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Competition between *Plasmodium falciparum* strains in clinical infections during *in vitro* culture adaptation

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Abstract

We evaluated the dynamics of parasite populations during *in vitro* culture adaptation in 15 mixed *Plasmodium falciparum* infections, which were collected from a hypoendemic area near the China-Myanmar border. Allele types at the *msp1* block 2 in the initial clinical samples and during subsequent culture were quantified weekly using a quantitative PCR method. All mixed infections carried two allele types based on the msp1 genotyping result. We also genotyped several polymorphic sites in the *dhfr*, *dhps* and *mdr1* genes on day 0 and day 28, which showed that most of the common sites analyzed were monomorphic. Two of the three clinical samples mixed at *dhps* 581 remained stable while one changed to wild-type during the culture. During *in vitro* culture, we observed a gradual loss of parasite populations with 10 of the 15 mixed infections becoming monoclonal by day 28 based on the *msp1* allele type. In most cases, the more abundant *msp1* allele types in the clinical blood samples at the beginning of culture became the sole or predominant allele types on day 28. These results suggest that some parasites may have growth advantages and the loss of parasite populations during culture adaptation of mixed infections may

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lead to biased results when comparing the phenotypes such as drug sensitivity of the culture-adapted parasites.

Keywords

Plasmodium falciparum; mixed infection; culture adaptation; in vitro competition; drug resistance; fitness cost

1. Introduction

Malaria remains a major public health problem in the tropical world. The recent substantial reduction in global malaria burden has motivated many malaria-endemic countries to consider and plan for malaria elimination (Feachem et al., 2010). As malaria control intensifies, there have been major changes in malaria epidemiology (Cotter *et al.*, 2013). Malaria involves intricate, tripartite interactions among humans, *Plasmodium* parasites and *Anopheles* mosquitoes. Thus, the overall reduced prevalence of malaria and increased proportions of vivax malaria in countries outside of Africa will affect the immune status of the host populations. Artemisinin-based combination therapies (ACTs), widely adopted for the treatment of falciparum malaria (White et al., 2014) and even vivax malaria (Sinclair et al., 2011), have played an important role in the reduction of malaria prevalence. However, the recent emergence in *Plasmodium falciparum* of resistance to artemisinin-related drugs in Southeast Asia (Dondorp et al., 2009; Noedl et al., 2008) is a great concern for malaria elimination. These factors will affect the intrahost competition, survival and transmission of the parasite strains, which ultimately influences the outcome of malaria epidemiology.

Human malarial infections, especially in hyperendemic areas, are often multiclonal, consisting of more than one genotype (Babiker et al., 1999; Druilhe et al., 1998; Soulama et al., 2009; Vafa et al., 2008). Even in areas of low endemicity and seasonality of malaria such as the Greater Mekong Subregion of Southeast Asia, mixed-species and mixed-strain infections are also common (Cui et al., 2003; Mayxay et al., 2001; Yuan et al., 2013). Mixed infections arise from inoculations of genetically diverse parasites by a single or multiple mosquitoes, which serve to generate genetic diversity of the parasite population. The number of parasite strains in a patient varies greatly and some could contain more than 15 clones (Juliano et al., 2010). Therefore, within-host competition between the co-infecting parasites can be intense and bears important evolutionary consequences. For immune-mediated apparent competition, immunity elicited by one parasite strain contributes to the suppression of population densities of the co-infecting strains (Raberg et al., 2006). Due to the highly polymorphic nature of parasite antigens, immunity against malaria parasites can be strain transcending, which may partially account for the temporal dynamics of parasite genotypes (Yuan et al., 2013).

Multiclonality and within-host competition also greatly affect the evolution and spread of drug resistance (Harrington et al., 2009; Wargo et al., 2007). Widespread use of antimalarial drugs has strongly affected the evolution of the malaria parasites. Drug resistance offers the parasites survival advantages in the presence of drug selection pressure, and drug selective sweep can lead to rapid spread of the resistant genotype across large geographical regions

(Roper et al., 2004; Wootton et al., 2002). At the same time, drug resistance is normally associated with a fitness cost (Rosenthal, 2013). In nature, the less fit drug-resistant parasites will gradually disappear from the parasite pool along time if active drug selection pressure is removed. For example, after the partial or complete withdrawal of chloroquine (CQ) from treatment of *P. falciparum* in many malaria-endemic countries, the prevalence of CQ-resistant parasites has declined in places such as Malawi and China's Hainan Island (Kublin et al., 2003; Wang et al., 2005). However, parasites sometimes acquire compensatory mutations to improve the fitness of the drug-resistant parasites. As a result, parasite fitness is not considerably reduced to affect the survival of the parasites in the absence of drug selection (Rodrigues et al., 2013). This may explain the persistence of parasites carrying the highly-resistant mutant genotypes of the dihydrofolate reductase (dhfr) and dihydropteroate synthetase (dhps) genes despite less extensive use of sulfadoxine and pyrimethamine (SP) (Marks et al., 2005). Taken together, switch of antimalarial drug use and emergence of new antimalarial resistance will have profound influences on malaria epidemiology.

Fitness cost is often measured using competition experiments to compare the growth between drug-resistant and wild-type parasites using either *in vitro* culture (Hayward et al., 2005; Peters et al., 2002) or animal models (Rodrigues et al., 2013; Walliker et al., 2005). The use of genetically defined transgenic parasites with similar genetic background and only a few mutations allows accurate determination of the fitness cost of individual mutations (Hayward et al., 2005; Peters et al., 2002). In nature, competition between parasite strains in clinical isolates is definitely much more complex. During culture adaptation of clinical isolates, some parasite clones are often lost and parasites with certain mutations in genes conferring drug resistance may be selected due to fitness advantages (Nsobya et al., 2008; Ochong et al., 2013). In this study, we collected *P. falciparum* clinical isolates in a malaria hypoendemic area and followed the dynamics of the parasite populations in the mixed infections during *in vitro* culture adaptation.

2. Materials and methods

2.1. Collection of parasite clinical samples

P. falciparum field isolates were collected in 2009 from symptomatic patients presenting with uncomplicated *P. falciparum* infections at a malaria clinic in Laiza Township near the China-Myanmar border. The current frontline ACT treatment for falciparum malaria in this region is dihydroartemisinin plus piperaquine. The human subject protocol for this study was approved by the Institutional Review Board of Kunming Medical University. Malaria infections were diagnosed by microscopic examination of Giemsa-stained thick and thin blood films. For *P. falciparum* cases, 1–3 ml of venous blood was collected in heparinized tubes and used for culture adaptation.

2.2. Genotyping of clinical isolates

For genotyping, parasite genomic DNA was extracted from the filter papers (day 0) by using a QIAamp DNA microkit (Qiagen, Germany) following the manufacturer's instructions. DNA was eluted in 80 μl of elution buffer. Parasite samples were then genotyped at three

polymorphic genes, *merozoite surface protein 1* (*msp1*), *msp2*, and *glutamate-rich protein* (*glurp*) as previously described (Meng et al., 2010).

2.3. Culture adaptation of mixed-population infections

To observe growth competition during *in vitro* culture, 15 clinical isolates with mixed parasite allele types were cultured *in vitro*. Frozen parasite samples were thawed, washed twice with RPMI 1640 medium at 37°C, and mixed with fresh type O human erythrocytes suspended at 5% hematocrit in a complete medium containing HEPES (5.94 g/liter), hypoxanthine (50 mg/liter), Albumax II (5 g/liter), RPMI 1640 (10.4 g/liter), gentamicin (5 mg/liter), $NaHCO_3$(2.1g/liter), and 6% AB human serum. Parasite cultures were kept at 37°C in 25 cm2 flasks (Costar) under a gas environment of 92% N, 5% CO_2, and 3% O_2. On day 8, 14, 21, and 28, 1 ml of the culture was collected and parasite DNA extracted from each culture using the QIAamp DNA microkit.

2.4. Quantitation of parasites based on *msp1* genotypes

To determine the relative abundance of individual parasite allele types in a sample, we used two allele types of the highly polymorphic block 2 of the *pfmsp1* gene, namely K1 and MAD20. To generate quantitative PCR (qPCR) standards for each of these allele types, the K1 and MAD20 fragments of *msp1* were amplified using primer pairs KF (AAATGAAGAAGAAATTACTACAAAAGGTGC) and KR (GCTTGCATCAGCTGGAGGGCTTGCACCAGA), and MF (AAATGAAGGAACAAGTGGAACAGCTGTTAC) and MR (ATCTGAAGGATTTGTACGTCTTGAATTACC), respectively. The PCR products were cloned into Pmd18 and the sequences were confirmed by sequencing. To generate qPCR standard curves, plasimds were purified, quantified, and serially (10×) diluted from a starting concentration of 3.5 × 107 copies/μl. qPCR was performed on an ABI7300 PCR machine in a final volume of 20 μl by using 2 μl of DNA, 0.6 μl each primer, and 10 μl of FastStart Universal SYBR Green Master Mix (Roche). Plasmid DNA was used to establish standard linear regression curves for the msp1 allele types.

Parasite DNA on day 0, 8, 14, 21 and 28 was used to quantify each of the two *msp1* types with the same K1 and MAD20 primer pairs by using the same qPCR procedure. Each allele form of *msp1* represents one homogeneous parasite population. The absolute number of each parasite population in a sample, represented by the copy number of each *msp1* allele as determined by qPCR (assuming *msp1* is a single-copy gene), was quantified using the standard curves established from plasmid DNA. The relative ratio of parasite populations carrying different *msp1* types in a mixed infection was then estimated based on the parasite numbers of individual populations in the sample.

2.5. Genotyping genes associated with drug resistance by PCR-restriction fragment length polymorphism (RFLP)

Parasite DNA on day 0 and 28 were used for the analysis of mutations in three genes associated with drug resistance in *P. falciparum*. Polymorphisms at codons 86, 184, 1034, and 1042 of *P. falciparum multidrug resistance 1* (*pfmdr1*) gene were determined by PCR-RFLP (Duraisingh et al., 2000). In the study parasite population, C59R and S108N

mutations in *pfdhfr* almost reached fixation, and A437G and K540E/N mutations also were also highly prevalent (>96%) (Yang et al., 2011). Therefore, we only analyzed polymorphisms at codons 51 and 164 of *pfdhfr* and codons 436 and 581 of *pfdhps* using a previously described method (Duraisingh et al., 1998).

3. Results

3.1. Allele dynamics during in vitro culture adaptation

Based on earlier genotyping results of clinical samples (Yuan et al., 2013), we selected 15 mixed infections to follow the dynamics of the parasite clones during *in vitro* culture adaptation. To reduce the complexity of the analysis, we only selected isolates with mixed alleles at the *msp1* locus, while *msp2* and *glurp* were both monomorphic (Table 1). The *msp2* genotypes were based on two allele types, IC/3D7 and FC27; five alleles were identified with FC27-300 being the predominant allele (8/15). There were seven *glurp* alleles and two allele sizes 800 bp and 900 bp accounted for 10/15 samples. Genotyping the *pfmsp1* block 2 revealed that all samples contained two parasite populations of the K1 and MAD20 allele types (Table 1). According to the lengths of the PCR fragments, there were two K1 alleles and three MAD20 alleles with a total of four allele type combinations; eleven samples had the K1-200:MAD20-200 combination.

We developed a qPCR method for the quantitation of the absolute abundance of each of the two allele type in a given sample using the standard linear regression curves generated from cloned fragments of the K1 and MAD20 allele types. This allowed us to calculate the ratio of the two allele types in each sample. The qPCR results of the day 0 DNA showed that in most (11/15) of the clinical samples the MAD20 allele type was more abundant than the K1 allele type with a K1:MAD20 ratio ranging from 1:3 to 1:123 (Table 1). Only in four samples was the K1 type more abundant than the MAD20 type with a K1:MAD20 ratio of 2-33:1. For all the 11 samples with higher proportions of the MAD20 allele, the cultures remained MAD20-predominant during the course of the study. Among them, the K1 type could not be detected by the qPCR method in three cultures by day 21 and in six cultures by day 28 (Table 1), which suggested that these cultures could be considered as monoclonal at these time points. In comparison, of the four clinical isolates containing higher proportions of the K1 type, three became monoclonal on day 28. However, in one clinical sample (A5) with a 2:1 K1:MAD20 ratio, the K1 allele type was rapidly outcompeted by the MAD20 allele type, and the culture became single infection of the MAD20 type on day 28. Taken together, of the 15 cultures that were mixed at the *msp1* locus on day 0, 10 (67%) were subsequently monoclonal on day 28. For the majority of the clinical isolates, the predominant allele type at the initiation of the culture remained predominant during the entire follow up period.

3.2. Selection of parasite drug resistance genotypes

At the beginning (day 0) and end (day 28) of the experiment, we also genotyped the parasites at several polymorphic sites in the *pfdhfr*, *pfdhps*, and *pfmdr1* gene to see whether certain drug resistant genotypes are selected during the culture period. Genotyping *pfdhfr* gene at the amino acid positions 51 and 164 showed that all 15 mixed infections had N51

and 164L. In *pfdhps*, all 15 samples had wild-type S436. In addition, 11 clinical samples had wild-type A581, one had 581G, and three were mixed at 581. After culturing for 28 days, two of the three mixed samples remained mixed, but one changed to A581, suggesting a potential fitness cost of the 581G. For *pfmdr1*, codons 86, 184 and 1034 were all monomorphic in the mixed clinical samples. Fourteen clinical samples contained N1042, and one was 1042D. All these sites were stable for 28 days.

4. Discussion

Within-host competition among malaria parasite strains greatly impacts the survivorship and transmission of individual parasite strains, and ultimately the spread of particular virulence types and drug resistance genotypes in parasite populations. Most studies on *in vitro* parasite competition performed thus far focused on genetically defined laboratory strains (Hayward et al., 2005; Peters et al., 2002) and clinical isolates from high-transmission areas where complexity of infection is high (Nsobya et al., 2008; Ochong et al., 2013). In this study, we assessed the competition between parasite populations in mixed clinical infections from a hypoendemic area near the China-Myanmar border during *in vitro* culture adaption. Our results showed that during the 28 day culture period, 10 of the 15 mixed infections grew to monoclonal. In most cases, the initial, more abundant allele types became the sole or predominant allele types on day 28, suggesting of probable growth advantages. While this is consistent with earlier studies showing selective growth of subpopulations of clinical samples during in vitro culture adaptation (Viriyakosol et al., 1994), some parasite isolates turned into monoclonal infections as rapidly as within 3 weeks.

MSP1 is essential for erythrocyte invasion and is involved in the initial attachment of merozoites to erythrocytes. MSP1 is the most abundant merozoite surface protein, and expressed as a large ~180 kDa protein but later proteolytically processed into fragments (McBride and Heidrich, 1987). Whereas the GPI-anchored C-terminal end is thought to be important for erythrocyte binding, the more N-terminal regions may also be involved in the early recognition and attachment events. While it is not understood whether the different allele types of msp1 block 2 affects the invasion efficiency of merozoites, our study suggests that some allele types in the *in vitro* growth competition assay may have invasion advantages. Moreover, the highly polymorphic block 2 of MSP1 appears to be under balancing selection and allele-specific antibodies to this region are associated with protection against clinical malaria (Cavanagh et al., 2004; Conway et al., 2000). Thus, it would be interesting to compare the *in vitro* competition of the strains in the presence of antibodies from the endemic host populations. As antibodies against *Plasmodium* antigens such as MSP1 can be long-lived in exposed host populations even in hypoendemic areas (Wipasa et al., 2010), they could be responsible for the observed temporal dynamics of the different msp1 allele morphs through the years (Yuan et al., 2013).

We also attempted to test how mutations in drug resistance genes affect the outcome of *in vitro* parasite cultures. During malaria treatment in patients, parasite populations are highly dynamic and disappearance and reappearance of parasite clones can occur within a few hours (Jafari et al., 2004). During in vitro culture adaptation, changes in parasites' sensitivity to certain antimalarial drugs and genotypes of drug target genes are also observed (Le Bras

et al., 1983; Nsobya et al., 2008). In our study, the choice of the genes for analysis was based on our earlier epidemiological survey of genes associated with drug resistance (Wang et al., 2012; Yang et al., 2011). Though the major CQ resistance determinant K76T of the *P. falciparum* CQ resistance transporter appears to have a fitness cost, it was not analyzed in this study because this mutation was essentially fixed in the parasite population under study. *Pfmdr1* is involved in resistance to a number of antimalarial drugs and five common SNPs (N86Y, Y184F, S1034C, N1042D, and D1246Y) have been identified in field isolates from different regions of the world (Foote et al., 1990). In co-culture experiments, the 184F, 1034C, 1042D, and 1246Y mutations all incur a fitness cost relative to the wild-type (Hayward et al., 2005). During *ex vivo* culture of African clinical samples, parasites carrying the mutant 86Y and the wild-type D1246 appeared to have fitness advantages (Ochong et al., 2013). Parasites with increased copy number of the *pfmdr1* gene such as those selected for resistance to mefloquine and artemisinin family drugs are less fit (Chen et al., 2010; Cui et al., 2012; Preechapornkul et al., 2009). However, in the parasite population under study, these pfmdr1 point mutations were rare and amplification of the *pfmdr1* gene was not detected (Wang et al., 2012). All of the 15 clinical samples were monomorphic at the four common mutation sites, making *in vitro* comparison of the fitness cost of these mutations in these field isolates impossible.

Resistance to the antifolate drugs SP is mediated by mutations in the *pfdhps* and *pfdhfr* genes. While a combination of three mutations in pfdhfr (S108N, C59R and N51I) defines a highly pyrimethamine-resistant haplotype, parasites carrying additional double mutant allele of *pfdhps* (A437G and K540E) are strongly associated with an increased risk of SP treatment failure in Africa (Gregson and Plowe, 2005). Outside Africa, additional mutations such as I164L in *pfdhfr* and A581G in *pfdhps* appear to mediate high-level SP resistance. In our study population, genetic analysis suggests high level resistance to antifolates since the *pfdhfr* C59R and S108N and *pfdhps* A437G and K540E were highly prevalent (Yang et al., 2011). In the 15 mixed infections tested, *pfdhfr* I164L was present in all samples, and three samples were mixed at *pfdhps* 581. During culture, one clinical sample mixed at 581 has changed to the wild-type on day 28, whereas two remained stable. From this result, it seemed that the wild-type A581 may have some growth advantages, but the advantages might be subtle. Alternatively, the mutant parasites that remained stable in the mixed infections might have gained compensatory mutations that improve the fitness of the parasites.

This study offers an assessment of the dynamics of parasite populations in mixed infections during in vitro culture using clinical samples from a hypoendemic area. We evaluated the complexity of infections using the msp1 block 2 allele types and several polymorphic sites of genes associated with drug resistance. Consistent with findings from other endemic areas (Nsobya et al., 2008; Ochong et al., 2013), we observed a trend of gradual decrease of parasite populations during culture, suggesting that some parasites are less fit. A large proportion of the parasites essentially became monoclonal during in vitro culture for 3–4 weeks. Thus, phenotypes such as drug sensitivity measured *ex vivo* and from culture-adapted parasites may have major differences. Accordingly, cultures adapted from monoclonal infections may offer less bias for the measurement of drug sensitivity. Yet, we did not

observe the emergence of new parasite strains during culture. This could be due to that the infections were much less complex than those from hyperendemic areas, and/or that the infections were only genotyped using only one polymorphic marker, which may have underestimated the complexity of infections. In addition, our study has limitations especially in measuring in vitro competition of parasites in terms of mutations for drug resistance. Although we did not observe loss of parasite populations immediately after thawing of the clinical samples, we could not exclude the possibility that freezing-thawing of parasites may influence strain selection. Most of the sites chosen for analysis had limited diversity and the small number of samples mixed at key alleles could not allow for a robust assessment of the effect of the mutations on parasite fitness. Further, the genetic backgrounds of the parasite populations present in the mixed infections are not known and could potentially differ significantly at other sites, thus masking the effects of the markers tested in this study. Future studies are needed to elucidate how host immunity affects the prevalence of parasite strains in the parasite population and how intrahost competition affects the evolution and spread of drug resistance.

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Highlights

- Strains in 15 mixed *P. falciparum* cases were evaluated for competition during culture for 28 days
- A trend of gradual loss of strains was observed during in vitro culture
- Most common mutations in *dhfr*, *dhps* and *mdr1* genes remained monomorphic and stable
- The more abundant *msp1* block 2 alleles in the clinical samples became the sole or predominant ones

Table 1

Dynamics of parasite strains during culture

Genotypes of isolates			Sample	Relative ratio of genotypes				
Mixed msp1	Msp2	Glurp		Day 0	Day 8	Day 14	Day 21	Day 28
K1-200:MAD20-150	FC27-300	800	N77	1:20	1:9.3×10^4	1:2.5×10^4	1:2.34×10^4	1:2.37×10^4
K1-200:MAD20-200	FC27-300	600	N36	3:1	300:1	8.5×10^4:1	1.78×10^5:1	K1
	FC27-350	700	A45	11:1	3.2×10^4:1	1.4×10^5:1	9.9×10^5:1	K1
	FC27-300	1000	A28	1:5	1:3.1×10^3	1:3.6×10^5	MAD20	MAD20
	FC27-400	900	A34	1:6	1:2.1×10^3	1:1.8×10^4	1:9.1×10^4	MAD20
	3D7-500	900	A16	1:7	1:2.6×10^4	1:3.5×10^4	1:3.47×10^4	1:1.59×10^5
	FC27-300	900	A32	1:13	1:3.3×10^3	1:5.4×10^3	1:9.55×10^3	1:2.82×10^4
	FC27-400	800	A12	1:20	1:4.2×10^3	1:3.6×10^4	1:2.29×10^4	1:9.54×10^4
	3D7-550	650	N17	1:25	1:1.9×10^4	1:8.3×10^3	1:4.17×10^4	MAD20
	FC27-300	900	A57	1:33	1:2.4×10^3	0:4.3×10^5	MAD20	MAD20
	FC27-300	800	A39	1:90	1:1.2×10^4	1:3.0×10^4	MAD20	MAD20
	FC27-300	900	N13	1:123	1:548	1:2.9×10^3	1:5.76×10^3	MAD20
K1-200:MAD20-250	FC27-300	800	A50	1:3	1:9.9×10^3	1:2.6×10^3	1:6.45×10^2	1:7.25×10^3
K1-250:MAD20-200	3D7-500	950	A5	2:1	1:1×10^4	1:1.9×10^4	1:7.42×10^4	MAD20
	FC27-350	900	A52	33:1	9.3×10^5:0	4.1×10^6:0	K1	K1

Note: Parasite msp1 genotypes have two major allele types K1 and MAD20, while msp2 genotypes have two allele types IC/3D7 and FC27. Numbers following the types indicate the sizes of the PCR products. Glurp alleles were based on the size of the PCR product only.

Table 2

Polymorphisms in *pfdhfr*, *pfdhps* and *pfmdr1* genes in clinical samples and after culture adaptation for 28 days.

Sample	dhfr				dhps				mdr1							
	N51I		I164 L		S436A		A581G		N86Y		Y184F		S1034C		N1042D	
	D0	D28	D0	D28	D0	D28	D0	D28	D0	D28	D0	D28	D0	D28	D0	D28
N77	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
N36	N	N	L	L	S	S	A/G	A	N	N	F	F	S	S	N	N
A45	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
A28	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
A34	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
A16	N	N	L	L	S	S	A/G	A/G	N	N	F	F	S	S	N	N
A32	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
A12	N	N	L	L	S	S	A/G	A/G	N	N	F	F	S	S	N	N
N17	N	N	L	L	S	S	A	A	N	N	F	F	S	S	D	D
A57	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
N13	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
A50	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
A39	N	N	L	L	S	S	G	G	N	N	F	F	S	S	N	N
A5	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N
A52	N	N	L	L	S	S	A	A	N	N	F	F	S	S	N	N

Note: Samples highlighted in bold had become monoclonal by day 28 based on genotyping of msp1 block 2.