multiple strategies to prevent and control malaria and provide an evidence base in favour of vector control measures to prolong efficacy of antimalarial drugs.

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