J. Parasitol., 96(5), 2010, pp. 908–913

RELATIVE CLONAL DENSITY OF MALARIA PARASITES IN MIXED-GENOTYPE INFECTIONS: VALIDATION OF A TECHNIQUE USING MICROSATELLITE MARKERS FOR *PLASMODIUM FALCIPARUM* AND *P. MEXICANUM*

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ABSTRACT: Quantifying the relative proportion of coexisting genotypes (clones) of a malaria parasite within its vertebrate host's blood would provide insights into critical features of the biology of the parasite, including competition among clones, gametocyte sex ratio, and virulence. However, no technique has been available to extract such data for natural parasite-host systems when the number of clones cycling in the overall parasite population is likely to be large. Recent studies find that data from genetic analyzer instruments for microsatellite markers allow measuring clonal proportions. We conducted a validation study for *Plasmodium mexicanum* and *Plasmodium falciparum* by mixing DNA from single-clone infections to simulate mixed infections of each species with known proportions of clones. Results for any mixture of DNA gave highly reproducible results. The relationship between known and measured relative proportions of clones was linear, with high regression r^2 values. Known and measured clone proportions for simulated infections followed over time (mixtures) were compared with 3 methods: using uncorrected data, with uncorrected data and confidence intervals constructed from observed experimental error, and using a baseline mixture of equal proportions to calibrate all other results. All 3 methods demonstrated value in studies of mixed-genotype infections sampled a single time or followed over time. Thus, the method should open new windows into the biology of malaria parasites.

Malaria parasites (*Plasmodium* and related genera) are diverse in both number of species and variety of genotypes within species (Vardo and Schall, 2007; Martinsen et al., 2008; Havryliuk and Ferreira, 2009). Because individual vertebrate and insect hosts are often infected with 2, or more, species or several clones within a species (Anderson et al., 2000; Mayxay et al., 2004; Vardo and Schall, 2007; Havryliuk and Ferreira, 2009), an important issue in the biology of these parasites is the possible interaction between species and conspecific clones when they infect a single host (Read and Taylor, 2001). Bruce et al. (2000) present intriguing data showing 3 species of human malaria parasite switched in their relative abundance within a child even when the overall parasitemia remained constant. If coexisting genetic clones within a *Plasmodium* species also alternate in relative abundance within individual infected hosts, this would have importance for studies on virulence (Mackinnon and Read, 2004; Vardo-Zalik and Schall, 2008), transmission (Taylor et al., 1997; Vardo-Zalik, 2009), and the parasite's life history, including gametocyte sex ratio (Reece et al., 2009; Schall, 2009).

Measuring the relative abundance of *Plasmodium* clones within a host presents formidable technical challenges. Using well-characterized laboratory isolates of *P. chabaudi*, quantitative PCR allows precise measure of the relative abundance of clones (Cheesman et al., 2003; Drew and Reece, 2007). However, as noted by Cheesman et al. (2003), this method requires knowledge of sequence variation to design clone-specific primers, so only a few genotypes of parasite could be studied in the laboratory model system. For ''wild'' parasite studies, and the potentially very large diversity of cycling parasite genotypes, quantitative PCR is not feasible because of lack of characterization of sequence variation among clones. Another method to measure clonal diversity uses microsatellite markers, short tandem repeats of a few nucleotides (typically 2 or 3 in length). This technique has been very useful in studies of population genetics (Anderson et al., 2000; Vardo and Schall, 2007), geographic differentiation of the parasite (Fricke et al., 2010), and life history traits (Vardo-Zalik and Schall, 2008, 2009), but measuring relative abundance of the coexisting clones requires calibration of the technique. Quantitative PCR is not useful for microsatellites because the sequence flanking the short repeat would have to differ (and be known).

A microsatellite marker is amplified by polymerase chain reaction (PCR) with 1 primer labeled with a florescent dye (Selkoe and Tooner, 2006). The product is then run through a DNA analyzer instrument that determines the length of each fragment when compared to a concurrently running set of size standards. The instrument presents data on the size of the amplified markers and the amount of the DNA present for each size allele as peaks on an electropherogram graph (Fig. 1). Malaria parasites are haploid when in the vertebrate host, so each peak represents a specific clone. A maximum of approximately 4–6 clones can be readily distinguished for a specific marker (Greenhouse et al., 2006; Vardo-Zalik et al., 2009). The peak heights could contain information on the relative abundance of the clones, but the competitive nature of PCR may favor some length alleles, and the vagaries of the instrument could likewise present variation in the peaks not related to the actual density of each clone (Liu et al., 2008; Vardo-Zalik et al., 2009).

Three studies have examined this issue using mixtures of DNA extracted from single-clone infections to simulate infected blood with differing relative abundances of the clones (Havryliuk et al., 2008; Liu et al., 2008; Vardo-Zalik et al., 2009). Although different in design, core findings of these studies show that some alleles (clones) amplify more efficiently than others even when in equal proportions, but the relationship between actual clone density and measured density is linear across different relative proportions. Thus, calibrating peaks' heights from a baseline of known equal proportions will give close estimates of the true relative proportions.

We have continued the study of this method of simulated mixed infections using DNA extracted from natural single-clone infections of *Plasmodium mexicanum* and DNA from cloned parasites of *Plasmodium falciparum*. We sought to determine the reproducibility, accuracy, and precision of the method (Gotelli and Ellison, 2004). First, we sought evidence of experimental artifact due to laboratory error, PCR bias, and errors by the DNA analyzer

Received 30 March 2010; revised 9 June 2010; accepted 11 June 2010.
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DOI: 10.1645/GE-2499.1

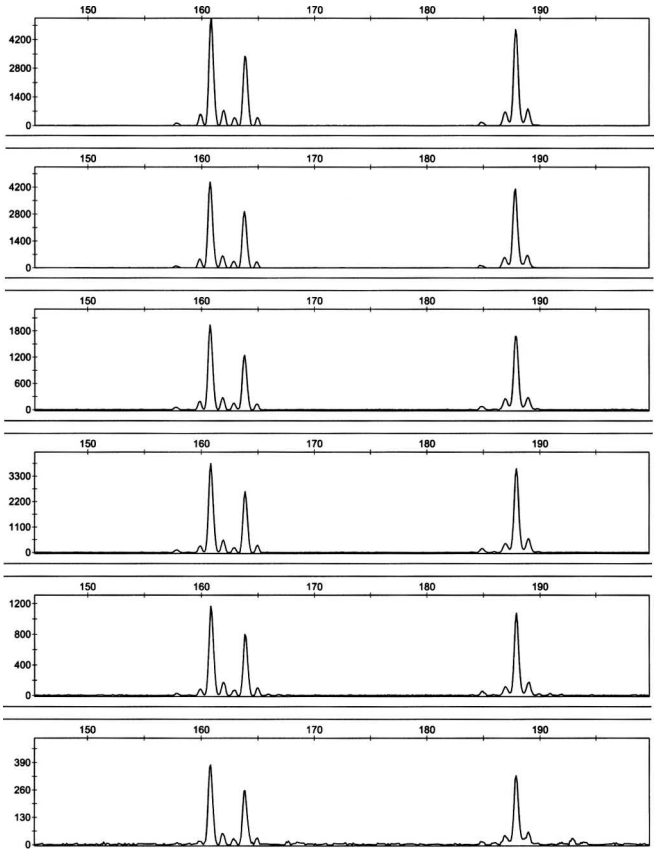

FIGURE 1. Electropherograms produced by GeneMapper 3.5 software for data from the ABI Prism genetic analyzer instrument for replicate simulated infections of *Plasmodium falciparum*, which mixed DNA from 3 single genetic clones of the parasite for the microsatellite marker A109. Each peak represents 1 allele; for the haploid *Plasmodium* genome, this would represent 1 clone of parasite cells in these mixtures. The horizontal axis shows the size of the DNA fragment that varies because of the number of 3 base repeats in the microsatellite. Peak height is generally proportional to density of each microsatellite allele in the PCR product, but varies because of both bias in the PCR and instrument function. Here DNA of each allele was mixed in equal proportions. Small peaks are artifact "stutter" seen in such electropherograms. High-quality results are indicated by a single sharp peak, with no flattened or jagged end.

instrument. That is, were results reproducible across replicates of the same proportions of clone DNA? Second, we asked if comparison of actual and measured relative proportions of clones in mixed simulated infections is linear across different proportions of each clone. The method would be accurate if this relationship had a slope of 1 and intercept of 0. Third, confidence intervals for relative proportions were constructed based on empirical results from the known versus measured proportions. Last, we compared results of uncorrected comparisons of known and measured relative proportions with results calibrated using mixtures of equal proportions of each clone's DNA. These comparisons will reveal the precision of the method. The results show this method is reproducible, accurate, and precise, and should be very useful in studies of natural infections of both species.

MATERIALS AND METHODS

Western fence lizards, *Sceloporus occidentalis*, were collected at the University of California Hopland Research and Extension Center field site in Mendocino County, California (Schall, 1996). Thin blood smears were made for each lizard and were treated with Giemsa stain and examined at ×1,000 to select individual lizards infected with the malaria parasite *P. mexicanum*. Several drops of blood from infected lizards were preserved on filter paper, and DNA was later extracted using the DNeasy kit (Qiagen, Valencia, California) and the provided protocol. Single-clone infections were identified by scoring 5 microsatellite markers (Schall and Vardo, 2007); a single peak on electropherogram for all 5 markers was regarded as evidence that the infection harbored only 1 genotype of parasite. Parasitemia for these infections was determined by counting parasites seen in 1,000 erythrocytes, and concentration of total extracted DNA measured using a NanoDrop (Thermo Scientific, Wilmington, Delaware) spectrometer. The DNA was then diluted to produce approximately equal concentrations of parasite DNA among samples, assuming that the concentration of host DNA was the same across samples. This gave only an approximation of parasite DNA concentration because *P. mexicanum* meronts could contain from 1 to 12 nuclei. *Plasmodium falciparum* DNA was obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, Virginia), from parasites grown in culture. All samples were already standardized to DNA concentration, and host DNA contamination was assumed to be minimal.

Three 3-nucleotide repeat microsatellite markers were examined for *P. mexicanum*: Pmex 306, Pmex 747, and Pmex 710s (Table I; Schall and Vardo, 2007). The PCR program for each was 94 C for 2 min, followed by 32 cycles of 94 C for 1 min, annealing (Pmx306: 60 C for 10 sec, 50 C for 20 sec; Pmx747: 51 C for 30 sec, 49 C for 20 sec; Pmx710s: 45 C for 50 sec), 60 C for 1 min, and a final extension of 60 C for 7 min. Two 3-nucleotide repeat microsatellite markers were examined for *P. falciparum*, PK2 and A109 (Table I; Anderson et al., 1999). The PCR conditions for these 2 markers were 94 C for 3 min, followed by 45 cycles of 92 C for 30 sec, 45 C for 30 sec, 65 C for 45 sec, and a final extension of 68 C for 5 min. All reactions used Ready-to-Go PCR beads (GE Healthcare, Piscataway, New Jersey) that contained DNA polymerase, dNTPs, and buffers. For a 25 ml reaction, water was added to the bead as well as 3 ml of the extracted DNA and 1 ml (*P. mexicanum*) or 0.25 ml (*P. falciparum*) each of the two 10 mM primers. PCR product was run on the ABI Prism genetic analyzer instrument, and the resulting data were examined using the GeneMapper 3.5 software (ABI, Foster City, California). PCR was conducted for each marker (and pair of primers) in a separate reaction.

To simulate mixed infections, DNA from single-clone infections of each parasite species was mixed in various proportions. These were the "known" proportions (the true proportions were approximations for *P. mexicanum*, but assumed accurate for *P. falciparum*). Six natural infections of *P. mexicanum* were labeled A–F. For each marker, the allele sizes differed by 2–13 repeat units. Three samples from single-genotype cloned infections of *P. falciparum* were used and labeled G, H, and I (MR4 lines 156G, 155G, and 152G). The alleles in these samples differed from 1 to 17 repeat units. Electropherogram typically reveal artifact "stutter" peaks for each microsatellite length allele that are peaks, 1 repeat unit larger and smaller than the target marker (Selkoe and Tooner, 2006). Most alleles present in our mixtures of DNA were chosen to be at least 2 repeat units different in size, did not fall within possible stutter peaks, and were thus obvious on the electropherogram. In 1 case, when only 1 repeat unit separated alleles (for *P. falciparum*), the second peak was clearly visible because of its height.

Three trials were performed for *P. mexicanum*. In Trial 1, DNA was mixed for samples A, B, and C in ratios of 1:1:1, 2:1:1, 1:5:1, 1:2:5, 10:1:2, and 1:10:1 for marker Pmx710s and marker Pmx747, with 2 replicates for most mixes. For marker 306, 3 replicates were performed each for mixes 1:1:1, 1:5:1, 1:2:5, 10:1:2, and 1:10:1. In Trial 2, the same 3 samples were used for all 3 markers at 1:1:1, 1:10:1, 10:10:1, 5:10:1, 2.5:2.5:1, 0:10:1, and 0:5:1, with 6 or 4 replicates for each mixture. For Trial 3, samples D, E, and F were mixed for all 3 markers at 1:1:1, 1:5:1, 1:10:1, 1:20:1, 1:50:1, 10:2:1, 5:1:5, with 3 replicates for each mixture.

Two trials were performed for *P. falciparum*. In Trial 1, DNA was mixed from 2 samples (infections), G and H, with 3 replicates of each mix for the 2 markers: 1:1, 1:2, 2:1, 1:5, 5:1, 1:10, 10:1. In Trial 2, the same 2 samples were used along with a third, I. Two replicate mixes were prepared for each of 8 combinations at ratios of I, G, and H of 1:1:1, 2:1:1, 5:1:1, 10:1:1, 1:2:5, 1:5:2; 1:10:10, 20:1:1.

Two comparisons were made between the known relative proportions of the clones and measured relative proportions based on peak heights seen in electropherograms (Fig. 1). These are called here the "uncorrected" and "calibrated" proportions. First, a known proportion of a clone in a mixture

TABLE I. Calibration of measured relative proportions of simulated mixed-genotype infections of 2 malaria parasites using microsatellite markers. Measured relative proportions were regressed against known proportions in mixtures of DNA from single clones. Peak heights on electropherograms produced by a genetic analysis instrument were used to estimate the relative proportions of clones in simulated mixed infections. Perfect measurement of relative proportions would result in a tight fit to the regression ($r^2 = 1.0$) and slope of 1.0. Empirical confidence intervals contained 90% of measured proportions.

	Plasmodium mexicanum			*Plasmodium falciparum*	
	Pmx306	Pmx747	Pmx710s	PK2	A109
Uncorrected peaks*					
N	172	175	163	87	88
r^2	0.927	0.93	0.933	0.887	0.819
Slope (intercept)	0.886 (0.040)	0.851 (0.052)	0.910 (0.031)	0.852 (0.061)	0.836 (0.067)
90% CI	±0.11	±0.11	±0.11	±0.15	±0.18
Corrected peaks†					
N	163	166	154	82	83
r^2	0.987	0.98	0.986	0.997	0.997
Slope (intercept)	0.964 (0.012)	0.914 (0.030)	0.953 (0.016)	0.966 (0.004)	0.982 (0.007)
90% CI (±%)	6	6	6	4	4
Primer sequences‡					
Forward	gatcacattttgctattttagtatt	cacaaattcaagataattcaaaag	ggtttcaaaatttgaagcg	ctttcatcgatactacga	ggttaaatcaggacaacat
Reverse	aacttttgattcttctataacag	tctttttcgagacatattattgc	cattttgctattttagtattttctagt	aaagaaggaacaagcaga	cctataccaaacatgctaaa

* Uncorrected peaks used the raw data from the electropherogram peak heights.
† Corrected peaks used peak heights for a mixture of known equal proportions of the clones to recalculate peak heights for mixtures with unequal proportions, thus correcting for any PCR, or instrument bias for specific alleles.
‡ Primer sequences are given for each microsatellite marker (forward was labeled with a fluorescent tag, 6FAM).

was compared to the proportion calculated as that allele's peak height/total combined peak height (=uncorrected proportions). Second, results were calibrated based on peaks heights seen in a known mixture of equal proportions of each clone, a 1:1:1 mixture that serves as the base sample (=calibrated proportions). Peak heights for each allele for a mixture were divided by the peak height for the base sample, and the ratios of these results calculated by fixing the lowest value as 1.0. For example, peak heights for 3 alleles for a 1:1:1 mixture were 8,251:4,048:5,448, and the peak heights for the next sample (mixture at 1:1:10) were 2,190:793:10,841. The resulting ratios were 2,190/8,251, 793/4,048, 10,841/5,448, or 0.2654:0.1958:1.99. Setting the lowest value (0.1958) as 1.0, the observed ratios were 1.36:1:10.16, to be compared to the known ratio of 1:1:10.

For the uncorrected proportions, each allele's proportion was plotted against its known proportion, the results modeled by regression to determine the linear fit of the data (measured compared to known proportions), and confidence intervals for the individual data calculated (JMP 8, SAS Institute, Cary, North Carolina).

Several factors could create variation in peak heights unrelated to true proportion of the clones: errors in mixing the template DNA, variation in the outcome of the PCR, variation in diluting the PCR product for processing by the DNA analyzer instrument, and functioning of the instrument itself. We teased out these possible sources of error by replicating the mixtures (this would combine all sources of error). For *P. falciparum* Trial 2, the PCR product was diluted twice for each sample, and for 1 of these dilutions, the sample was run twice on the instrument. These results would test for laboratory errors in diluting the product and instrument variation in reading the concentration of each clone's DNA. Another important source of error in reading the peak heights of electropherograms is the quality of the PCR product that is injected into the instrument. This is apparent from the form of the peaks; those that are cut off at the top or seen as double peaks at their tips will give spurious measures of the relative density of the PCR product for each allele. Therefore, we discarded any result with such defects, and reprocessed the samples taking care to dilute the PCR product to a lower concentration. Therefore, all results are based on clean results from the instrument (examples in Fig. 1).

RESULTS

Almost all of the expected peaks were visible for all mixtures. The single exception was the 1:50:1 mix in Trial 3 for *P. mexicanum*. All 3 alleles were seen for marker Pmx747, but 1 peak was missing for the other 2 markers. This result agrees with previous studies that show very different clonal densities can give poor results (Greenhouse et al., 2006; Vardo-Zalik et al., 2009); therefore, these few data were excluded from subsequent analysis.

Peak heights seen in mixtures with equal proportions of DNA for each allele often differed (example in Fig. 1), but, in some cases, peaks were very similar in height (also seen in Fig. 1). There was no general trend of this effect by length of the allele fragment; that is, some shorter alleles (fewer repeats) produced the taller peak, whereas other longer alleles yielded the taller peaks. Replicate mixes (with same proportions of DNA) showed very similar results. An example shown in Figure 1 reveals very similar patterns even when the overall concentration of the PCR product varied (fluorescent unit scale varied substantially). For Trial 2 of *P. falciparum*, the template DNA was prepared twice for each mixture, and for one of those the PCR product was inserted twice into the ABI genetic analyzer instrument. These replicates (not included in the analysis below) gave almost identical results (peak heights).

For each parasite species, and for each marker, data for known proportions of clones were compared to those calculated from peak heights on the electropherograms (uncorrected proportions). For the *P. mexicanum* trials, the concordance was strong, with r^2 values close to one ($P < 0.05$), slopes not significantly different from one, and intercepts not significantly different from 0 ($P > 0.05$; Table I). For the *P. falciparum* trials, the results were less tight, with somewhat lower regression r^2 (Table I), but also with slopes not significantly different from 1.0. Intercepts, though, were greater than 0 (Table I). The calibrated results were only slightly improved relative to the uncorrected proportions for the *P. mexicanum* markers (Table I). For *P. falciparum*, measured proportions were closer to known proportions, with regression r^2 values substantially greater and slopes not significantly different from 1 and intercepts of 0 (Table I). Figures 2 and 3 present

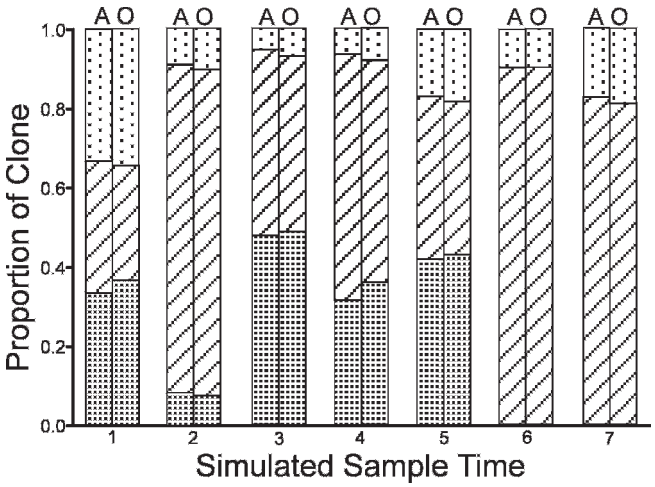

FIGURE 2. Comparison of mixtures of 3 alleles of marker Pmx306 for *Plasmodium mexicanum* for the actual proportion of DNA from single-clone infections (A) and the observed proportions (O) after calculated from a base-line infection (calibrated results). Three alleles (clones) are shown with different fill patterns. The time series is a simulation as though the samples were taken over a period of time from the same infection with alleles changing in proportion over time. The 7 mixtures are placed on the simulated time axis in an arbitrary order. The mixtures were chosen to show the pattern expected if the relative proportions of clones in an infection of a malaria parasite were changing over time.

comparisons of known and calibrated measured proportions for 3 alleles of *P. mexicanum* in Trial 2 and *P. falciparum* for Trial 2 for different mixtures, with close concordance between known and calibrated measured proportions.

A confidence interval was calculated for the results comparing known and measured relative proportions; a 90% confidence interval would include 90% of the data (JMP, SAS, Cary, North Carolina). For each species, confidence intervals were very similar across markers (Table I). The confidence intervals shown in Table I were used to compare known and uncorrected measured proportions of the alleles from Trial 2, with various mixtures simulating a series of samples taken from an infection over time (Fig. 4). In most cases, confidence intervals of the measured proportions overlapped with known proportions (again indicating the measured proportions closely matched the known proportions), and known differences in proportions among mixtures were detected by nonoverlapping confidence intervals.

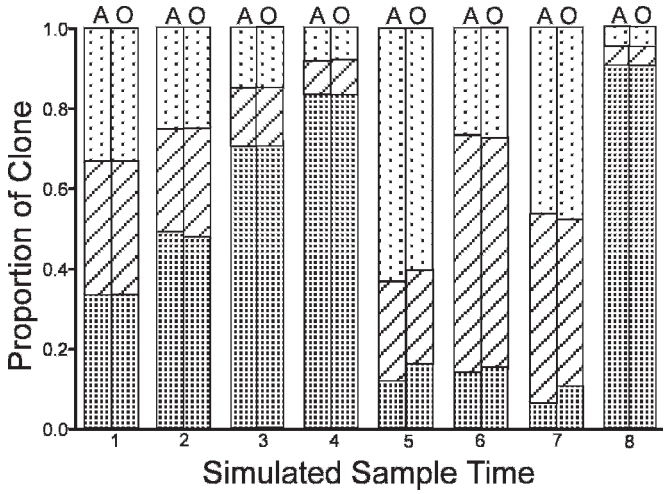

FIGURE 3. Comparison of mixtures of 3 alleles of marker A109 for *P. falciparum* for the actual proportion of DNA from single-clone infections (A) and the observed proportions (O) after calculated from a base-line infection (calibrated results). Three alleles (clones) are shown with different fill patterns. The time series is a simulation as though the samples were taken over a period of time from the same infection with alleles changing in proportion over time. The 8 mixtures are placed on the simulated time axis in an arbitrary order. The mixtures were chosen to show the pattern expected if the relative proportions of clones in an infection of a malaria parasite were changing over time.

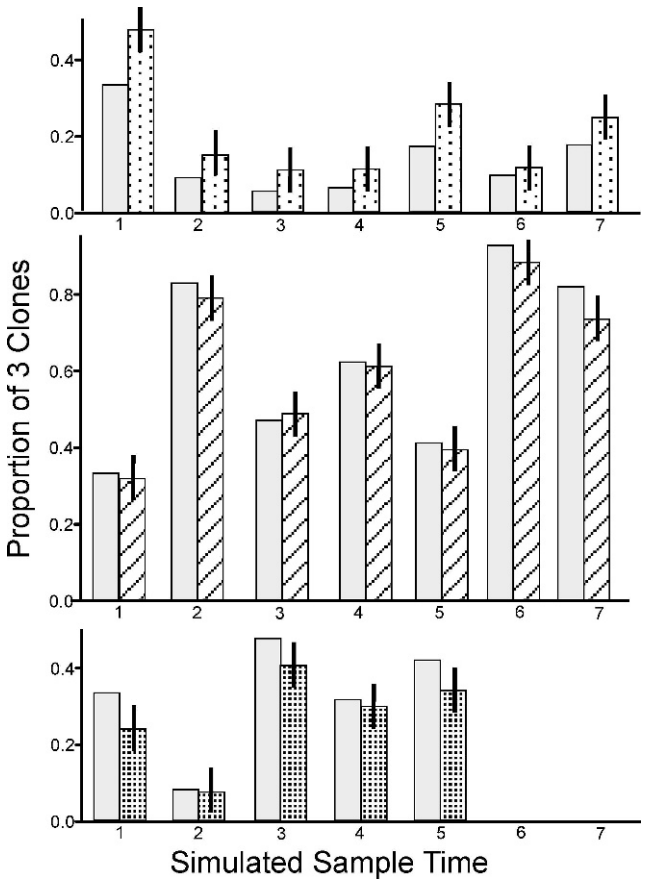

FIGURE 4. Relative proportion of 3 clones of *P. mexicanum* in a simulated mixed infection based on microsatellite marker Pmx306. The known proportion of each allele is shown with shaded bars and the measured proportions (from peak heights on electroelectropherogram graphs) shown with different fill patterns for each of the 3 alleles. Data are from same series of mixtures shown in Figure 2 (with same fill pattern for each allele), but here are not corrected. The 90% confidence interval is shown. Mixtures are used to simulate a series of samples taken from the infection over time in which only raw data on peak heights were available.

DISCUSSION

In mixed-clone infections of malaria parasites, changes in relative proportion of coexisting clones may be relevant for life history events such as growth rate of the infection (Vardo-Zalik and Schall, 2009), competition among clones (de Roode et al., 2005), gametocyte sex ratio (Reece et al., 2005; Drew and Reece, 2007; Schall, 2009), transmission to the insect vector (Vardo-Zalik, 2009), and virulence (Mackinnon and Read, 2004; Vardo-Zalik and Schall, 2008). Also, any changes in relative propor-

tions could indicate complex interactions among parasite genotypes, perhaps driven by competition, but also positive interaction among clones to elude the host immune system. Studies using laboratory clones of *Plasmodium chabaudi* in the white mouse model host system have revealed complex facets of the biology of this parasite when in mixed-clone infections (de Roode et al., 2005). However, the methods used in these studies would be difficult, or even impossible, for natural parasite-host systems. We, therefore, sought to validate another research method, the relative PCR amplification of microsatellite markers that would indicate changes in the relative proportions of coexisting clones.

First, the results show the method is highly reproducible. That is, variation due to laboratory error, and the PCR and instrument function was nil, with almost identical results for replicate trials. Second, the method is accurate, with no systematic bias for *P. mexicanum* (regression of known and measured proportions with slope of 1 and intercept of 0). For *P. falciparum*, there was a slight bias in the uncorrected data (in intercept) that vanished when the data were corrected using a mixture of equal proportions. Third, the method is fairly precise, with the ability to measure actual proportions to ±0.04–0.06 for the 2 parasites.

Three types of studies on malaria parasites can use microsatellite markers to monitor relative proportions of coexisting clones: experimental infections with known combinations of clones (Drew and Reece, 2007; Vardo-Zalik and Schall, 2008, 2009), natural infections sampled a single time, and natural infections followed over time. Calibrated results as done here are easily achieved only for the experimental infections (by mixing equal proportions of DNA from the clones). Figures 2 and 3 present simulations of the results of this kind of study and show that measured proportions match actual proportions of clones very closely. Thus, the method should be very useful for experimental studies, and events within infections, such as switches in the dominant allele, can be readily observed with high accuracy. All 3 types of studies can use uncorrected proportions for which no base-line mixtures are required. The linear results comparing known with measured proportions, with high proportion of variance explained (r^2 values), for both parasite species show that relative proportions of clones in infections sampled a single time and significant changes in relative proportions in infections followed over time will be correctly identified from peak heights (simulation shown in Fig. 4). The most reliable conclusions, however, are possible if confidence intervals can be constructed. This would be straightforward for the experimental studies, but for the natural infections, single-clone infections of each cycling allele must be identified to produce the mixtures of DNA and to calculate the confidence intervals. In practice, this would be arduous, if even possible. However, we were struck that the confidence intervals for all the alleles studied here were similar within parasite species and among all markers. Only a sample of alleles may be needed to discover the error typical for a particular parasite and microsatellite marker, and perhaps for a range of markers for each species. Results were far cleaner for *P. mexicanum* than *P. falciparum*, which displayed broader confidence intervals. We suspect that the differences seen are a result of the primer design and could be improved. Liu et al. (2008) also noted that specific makers and associated PCR primers varied in accuracy of results, with slopes of known versus measured proportions differing among markers.

We entered this study not expecting such useful results. In particular, we were concerned with the reproducibility of results, being aware of all the possible sources of error including error in preparing mixtures, variation in the PCR, preparing samples for injection into the instrument, and the instrument function. These possible errors were examined by replicating samples. Replicated samples, either replicates in the PCR or samples injected twice into the instrument, were almost identical in outcome (example in Fig. 1). Three sources of error were identified. First, if 1, or more, clones had 50-fold greater density than another clone or clones, the lower density clones were sometimes missed on the electropherogram peaks. In practice, the biological effect of such very-low-density clones may be nil, for example, in alteration of gametocyte sex ratios (Reece et al., 2009; Schall, 2009). Second, low-quality PCR product or overloading the capillary gels resulted in cutoff peaks or double peaks at the tips that produced highly variable data, and thus whenever such peaks were observed, the samples were reprocessed (often with greatly diluted PCR product) producing good results typical of those shown in Figure 1. Third, equal amounts of DNA used as template in the PCR can produce different peak heights on electropherograms. This result, however, was consistent across replicates, and linear across different proportions of DNA. This effect does not affect detection of major changes in relative abundance, and results are more reliable if calibrated or if the error is defined by confidence intervals based on empirically determined measurement error.

An obvious limitation of the method described here is its restriction to known single-species infections. When 2, or more, parasite species coexist in the vertebrate or invertebrate host, primers may co-amplify all species (and perhaps with differing efficiency), and biases of the PCR are likely. Even cryptic infection with 1, or more, additional species in apparently single-species infections would confound the results.

The results of this and previous studies argue that both experimental infections and natural *Plasmodium* infections followed over time can supply important information on relative abundance of clones, and these data can be used in studies of a wide variety of issues in the biology of malaria parasites that will open novel avenues of research in malaria studies. The method should be useful for studies of other microparasites as well.

ACKNOWLEDGMENTS

The staff of the Hopland REC welcomed us and provided logistical support during the field studies. The research was funded by an NSF grant to J.J.S., and the MR4 samples funded by an NIH grant to Guiyun Yan. We thank Lori Stevens for facilitating funding for AFF from the UVM NSF Mathematics-Biology scholars program. A. Neal offered important assistance in the field and laboratory studies.

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