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Impact of Multiplicity of *Plasmodium falciparum* Infection on Clinical Disease in Malawi

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Abstract. Multiplicity of infection (MOI), the number of unique *Plasmodium falciparum* parasite genotypes found in one infected individual, may contribute to the development of clinical malaria disease. However, the independent contribution of MOI and parasite density to clinical disease has not been well characterized. We conducted a two-year longitudinal cohort study of adults and children in a high-transmission setting in Malawi to test the hypothesis that increased MOI was independently associated with clinical disease, after accounting for parasite density. Of 1,062 episodes of infection, 477 (44.9%) were associated with symptoms. After controlling for repeated measures within an individual, key demographic factors, and parasite density, there was no association between MOI and clinical disease (OR = 1.02, 95% CI: 0.70–1.51). Although the limited ability to discern MOI in low-density asymptomatic infections may have impacted our results, we conclude that MOI is not an independent risk factor for clinical disease.

Plasmodium falciparum infection is common, but clinical disease is less frequent. A lack of preexisting immunity and increased parasite density are well-characterized predictors of clinical disease.[1–3] However, other factors may play a role in determining whether an infection produces symptoms. We have recently demonstrated that clinical disease most often occurs when an individual becomes infected with a new parasite, rather than developing symptoms after a prolonged chronic asymptomatic infection with the same parasite.[4] This led us to explore the possibility that multiplicity of infection (MOI) may be an important risk factor for symptomatic illness. Having a greater number of unique genotypes at a given time may increase the likelihood that parasites expressing unrecognized surface proteins are present and can cause clinical disease.

Most previous studies of the impact of MOI on clinical disease have been cross-sectional in nature, examining the association between higher MOI and risk of any symptoms at a single point in time.[5,6] There are limited conclusions that can be made from these studies, as symptoms might have occurred just before or just after the observation and accurate information about treatment history is often not available. Also, studies have generally focused exclusively on young children, who have high rates of clinical disease.[7] Generalizable conclusions are also limited by differing epidemiological context and methods for measuring MOI.

We had the opportunity to evaluate the impact of MOI on risk of clinical disease in the context of a longitudinal study in a high-transmission setting in southern Malawi. We enrolled adults and children at the time of an uncomplicated malaria illness and followed them up for 2 years. We measured the risk of clinical disease by MOI and also assessed the impact of MOI on the parasite density that elicits fever, referred to as the pyrogenic threshold. We hypothesized that higher MOI would be associated with an increased risk of clinical disease.

The study was conducted at the Mfera Health Center in Chikwawa, Malawi, a rural area with perennial high transmission of *P. falciparum*. The study has been described in detail previously.[4] Briefly, we enrolled 120 adults and children aged more than one year who presented to the health center with clinical malaria confirmed by blood smear microscopy. Active detection of infection occurred in routine monthly visits, and participants were encouraged to attend the study clinic any time they were ill. Dried blood spots on filter paper and blood smears were collected at all visits. Participants with symptoms suggestive of malaria (including fever, headaches, musculoskeletal pain, and vomiting) were tested using malaria rapid diagnostic tests (mRDT) and treated with a three-day course of artemether–lumefantrine if the test was positive. Participants were followed up in the study for up to two years. At the end of the study, all dried blood spots on filter paper underwent quantitative PCR (qPCR) for the detection of submicroscopic malaria infections. Specimens with positive qPCR results underwent genotyping. Merozoite surface proteins (msp1 and msp2) and glutamate-rich protein (glurp) genotyping used nested PCR to amplify polymorphic antigens, followed by gel electrophoresis to assess fragment lengths. Our methods for detecting, quantifying, and genotyping *P. falciparum* infection using microscopy and molecular methods were published previously.[8–10] The study was approved by the ethical review committee of the University of Malawi College of Medicine and the University of Maryland School of Medicine Institutional Review Board.

Observations in this analysis include all enrollment samples and any follow-up visits with *P. falciparum* infection detected by qPCR. A clinical disease episode was defined as a *P. falciparum* infection detected by mRDT at the time of reported malaria symptoms and confirmed by qPCR. This included the enrollment visit. An asymptomatic infection episode was defined as a *P. falciparum* infection detected by qPCR with no symptoms indicative of clinical disease reported at the time or within two weeks. Participants were analyzed according to their age at the time of each visit. Infection was categorized as submicroscopic infection (microscopy negative and qPCR positive) or microscopically detectable infection. Age was categorized as children aged less than 15 years. Multiplicity of infection was either a continuous variable or categorized as monoclonal or polyclonal infections.

Statistical analysis was conducted using RStudio1.1.383 (RStudio, Inc., Boston, MA) and SAS 9.4 (SAS Institute Inc.,

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TABLE 1
Participant characteristics

	Total observations, *N*	Clinical disease, *N* (%)	*P*-value*
Age (years)			0.060
1–5	263	131 (49.8)	–
5–15	593	266 (44.9)	–
≥ 15	206	80 (38.8)	–
Season			0.427
Rainy	299	128 (42.8)	–
Dry	763	349 (45.7)	–
Parasite density			< 0.001
Submicroscopic	142	22 (15.5)	–
Low density	175	73 (41.7)	–
High density	401	319 (79.6)	–
MOI			< 0.001
Not evaluable†	343	62 (18.1)	–
Monoclonal infection	237	113 (47.7)	–
Polyclonal infection	482	302 (62.7)	–

MOI = multiplicity of infection; *N* = number of pcr-positive observations. Rainy season: November–April; dry season: May–October.
* *N* = 1,062; chi-squared tests determined *P*-value for bivariate analysis.
† Number of MOI with not evaluable observations.

Carey, NC). Parasite density and MOI were compared using Wilcoxon rank-sum tests. Chi-squared tests were used for bivariate analyses of categorical variables and both MOI and clinical disease. Parasite density was either log-transformed or categorized as low (< 2,000 parasites/uL) versus high (≥ 2,000 parasites/uL).

Mixed effect logistic regression models were used for all adjusted analyses to account for nonindependence of observations due to repeated measures. Continuous and categorical variables for MOI and parasite density were considered in the models, and the variable that yielded the best fit model was selected, as assessed by Akaike's information criterion. Covariates that were significantly associated with either MOI or symptom status were explored for inclusion in the final multivariable models.

Pyrogenic threshold was defined as the level of parasitemia which best predicted the presence of measured temperature at the time of the visit ≥ 37.5°C. To assess this, the parasite density range was divided into 35 parasite density cutoff values based on the parasite density distribution. For each parasite density cutoff, sensitivity and specificity were calculated.[11] The pyrogenic threshold was determined at the parasite density cutoff value at which the difference in sensitivity and specificity was minimized. No statistical test of significance is available.

A total of 120 participants were included in the study, with a total of 1,062 infections detected (Table 1). The median parasite density of microscopically detected infections was 8,480 parasites/uL (interquartile range [IQR] 1,020–51,027). Multiplicity of infection was evaluable for 67.7% of all observations, including 415/477 (87%) of clinical disease episodes and 304/585 (52%) of asymptomatic infections. More than 82% of submicroscopic infections did not have evaluable MOI. The median MOI was 2 (IQR 1–3), with a range of 1–10.

Polyclonal infection was more common in clinical malaria disease (302/477, 63.3%) than asymptomatic infections (180/585, 30.8%, $P < 0.001$), in children (416/856, 48.6%) than adults (66/206, 32%, $P < 0.001$), and in the dry season (355/763, 46.5%) than the rainy season (127/299, 42.5%, $P < 0.05$). The median parasite density was lower for monoclonal infections (3,030 parasites/uL, IQR 660–26,470) than polyclonal infection (15,200 parasites/uL, IQR 2,035–65,230, $P < 0.001$).

In the final mixed effects logistic regression model accounting for repeated measures and controlling for parasite density, age, and season, there was no association between MOI and clinical disease (OR = 1.02, 95% CI: 0.70–1.51) (Table 2). There was no evidence of interaction between MOI and parasite density or between age and MOI, suggesting that our final model does not require stratification.

Table 3 shows the results of our pyrogenic threshold analysis. The pyrogenic threshold was lower for monoclonal infections (7,428 parasites/uL) than polyclonal infections (32,100 parasites/uL) and adults (17,708 parasites/uL) than children aged between 5 and 15 years (23,876 parasites/uL).

TABLE 2
Unadjusted and adjusted multivariate logistic regression models to assess the association between exposures (categorical MOI and categorical parasite density) and odds of symptoms

	Symptomatic infection, *N* (%)*	Unadjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)†
Monoclonal infection	112 (47.5)	REF	REF
Polyclonal infection	302 (62.7)	1.86 (1.36–2.55)	1.02 (0.70–1.51)
Submicroscopic parasite density	22 (15.5)	REF	REF
Low parasite density	73 (41.7)	5.49 (3.68–8.19)	4.19 (2.40–7.30)
High parasite density	319 (79.6)	28.8 (19.7–42.0)	23.10 (13.38–39.88)

MOI = multiplicity of infection. Using mixed effects to account for repeated measures among individuals, age, and season among all pcr-positive observations (*N* = 718).
* Number and proportion of observations that were symptomatic.
† Model adjusted for age and season.

TABLE 3
Pyrogenic threshold with sensitivity and specificity of parasite densities to predict fever

Infections	Pyrogenic threshold (parasites/uL)	Sensitivity	Specificity
Monoclonal	7,428	0.69	0.71
Polyclonal	32,100	0.81	0.70
Age (years)			
0–5	32,100	0.75	0.76
5–15	23,876	0.74	0.75
> 15	17,708	1.00	0.91

In this longitudinal cohort study of Malawian children and adults, we found the parasite density, but not MOI, was highly associated with the presence of clinical disease in this high-transmission setting. Our results are among the most rigorous exploration of this topic because we included long-term follow-up of participants of all ages throughout both transmission seasons, allowing us to account for age and seasonal effects on the outcomes. The retrospective detection of asymptomatic infections through qPCR allowed us to observe the natural history of chronic malaria infections. In our study, like most high-transmission settings, most of the infections detected were asymptomatic and frequently submicroscopic.

The limited ability of the genotyping assay to perform at the lowest parasite densities may have biased our results, leading to the failure to detect minor alleles present or even failing to yield evaluable results in the lowest density infections, which were always asymptomatic. This limitation was expected.[12,13] The limit of detection likely caused us to systematically underestimate MOI and prevented inclusion of a large number of low parasitemia asymptomatic infections in the analysis. Although more sensitive techniques are available to detect all parasite genotypes, we suspect that sequencing methods would lead to higher MOIs in all infections and would be unlikely to alter our conclusions.[14]

The results of the pyrogenic threshold, showing a lower pyrogenic threshold for monoclonal versus polyclonal infections and adults versus children, were surprising. Assessing the impact of MOI on clinical presentation in a high-transmission setting where exposure and age-acquired immunity provide protection from disease is complex.[15,16] Host immune responses alter the ability of parasites to replicate in blood, thus lowering parasite density even when symptoms occur.[17,18] Previous studies have demonstrated an association between MOI and disease severity,[19] but our cohort included only uncomplicated cases.

Our study suggests that MOI is not a key determinant of clinical disease on the individual level. However, recent studies have demonstrated changes in parasite diversity and population structure can occur in response to malaria control and elimination activities.[20] Thus, measuring parasite diversity on a population level may provide valuable information.

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