CAMBRIDGE UNIVERSITY PRESS

Quantification of *Plasmodium falciparum* malaria from complex infections in the Peruvian Amazon using quantitative PCR of the merozoite surface protein 1, block 2 (*Pf*MSP1-B2): *in vitro* dynamics reveal density dependent interactions

Journal:	*Parasitology*
Manuscript ID:	PAR-2011-0213.R2
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Zervos, Thomas; New York University School of Medicine, Medical Parasitology Hernandez, Jean; Universidad Nacional de la Amazonia Peruana, Laboratorio de Investigaciones Productos Naturales Anti-Parasitarios Sutton, Patrick; New York University School of Medicine, Medical Parasitology Branch, OraLee; New York University School of Medicine, Medical Parasitology
Key Words:	Plasmodium falciparum, merozoite surface protein one, block 2, quantitative PCR, density regulation

Title: Quantification of *Plasmodium falciparum* malaria from complex infections in the Peruvian Amazon using quantitative PCR of the merozoite surface protein 1, block 2 (*Pf*MSP1-B2): *in vitro* dynamics reveal density dependent interactions

Thomas M. Zervos[1], Jean N. Hernandez[2], Patrick L. Sutton[1] and OraLee H. Branch[1*]

[1]Department of Medical Parasitology, New York University School of Medicine, 341 E 25th St, OPH, New York, NY 10010, USA

[2]Universidad Nacional de la Amazonia Peruana, Laboratorio de Investigaciones Productos Naturales Anti-Parasitarios, Iquitos, Peru

Short Title: *PF*MSP1-B2 QPCR and DENSITY DEPENDENT INTERACTIONS IN CULTURE

*Corresponding Author. Department of Medical Parasitology, New York University School of Medicine, 341 E 25th St, OPH, New York, NY 10010, USA. Tel: +011 212 263 4364. Fax: +011 212 263 8116. Email: OraLee.Branch@nyumc.org

SUMMARY

The majority of *Plasmodium falciparum* field isolates are defined as complex infections because they contain multiple genetically distinct clones. Studying interactions between clones in complex infections *in vivo* and *in vitro* could elucidate important phenomenon in malaria infection, transmission and treatment. Using quantitative PCR (qPCR) of the *P. falciparum* merozoite surface protein 1, block two (*Pf*MSP1-B2), we provide a sensitive and efficient genotyping method. This is important for epidemiologic studies because it makes it possible to study genotype specific growth dynamics. We compared three *Pf*MSP1-B2 genotyping methods by analyzing 79 field isolates from the Peruvian Amazon. *In vivo* observations from other studies using these techniques led to the hypothesis that clones within complex infections interact. By co-culturing clones with different *Pf*MSP1-B2 genotypes, and measuring parasitemia using qPCR, we found that suppression of clonal expansion was a factor of the collective density of all clones present in a culture. *Pf*MSP1-B2 qPCR enabled us to find *in vitro* evidence for parasite-parasite interactions and could facilitate future investigations of growth trends in naturally occurring complex infections.

Key Words: *Plasmodium falciparum,* merozoite surface protein one, block 2, quantitative PCR, density regulation.

INTRODUCTION

In *Plasmodium falciparum* malaria infections, symptoms, immunity, parasite genetic diversity, and interactions of parasites co-infecting a given individual are all likely interrelated. The number of parasites in a given infection, detected by genetic typing of a polymorphic single copy gene, is called the complexity of infection (COI), and is also referred to as the multiplicity of infection. The merozoite surface protein 1, block two region (*Pf*MSP1-B2) has often been used to classify parasites. The traditional method of genetic typing uses a nested primer PCR approach, which provides a non-quantitative but sensitive method to determine at a minimum how many different *P. falciparum* parasite clones are within an infected host (Robert *et al*., 1996). Each polymorphism generally represents a distinct population of parasites and these clones can be mixed within both the *Anopheles spp*. vector and *Homo sapiens* host.

In high transmission regions, such as those in Sub-Saharan Africa, COI is high due to mosquitoes carrying more than one *P. falciparum* genotype and frequent mosquito biting causing overlapping complex co-infections in the human host. There are three major *Pf*MSP1-B2 allelic families detected worldwide: K1, Mad20 and RO33 (Certa *et al.* 1987; Tanabe *et al.* 1987), but the *Pf*MSP1-B2 genotyping method also detects subtypes within these three major allelic families. For example, we studied COI in Western Kenya and found that up to 85% of infections had more than five different *Pf*MSP1-B2 genotypes in one infection (Branch *et al.*, 2001).

Within host COI and parasite genotype are critical variables for *in vitro* experiments testing the efficacy of chemotherapy and vaccines in the developmental pipeline (Trager & Jensen, 1997). For example, an isolate containing drug sensitive and drug resistant clones will appear unaffected by drug treatment, when in reality, drug sensitive clones are lost (Huijben *et al.* 2011). Experiments designed to study ligand/receptor mediated invasion pathways utilized by merozoites are commonly performed on field isolates from complex infections. If clones in complex infections utilize different invasion pathways, results would show a composite of all pathways present in an infection (Bei *et al.*, 2007). Knowing the COI of *P. falciparum* isolates, even if not directly related to the phenotype being tested, provides valuable information for interpreting *in vitro* experiments (Farnert *et al.*, 2001).

A low malaria transmission setting in the Peruvian Amazon was chosen to avoid the high parasite genetic diversity and COI observed in high transmission settings. In this site, the transmission rate is <1 *P. falciparum* infection/person/year (Branch *et al*. 2005). Although lower than in high transmission settings, there is considerable genetic diversity (Chenet *et al*., 2008; Sutton *et al.*, 2009; Sutton *et al*., 2010; Branch *et al*., 2011). Approximately

20% were mixed genotype infections, most of which appeared to be caused by a mosquito bite containing more than one genotype (Sutton *et al.*, 2009). We detected mixed genotype infections with oscillating genotypes, where there was one clone detected and then additional or different clone(s) detected in the next days' blood sample(s) (Sutton *et al.*, 2009). This is consistent with many prior studies following genotype dynamics of higher transmission (Smith *et al.*, 1999; Bruce *et al.*, 2000; Farnert *et al.*, 2008). Therefore, even in our low transmission region, we need a sensitive, reliable, robust and high-throughput method to evaluate parasite genotype and within-host COI over time.

In this study, we compared a method of quantitative PCR (qPCR) with traditional PCR (McBride & Heidrich, 1987) and capillary gel electrophoresis (CGE) (Liljander *et al.*, 2009). Natural infections from our Peru cohort were used to compare sensitivity of these methods. qPCR was then used to quantify mixed clones in an *in vitro* culture model system and track growth dynamics over time. A *Pf*MSP1-B2 method was previously developed introducing a highly sensitive diagnosis of *P. falciparum* amongst a batch of various infectious diseases (Colborn *et al*, 2006). We developed our method specifically to quantify different genotypes over time.

In addition to demonstrating sensitivity of the qPCR method, the experimental co-infections showed evidence of density dependent dynamics. Previously, rodent malaria model infections were used to investigate experimental co-infections of different *Plasmodium chabaudi* genotypes (Taylor *et al.*, 1997). Recently, a qPCR method was developed to investigate density dependent parasite-parasite interactions in the rodent malaria experimental infections, using an analogous gene for genotyping (Cheesman *et al.*, 2003). We used recently isolated, single-clone, *P. falciparum* field isolates to make experimental genotype mixtures and found that density was associated with genotype dynamics. This method of qPCR appears effective and can be utilized in conjunction with traditional PCR and CGE to track genotypes *in vitro* and in the field.

MATERIALS AND METHODS

Sample collection

Isolates in this study were collected from 2003-2009 within the State of Loreto in communities surrounding the central city of Iquitos, Peru. Study design, human subjects ethical approval (2003-2005), sample collection and field site description were previously described (Branch *et al.*, 2005). All protocols were reviewed and approved by the Institutional Review Boards at New York University and the Peruvian Ministry of Health Institutes of National

Health during the period of 2005-2009 and the University of Alabama prior to 2005. We detected *Plasmodium falciparum* malaria infections by weekly active case sampling. Infections were treated immediately if the participant had a fever (reported or measured >37.5^{o}C) or a hematocrit level of >30.0%. Approximately 30% remained asymptomatic for seven days at which time asymptomatic infections were treated (Branch *et al.*, 2005; Torres *et al.*, 2008). In brief, when a positive case (asymptomatic or symptomatic) was detected, 5.0-8.0mL of whole blood was taken intravenously before treatment, and stored in a vacutainer containing EDTA. DNA was extracted from 200.0uL of whole blood for genotype characterization and the remaining blood was used for the *in vitro* culture of *P. falciparum* parasites. After expanding cultures in our Peruvian field site, they were frozen in a glycerolyte solution and transported on dry ice to New York University Medical Center.

P. falciparum isolate culture and competition assay design

P. falciparum isolates K1 (MRA-159), FCR3 (MRA-737) and Dd2 (MRA-150) were obtained from the MR4 repository. These are commonly used reference strains with robust growth and similar multiplication rates. Clones designated 6390 and MZ1187 were isolated in our field site near Iquitos, Peru. After cultivation of field isolates for seven consecutive days, they were cryo-preserved and transported on dry ice. Prior to experimentation, field isolates were cultured for two weeks in continuous culture to ensure parasite viability between generations. Isolates were cultured according to previously described methods (Trager & Jensen, 1976) at 5% hematocrit using Albumax II (Invitrogen, CA) as a substitute for human serum and gassed in a modular incubator chamber (Billups-Rothenberg inc., CA). Uninfected, leukocyte reduced O+ erythrocytes, obtained from anonymous donors were washed three times in RPMI. Donor erythrocytes were consistent between experiment duplicates. Gelatin flotation was used to synchronize cultures during expansion (Goodyer *et al.*, 1994). It was not possible to synchronize Dd2 using gelatin flotation. Prior to competition assays, cultures were synchronized using a sorbitol lysis technique (Lambros & Vanderberg, 1979). After experimentation, cultures were confirmed negative for *Mycoplasma spp*. and *Acholeplasma spp.* contamination using PCR (Tang *et al.*, 2000).

During the first *in vitro* experiment, 4.0mL cultures were grown in duplicate within tissue culture treated culture dishes (Corning, Lowell, MA). Media was changed daily. The fastest growing clone, Dd2, was previously determined to expand at an approximate rate of four times per division, so to maintain a parasitemia less than four percent over 10 days, one fourth of all infected erythrocytes were replaced every other day. Erythrocytes from the

same donor were added throughout the experiment. Parasitemia was counted daily using microscopy and 0.5mL of sample taken in 24-hour intervals for DNA extraction and subsequent qPCR analysis (Fig. 2).

In the second *in vitro* experiment (Fig. 3), cultures were grown in duplicate in multi-well cell culture plates for four days with daily media changes. Giemsa stained slides were made every day and 0.5mL of culture was taken every other day for DNA extraction starting with 4mL cultures. No additional erythrocytes were added after day zero during the second experiment. Unlike the first experiment, FCR3, which has a greater multiplication rate than the field isolates, was permitted to reach a maximum density since infected erythrocytes were not replaced.

PfMSP1-B2 amplification by nested PCR: gel and capillary electrophoresis

Amplification of sufficient target gene(s) for visualization on a 1.5% Invitrogen UltraPure™ agarose gel with ethidium bromide requires a nested amplification. Genomic DNA from reference strains 7C424, Dd2 (Mad20 genotypes), K1, 3D7 (K1 genotypes), RO33 and 7G8 (RO33 genotypes) were obtained from MR4 and used as PCR controls. A region flanking *Pf*MSP1-B2 is first amplified using external primers 5' AAG CTT TAG AAG ATG CAG TAT TGA C and 3' ATT CAT TAA TTT CTT CAT ATC CAT C (Branch, 2001). The internal primers are specific to three allelic families: K1-5' GAA ATT ACT ACA AAA GGT GCA AGT G, K1-3' AGA TGA AGT ATT TGA ACG AGG TAA AGT G; Mad20-5' GCT GTT ACA ACT AGT ACA CC, Mad20 3' TGA ATT ATC TGA AGG ATT TGT ACG TC; RO33-5' GCA AAT ACT CAA GTT GTT GTT GCA AAG C, RO33-3' AGG ATT TGC AGC ACC TGG AGA TCT. Reactions were performed on an Eppendorf Mastercycler (Westbury, NY).

Capillary gel electrophoresis was conducted according to methods described previously (Liljander *et al.*, 2009) with minor modifications. Following nested PCR reactions, products from independent Mad20, K1 and RO33 reactions were analyzed on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster, CA). K1 probes were labeled with NED™ (yellow), MAD 20 with PET® (red), and RO33 with VIC® (green) fluorophores to allow multiplexing of reactions. Allele length was determined using an internal size standard (GeneScan 500 LIZ Size standard, Applied Biosystems). Data was analyzed using GeneMapper v4.0 software.

QPCR of PfMSP1-B2

Taqman probes® for quantitative PCR were designed in order to anneal with conserved polymorphisms specific to each allele allowing product-specific detection and multiplexing of reactions. Reactions for qPCR implemented the same internal primers used during nested PCR at a final concentration of 900.0nM. K1 and Mad20 alleles, from the most common size polymorphisms (170 base pairs (bp), 195bp, 200bp and 210bp, respectively)

were sequenced. Dual labeled probe oligo sequences were chosen through the alignment of 11 K1-170 (GenBank accession numbers: FJ612009–FJ612019), 18 K1-195 (FJ612020–FJ612037), 2 Mad20-200 (FJ612038 and FJ612039) and 24 Mad20-210 (FJ612040–FJ612063) allele sequences. K1-probe 6FAM CAA GTG GTA CAA GTC CAT CAT CTC GTT C MGBFQ and Mad20-probe VIC CAG GTG GTT CAG TTA CTT CAG GTG GTT CAG MGBNGQ were used at a final concentration of 250.0nM. Rotor-Gene multiplex kit (Qiagen, CA) was used at a final buffer concentration of 1X. 25.0uL reactions performed in triplicate contained 5.0uL of DNA each extracted using a DNeasy Blood and Tissue Kit (Qiagen, CA) according to manufacturer's instructions. Amplification was performed on a Rotor-Gene, RG-3000, qPCR machine (Corbet research, CA) and data was processed using Rotor Gene 6 software. Temperature cycling occurred as follows: denature for 10.0 minutes at 95.0^{o}C; cycle 40 times at 95.0 ^{o}C for 10 seconds, anneal at 62.0 ^{o}C for 15 seconds and extend at 72.0 ^{o}C for 25 seconds; extend for five minutes at 72.0^{o}C. Ct values were converted to ng of DNA /uL through standard curve analysis of K1 and Dd2 genomic DNA provided and quantified by MR4. Parasitemia was also associated with Ct values using microscopy counts from cultured isolates as standards.

SYBR green fluoresces when bound to double-stranded DNA, which allows non-specific detection of DNA amplification during qPCR. Internal primers described above were used at a final concentration of 500.0nM in separate reactions. SYBR buffer (Applied Biosystems, Warrington, UK) was used at a final 1x concentration. 25.0uL reactions contained 5.0uL of DNA and were performed in triplicate.

RESULTS

A new method of quantitative PCR

Quantitative PCR was the most sensitive method for detecting low density alleles compared with gel and capillary electrophoresis; however, it is limited by its inability to determine sequence length (Table 1). Mixed K1-K1 and Mad20-Mad20 complex infections of differing base pair length appear as single clone infections. Within the 79 non-cultured, clinical samples tested, qPCR detected 17 complex infections whereas CGE detected seven and gel electrophoresis detected four (Table 1). In seven of the samples, there was no detectable amplification of product using CGE or gel electrophoresis, but alleles were detected in all samples using qPCR. No RO33 alleles were detected using CGE or gel electrophoresis. Different CGE and gel electrophoresis methods were not tested for variation in sensitivity. Using SYBR green, non-specific binding with human DNA and primer-dimer formation

produced a separate melt peak occurring between 72-73^{O}C. It was also not possible to multiplex reactions using this method, which leads to increased requirements for reagents and time compared with the Taqman Probe® technique.

The Taqman® multiplex approach was verified by combining genomic DNA from K1 and 7C424 reference strains in proportions of 0:1, 1:4, 2:3, 1:1, 3:2, 4:1 and 1:0. Data for FAM and VIC fluorescence channels yielded a standard curve with R^2 >0.99 and an efficiency (E) > 0.98, which was similar to R^2 and E values obtained during separate ten-fold dilutions of either independent DNA standard, validating the multiplex assay (data not shown).

Interactions in vitro, considering reference laboratory strains:

We tested for the loss of either clone during ten days of co-culture when originally mixed in proportions of 0:1, 1:19, 1:4, 1:1, 4:1, 19:1 and 1:0. Controls started with the same parasitemia (1.0%) as the mixture group's total (e.g. 0.5% for each clone in the 50:50 mixture). Growth of each control group was multiplied by the fraction of same-genotype clones in each mixture and graphed as dotted lines (Fig. 2). Fig. 2 plots the growth of each individual clone in a mixed culture, represented by solid lines in comparison with adjusted controls. By graphically representing adjusted growth rates of control cultures with respective clones in experimental groups, it is possible to directly compare the growth of each clone when they are in competition with genetically different verses single clone growth.

Dd2 parasites grew more quickly and had a shorter life cycle than K1 parasites. Dd2 and K1 control cultures had the same overall parasitemia as the respective clones in mixed cultures during the first 48 hours. Likewise, the growth of controls was statistically the same as mixed groups during this time (Fig. 2). After the first round of replication, the adjusted parasitemia for Dd2 controls was lower than Dd2 in mixed cultures (Fig. 2). This was because K1 grows more slowly than Dd2, so Dd2 parasites in mixed cultures were able to outgrow their single clone counterpart even though the total density of both cultures was the same. On day ten we saw a dramatic increase in the proportion of Dd2 relative to K1 in mixed cultures. Dd2 has a shorter lifecycle than K1, and by day ten Dd2 had undergone an extra round of division. Supporting this further, K1 controls relative to K1 mixed cultures had the opposite trend of Dd2. These data suggest that growth was mediated by the additive density of all clones in a culture as opposed to genotype specific densities.

The increase in ng/uL of DNA from day zero to day one results from the replication of DNA during the trophozoite stage. This pattern is repeated on a 48 hour interval. The difference in average DNA content increase between K1 and Dd2 during trophozoite development is likely explained by variation in the number of merozoites

per schizont between these two strains. We found that Dd2 had an average of 30 merozoites/schizont whereas K1 had 24. It was not possible to represent parasitemia on the y-axis of Fig. 2 due to this increase in copy number per parasite during development. However, when sampling is conducted every other day during the same stage of development, parasite counts can be correlated with Ct values.

Interactions in vitro, considering native Peruvian strains:

Next, we mixed various K1 genotype clones (K1, 6390 and MZ1187) with a Mad20 genotype clone (FCR3) and allowed the cultures to grow exponentially until FCR3 reached a maximum density and parasites failed to consistently mature into late stage trophozoites, thus growth significantly decreased. The time to maximum parasite density was consistent in all experiments. We selected these isolates based on their genotypes (single clone) and being outside the host for less than fifteen generations, which permits the study of density sensing using clones that have not lost natural phenotypes due to laboratory propagation.

Unlike the previous experiment, single clone controls started with the same parasitemia (0.5%) as each respective clone in the 50:50 mixtures. Under these conditions we found that clones grew more quickly alone than when they were mixed with FCR3 (Fig. 3). This effect was independent of multiplication rate and isolate region of origin. We also found that all three clones suppressed FCR3 to the same extent despite growing at different rates (not shown). Therefore, the overall parasitemia in mixtures varied according to the growth of K1 genotype clones. In both experiments, the additive density of all clones present in a given culture influenced growth rate.

DISCUSSION

qPCR of *Pf*MSP1-B2 can be used to explore complex infections in greater detail than with other techniques since it is quantitative. The conventional nested, end-point PCR method is long, prone to error and stochastic; moreover, band intensity is a poor indicator of DNA concentration in the original sample during nested PCR. Our qPCR method provides quantitative data and increased sensitivity. However, while it discriminates between allelic families (e.g. MAD20, K1 and RO33), it is unable to characterize sub-families (e.g. K1-170 and K1-195), which represent allele length in base pairs. This limitation makes qPCR appropriate for controlled experiments where parasite genotype is selected for or clinical isolates where methods like gel electrophoresis, CGE, MSP-2 genescan-based genotyping are first used to establish the lack of sub-family alleles (Falk *et al.*, 2006). Our *Pf*MSP1-B2 qPCR was developed as a high throughput assay specifically to evaluate co-infections of *P. falciparum* infections. Colborn

et al. developed a qPCR method to detect malaria infection when testing batches of samples for potential bioterroristic agents, but this was not intended to be genotype specific (Colborn *et al.*, 2006). *Pf*MSP1-B2 has been shown to be a valuable epidemiologic marker when comparing one *P. falciparum* genotype to another. This quantitative method can be used to study *in vivo* intra-species growth competition dynamics. In conjunction with CGE and gel electrophoresis *Pf*MSP1-B2 methods, the qPCR method can also be used to study natural infection in field samples.

The application of qPCR to study within-host competition between distinct clonal populations within complex infections may lead to a better understanding of disease severity, drug treatment, vaccine efficaciousness, and malaria transmission (Smith *et al.*, 1999; Farnert *et al.*, 2008; Sutton *et al.*, 2009). For example, drug treatment of complex infections leads to the out-competition of drug-sensitive clones by drug resistant ones (Harrington *et al.*, 2009). It has been shown that overall density increases after treatment due to the expansion of drug resistant clones "released from competitive facilitation" (Harrington *et al.*, 2009). qPCR of *Pf*MSP1-B2 could be used alongside other methods to accurately enumerate and quantify clones in an infection during vaccine trials where a host may have genotype-specific immunity.

Using a murine-*Plasmodium chabaudi* model and genotype-specific qPCR method analogous to *Pf*MSP1-B2, it was shown that within-host competition between clones occurs in complex infections (Bell *et al.*, 2006; de Roode *et al.*, 2004; de Roode *et al.*, 2005). This competition was shown to select for virulence and increased transmission (Bremermann & Pickering, 1983; Mosquera & Adler, 1998; Read & Taylor, 2001). By using an *in vitro* system we are able to study inter-clone competition while restricting host immunity to intrinsic erythrocyte factors. It also permits the study of *P. falciparum* competition as opposed to using murine surrogate models. Within this *in vitro* system, we found that density suppression is non-specific, that is, there was no evidence for targeted growth inhibition between genetic types. These results suggest that the total parasite density governs culture expansion in a non clone-specific manner. However, these observations apply only to parasite behavior *in vitro* since the host may influence parasite interactions.

Studying COI, genotype dynamics and parasite density, many longitudinal field studies in high transmission have found evidence for non-random genotype dynamics suggesting interactions during *P. falciparum* genotype co-infections (Smith *et al.*, 1999; Bruce *et al.*, 2000; Farnert *et al.*, 2008). Whether or not this is *P.*

falciparum parasite-parasite interaction is open to interpretation. Density regulation of parasitemia was first suggested as an explanation for the inverse oscillation observed when more than one malaria species were present in a co-infection (Bruce *et al.*, 2000). In high transmission, genetically diverse *P. falciparum* transmission settings, the dynamics could be attributed to the high rate of super-infection, overlapping complex co-infections, randomly inoculating different genotypes or be attributed to a stochastic loss of detected clones over time. Even if there are co-infection interactions, this could be indirect through resource competition, immune-mediated variant replacement and/or immune evasion (Branch *et al.* 2001; Bell *et al.*, 2006; Farnert *et al.*, 2008; Orjuela-Sanchez *et al.*, 2009; Liljander *et al.*, 2011).

In our *in vitro* competition assays, we found that the co-culture of genetically distinct clones was associated with decreased growth rate. The suppression of growth may have been caused by (1) resource limitations (2) increased production of metabolic waste or (3) density regulation. The influence of limiting resources on parasite growth cannot be negated (Hellriegel, 1992; Mutai & Waitumbi), but we believe that this does not completely explain the suppression of growth in *P. falciparum* cultures containing a low number of infected erythrocytes (0.5-4.5% in this experiment) undergoing daily media changes. Cultures grown at low hematocrit can reach a parasitemia near 100%, so erythrocytes are not a likely limitation on growth for cultures <4.0% parasitemia, but nutrient depletion or secretion of harmful metabolites may slow growth. The percent reduction in growth of clones mixed with FCR3 relative to single clone controls by day four was: K1 (57.5%), 6390 (58.9%) and MZ1187 (68.8%) and the overall culture parasitemias, including FCR3 in the mixed cultures, reached 3.5, 1.6 and 2.1, respectively (Fig. 3). Despite having a lower overall parasitemia than the K1/FCR3 mixed culture, 6390/FCR3 and MZ1183/FCR3 had greater reduction in growth. If the rate of metabolite production is consistent across clones, our results suggest an alternative cause for growth limitation.

Currently, the only proposed mechanism for *P. falciparum* genotype co-infection density sensing supported by experimental data is via a density modulated apoptosis pathway (Mutai & Waitumbi, 2009). Another possibility is reduction in parasite density due to the conversion of trophozoites, required for asexual replication, into gametocytes. Gametogenesis might be regulated by small diffusible molecules (Dyer & Day 2003). Others have reported density dependent switching of parasites from the blood-stage trophozoite stage to the gametocyte stage (Richard Carter, personal communication), which might result in a lower overall parasite density count.

Competition between Peruvian isolates with reference strains supports the possibility of a density dependent interaction between genetically diverse mixed clone cultures. The growth of laboratory and field isolates was dependent on the additive density of clones in a culture, not the individual clone densities. This suggests that density is regulated by a soluble factor that is not clone specific.

We will continue to investigate density and genotype dynamics by testing permutations to further support our hypothesis. *In vivo* studies of genotype competition are possible, but even in low transmission, the qPCR method would have to be joined with the traditional methods to be certain that sub-alleles are being considered. If there are density dependent interactions *in vivo*, targeting the density-sensing pathway could be a favorable chemotherapeutic target. In conclusion, qPCR of *Pf*MSP1-B2 could potentially enhance knowledge gained from vaccine/drug trials, model influences on parasite population evolution, and advance efforts to reduce transmission of malaria worldwide.

ACKNOWLEDGMENTS

Field sample collection was made possible by the dedicated work of many members of the Malaria Immunology and Genetics in the Amazon, MIGIA Project in Iquitos, Peru. We thank Dr. Lastenia Ruiz, Peru laboratory director, for her coordination and facilitation of culture in Peru and shipment of clones to NYU for further testing. The authors thank Drs. Julian Rayner and Marian Nyako for sharing their expertise in *P. falciparum* culture.

FINANCIAL SUPPORT

Funding for sample collection and genetic analysis was from RO1 grant AI064831. *P. falciparum* culture was supported by Fogarty International (FIRCA)-RO3-TW008064 funding.

REFERENCES

Bei, A. K., Membi, C. D., Rayner, J. C., Mubi, M., Ngasala, B., Sultan, A. A., Premji, Z. and Duraisingh, M. T. (2007). Variant merozoite protein expression is associated with erythrocyte invasion phenotypes in Plasmodium falciparum isolates from Tanzania. *Molecular and Biochemical Parasitology,* **153**(1), 66-71. doi: S0166-6851(07)00034-5 [pii] 10.1016/j.molbiopara.2007.01.007.

Bell, A. S., de Roode, J. C., Sim, D. and Read, A. F. (2006). Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution,* **60**(7), 1358-1371.

Branch, O.H., Casapia, M.W., Gamboa, D.V., Hernandez, J.N., Alava, F.F., Roncal, N., Alvarez, E., Perez, E.J. and Gotuzzo, E. (2005). Clustered local transmission and asymptomatic Plasmodium falciparum and Plasmodium vivax malaria infections in recently emerged, hypoendemic Peruvian Amazon community. *Malaria Journal,* **4**(27).

Branch, O.H., Takala, S., Kariuki, S., Nahlen, I.B., Kolczak, M.K. and Lal A.A.* (2001). Plasmodium falciparum Genotypes, Low Complexity of Infection, and Resistance to Subsequent Malaria in Participants in the Asembo Bay Cohort Project. *Infection and Immunity,* **69**(12).

Branch, O.H., Sutton, P.L., Barnes, C., Castro, J.C., Hussin, J., Awadalla, P. and Hijar, G. (2011). Plasmodium falciparum genetic diversity maintained and amplified over 5 years of a low transmission endemic in the Peruvian Amazon. Molecular Biology and Evolution,**28**(7), 1973-86.

Bremermann, H. J. and Pickering, J. (1983). A game-theoretical model of parasite virulence. *Journal of Theoretical Biology,* **100**(3), 411-426. doi: 0022-5193(83)90438-1.

Bruce M.C., Galinski M.R., Barnwell J.W., Donnelly C.A., Walmsley M., Alpers M.P., Walliker D. and Day K.P. (2000). Genetic diversity and dynamics of plasmodium falciparum and *P. vivax* populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology,* **121**(Pt 3), 257-272.

Certa, U., Rotmann ,D., Matile, H., Reber-Liske, R. (1987). A naturally occurring gene encoding the major surface antigen precursor p190 of *Plasmodium falciparum* lacks tripeptide repeats. *EMBO Journal*, **6**(13), 4137-4142.

Cheesman, S.J., de Roode, J.C., Read, A.F. and Carter R. (2003) Real-time quantitative PCR for analysis of genetically mixed infections of malaria parasites: technique validation and applications. *Molecular and Biochemical Parasitology,* **131**(2), 83-91.

Chenet, S. M., Branch, O. H., Escalante, A. A., Lucas, C. M. and Bacon, D. J. (2008) Genetic diversity of vaccine candidate antigens in Plasmodium falciparum isolates from the Amazon basin of Peru. *Malaria Journal,* **27**(7), 93. doi: 10.1186/1475-2875-7-93

Colborn, J. M., Koita, O. A., Cisse, O., Bagayoko, M. W., Guthrie, E. J. and Krogstad, D. J. (2006). Identifying and quantifying genotypes in polyclonal infections due to single species. *Emerging Infectious Diseases,* **12**(3), 475-482.

De Roode, J.C., Culleton, R., Bell, A.S. and Read, A.F. (2004) Competitive release of drug resistance following drug treatment of mixed Plasmodium chabaudi infections. *Malaria Journal,* **3**(33). doi: 10.1186/1475-2875-3-33

De Roode, J. C., Pansini, R., Cheesman, S. J., Helinski, M. E., Huijben, S., Wargo, A. R., Bell, A. S., Chan, B. H., Walliker, D. and Read, A. F. (2005) Virulence and competitive ability in genetically diverse malaria infections. *Proceedings of the National Academy of Sciences of the United States of America,* **102**(21), 7624-7628. doi: 0500078102 [pii] 10.1073/pnas.0500078102.

Dyer, M. and Day, K.P. (2003) Regulation of the rate of asexual growth and commitment to sexual development by diffusible factors from *in vitro* cultures of *Plasmodium falciparum*. *The American Journal of Tropical Medicine and Hygiene,* **68**(4), 403-409.

Falk, N., Maire, N., Sama, W., Owusu-Agyei., Smith, T., Beck, Hans-Peter., Felger, I. Comparison of PCR-RFLP and Genescan-based genotyping for analyzing infection dynamics of *Plasmodium falciparum. The American Journal of Tropical Medicine and Hygiene,* **74**(6), 944-950.

Farnert, A., Arez, A. P., Babiker, H. A., Beck, H. P., Benito, A., Bjorkman, A., Bruce, M. C., Conway, D. J., Day, K. P., Henning, L., Mercereau-Puijalon, O., Ranford-Cartwright, L. C., Rubio, J. M., Snounou, G., Walliker, D., Zwetyenga, J. and do Rosario, V. E. (2001). Genotyping of Plasmodium falciparum infections by PCR: a comparative multicentre study. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **95**(2), 225-232.

Farnert, A., Lebbad, M., Faraja, L. and Rooth, I. (2008). Extensive dynamics of Plasmodium falciparum densities, stages and genotyping profiles. *Malaria Journal,* **7**(241). doi: 1475-2875-7-241 [pii] 10.1186/1475-2875-7-241.

Goodyer, I. D., Johnson, J., Eisenthal, R. and Hayes, D. J. (1994). Purification of mature-stage Plasmodium falciparum by gelatine flotation. *Annals of Tropical Medicine and Parasitology,* **88**(2), 209-211.

Harrington, W. E., Mutabingwa, T. K., Muehlenbachs, A., Sorensen, B., Bolla, M.C., Fried, M. and Duffy, P. E. (2009). Competitive facilitation of drug-resistant Plasmodium falciparum malaria parasites in pregnant women who receive preventive treatment. *Proceedings of the National Academy of Sciences of the United States of America,* **106**(22), 9027-9032. doi: 0901415106 [pii] 10.1073/pnas.0901415106.

Hellriegel, B. (1992). Modelling the immune response to malaria with ecological concepts: short-term behaviour against long-term equilibrium. *Proceedings of the Royal Society B: Biological Sciences*, **250**(1329), 249-256. doi: 10.1098/rspb.1992.0156.

Huijben, S., Sim, D.G., Nelson, W.A. and Read, A.F. (2011). The fitness of drug-resistant malaria parasites in a rodent model: multiplicity of infection. *Journal of Evolutionary Biology* **24**(11), 2410-2422. doi: 10.1111/j.1420-9101.2011.02369.x.

Lambros, C. and Vanderberg, J. P. (1979). Synchronization of Plasmodium falciparum erythrocytic stages in culture. *The Journal of Parasitology,* **65**(3), 418-420.

Liljander, A., Bejon, P., Mwacharo, J., Kai, O., Ogada, E., Peshu, N., Marsh, K. and Farnert, A. (2011). Clearance of asymptomatic P. falciparum Infections Interacts with the number of clones to predict the risk of subsequent malaria in Kenyan children. *PLoS One,* **6**(2), e16940. doi: 10.1371/journal.pone.0016940.

Liljander, A., Wiklund, L., Falk, N., Kweku, M., Martensson, A., Felger, I. and Farnert, A. (2009). Optimization and validation of multi-coloured capillary electrophoresis for genotyping of Plasmodium falciparum merozoite surface proteins (msp1 and 2). *Malaria Journal,* **8**(78), doi: 1475-2875-8-78 [pii] 10.1186/1475-2875-8-78.

McBride, J. S. and Heidrich, H. G. (1987). Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated Plasmodium falciparum merozoites form an antigenic complex. *Molecular and Biochemical Parasitology,* **23**(1), 71-84. doi: 0166-6851(87)90189-7.

Mosquera, J. and Adler, F. R. (1998). Evolution of virulence: a unified framework for COInfection and superinfection. *Journal of Theoretical Biology,* **195**(3), 293-313. doi: S0022-5193(98)90793-7 [pii] 10.1006/jtbi.1998.0793.

Mutai, B. K. and Waitumbi, J. N. (2010). Apoptosis stalks Plasmodium falciparum maintained in continuous culture condition. *Malaria Journal,* **9** (Suppl 3), S6. doi: 1475-2875-9-S3-S6 [pii] 10.1186/1475-2875-9-S3-S6.

Orjuela-Sanchez, P., Da Silva-Nunes, M., Da Silva, N. S., Scopel, K. K., Goncalves, R. M., Malafronte, R. S. and Ferreira, M. U. (2009). Population dynamics of genetically diverse Plasmodium falciparum lineages: community-based prospective study in rural Amazonia. *Parasitology*, **136**(10), 1097-1105. doi: 10.1017/S0031182009990539.

Robert, F., Ntoumi, F., Angel, G., Candito, D., Rogier, C., Fandeur, T., Sarthou, J.L., Mercereau-Puijalon, O. (1996). Extensive genetic diversity of Plasmodium falciparum isolates collected from patients with severe malaria in Dakar, Senegal. Transactions of the Royal Society of Tropical Medicine and Hygiene, **190**(6), 704-711.

Sutton, P. L., Neyra, V., Hernandez, J. N. and Branch, O. H. (2009). Plasmodium falciparum and Plasmodium vivax infections in the Peruvian Amazon: propagation of complex, multiple allele-

type infections without super-infection. *The American Journal of Tropical Medicine and Hygiene,* **81**(6), 950-960. doi: 81/6/950 [pii] 10.4269/ajtmh.2009.09-0132.

Sutton P.L., Clark, E.H., Silva, C., Branch, O.H. (2010). The Plasmodium falciparum merozoite surface protein-1 19 KD antibody response in the Peruvian Amazon predominantly targets the non-allele specific, shared sites of this antigen. *Malaria Journal*, **9**(3). doi: 1475-2875-9-3 [pii]10.1186/1475-2875-9-3

Smith, T., Felger, I., Kitua, A., Tanner, M. and Beck, H.P. (1999). Dynamics of multiple Plasmodium falciparum infections in infants in a highly endemic area of Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93** (Suppl 1), 35-39.

Tanabe, K., Mackay, M., Goman, M., Scaife, J.G. (1987). Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *Journal of Molecular Biology,* **195**(2), 273-287.

Tang, J., Hu, M., Lee, S. and Roblin, R. (2000). A polymerase chain reaction based method for detecting Mycoplasma/Acholeplasma contaminants in cell culture. *Journal of Microbiological Methods,* **39**(2), 121-126. doi: S0167-7012(99)00107-4.

Taylor, L. H., Walliker, D. and Read, A. F. (1997). Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones. *Proceedings of the Royal Society B: Biological Sciences,* **264**(1383), 927-935. doi: 10.1098/rspb.1997.0128.

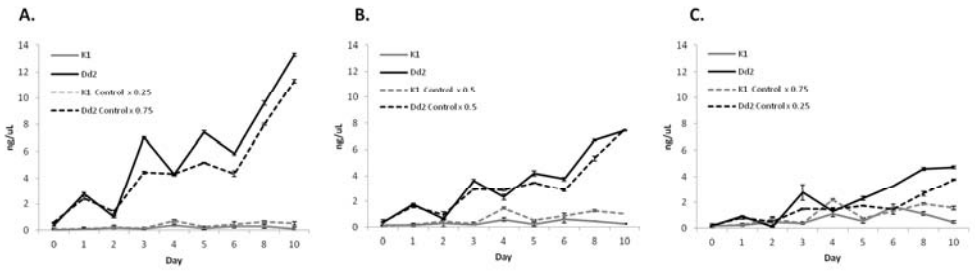
Torres, K. J., Clark, E. H., Hernandez, J. N., Soto-Cornejo, K. E., Gamboa, D. and Branch, O. H. (2008). Antibody response dynamics to the Plasmodium falciparum conserved vaccine candidate antigen, merozoite surface protein-1 C-terminal 19kD (MSP1-19kD), in Peruvians exposed to hypoendemic malaria transmission. *Malaria Journal,* **7**(173), doi: 1475-2875-7-173 [pii] 10.1186/1475-2875-7-173.

Trager, W. and Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science,* **193**(4254), 673-675.

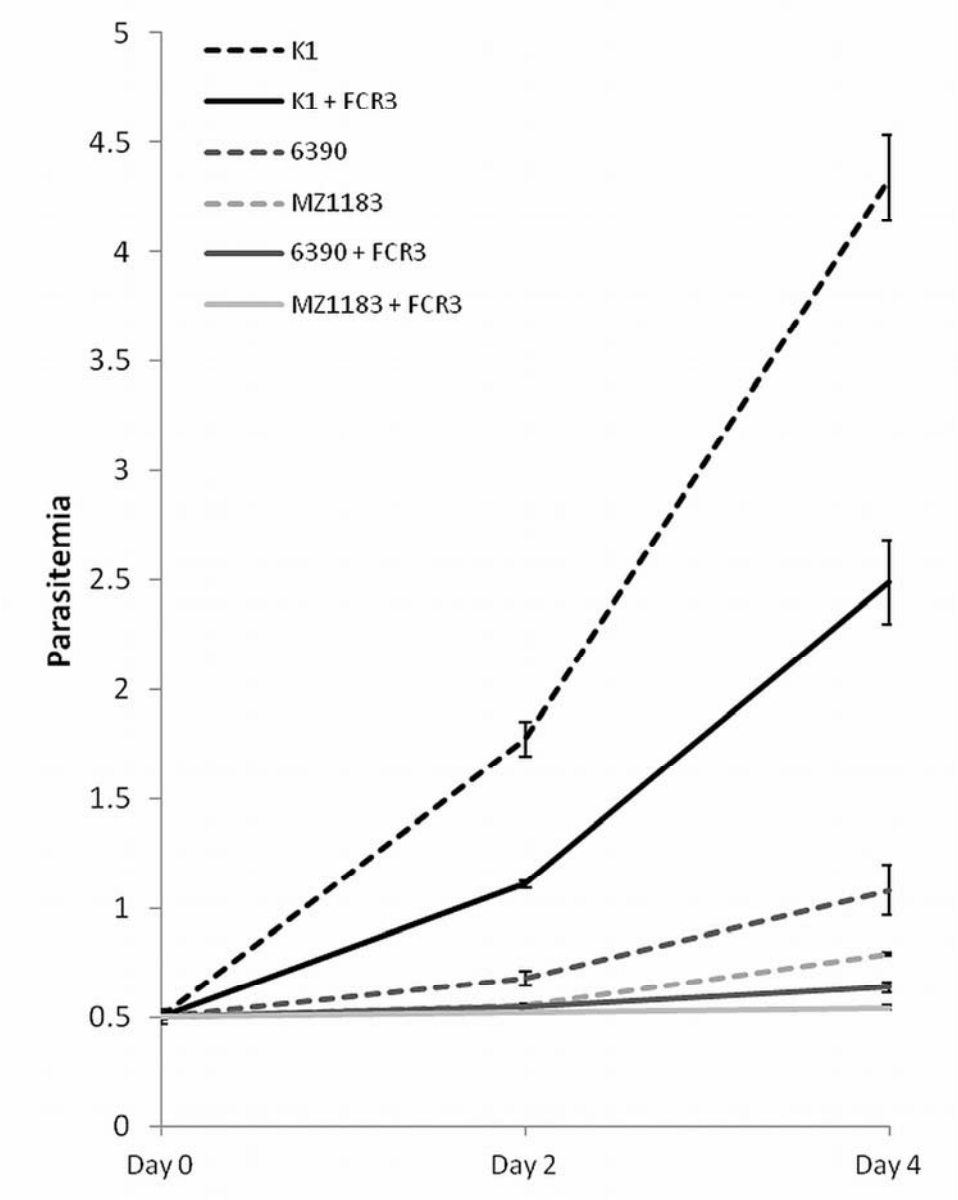
448 **Trager, W. and Jensen, J. B.** (1997). Continuous culture of Plasmodium falciparum: its impact on malaria
449 research. *International Journal for Parasitology,* **27**(9), 989-1006. doi: S0020751997000805.

Features	Gel Electrophoresis	CGE	qPCR
determination of sub-family at resolution of <30 base	yes	most accurate	no
multiplex	no	no	yes
DNA volume/reaction	1.2	1.2	5
quantification	no	no	yes
expense	second most expensive	most expensive	least expensive
time to completion	second longest	longest	shortest
Single clone Mad20 isolates detected out of 79	34	29	24
Single clone K1 isolates detected out of 79	34	35	37
Mad20 + K1 complex infections detected out of 79	4	7	17
RO33 alleles detected out of 79 samples	0	0	not tested
Total alleles detected	76	78	95
sensitivity	least	second most	most

Compares gel electrophoresis, capillary gel electrophoresis (CGE) and quantitative PCR (qPCR) methods for detecting MSP1-B2 alleles.
73x32mm (600 x 600 DPI)



Laboratory strains in mixed clone assays. Results were independent of clonal proportions (A: K1 25% with Dd2 75%, B: K1 50% with Dd2 50%, and C: K1 75% with Dd2 25%). Solid lines represent clone growth from mixed cultures. Dotted lines are adjusted values from single-clone controls. Dd2 single-clone controls had a greater overall parasitemia (not shown) but a lower adjusted parasitemia than Dd2 in mixture groups. K1 showed the opposite trend. This is explained by a density dependent growth rate and the shorter life-cycle and faster multiplication rate of Dd2 verses K1.
165x47mm (300 x 300 DPI)



Native Peruvian strains in competition assays; comparative growth of clones when alone versus co-cultured with FCR3. FAM fluorescence, shows the growth of K1 genotype clones K1, 6390 and MZ1183. Solid line colors illustrate clones in a 50/50 co-culture with FCR3 whereas dotted lines represent single clone controls.
80x100mm (300 x 300 DPI)