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COMPARISON OF PCR-RFLP AND GENESCAN–BASED GENOTYPING FOR ANALYZING INFECTION DYNAMICS OF *PLASMODIUM FALCIPARUM*

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Abstract. Parameters describing the infection dynamics of *Plasmodium falciparum* are important determinants of the potential impact of interventions and are potential outcome measurements for malaria intervention trials. Low parasite densities, periodic sequestration of parasites, and the presence of multiple concurrent infections make it essential to use molecular techniques to estimate the force of infection and duration of infections in endemic areas. We now compare two approaches for tracking individual genotypes of the highly polymorphic merozoite surface protein 2: 1) fluorescence-labeled polymerase chain reaction (PCR) and GeneScan-sizing and 2) restriction fragment length polymorphism (RFLP). We analyze samples from a longitudinal field study in Ghana and use statistical approaches that allow for imperfect detectability. The two methods gave broadly similar estimates of parasite dynamics, but GeneScan is more precise and can achieve a higher throughput. The analysis of parasite dynamics indicated an average duration of infection of 210 days by GeneScan versus 152 days by PCR-RFLP in the study population in Kassena-Nankana, Northern Ghana. This reflects the good performance of GeneScan-based genotyping for studies of parasite infection dynamics.

INTRODUCTION

Plasmodium falciparum parasite densities fluctuate over time in the course of an infection, and the late stages of the parasite cytoadhere to endothelial receptors and sequester in deep tissues, leading to a 48-hour cycle in the appearance of parasites in the peripheral circulation. These features of malaria parasite biology complicate the analysis of infection dynamics. This background of persisting but transiently detectible parasitemia makes it inappropriate to use standard microscopy techniques to determine the rates of infection or the clearance rates of infections in the absence of interventions. These are essential parameters for understanding the transmission dynamics of the parasite.[1]

Allelic discrimination is possible by molecular means and multiple concurrent infections are usually found in blood samples from areas endemic for malaria.[2–4] Highly polymorphic marker genes amplified by polymerase chain reaction (PCR) can be used to track individual clones in longitudinal sample sets. Despite the lower detection limit of PCR relative to microscopy, some clones still seem to disappear but are in fact persisting, and genotyping often detects clones recurring periodically at intervals of 48 hours.[5,6] We have previously analyzed infection dynamics of individual clones as determined by PCR-restriction fragment length polymorphism (RFLP) using transition models based on the relative frequencies of different patterns of infection defined by presence/absence at successive surveys.[7,8] These models take into account the imperfect detectability of clones sequestered at the time of sampling.

We now compare two molecular techniques for identifying individual clones. The first is RFLP of PCR fragments (PCR-RFLP), which has been used in a number of studies to track parasite clones longitudinally. The marker gene merozoite surface protein 2 (*msp2*) is highly polymorphic because of intragenic repeats, with > 50 genotypes identified by RFLP in each of its two allelic families 3D7 and Fc27.[3,9] The pattern of restriction fragments clearly identifies individual clones, and particular patterns are recognized also within mixed infections. However, in samples with more than five concurrent genotypes, the superimposed patterns are increasingly more difficult to analyze. The second technique is based on sizing PCR fragments by an automated sequencer using the GeneScan program. This technique uses fluorescent-labeled PCR primers specific for the *msp2* allelic families. FC27- and 3D7-type *msp2* PCR fragments are identified by the two fluorescent markers 6-Fam and VIC, respectively. The use of a different dye for each allelic family increases resolution of fragment sizing. GeneScan analysis software uses a size standard added to each sample after PCR to create an internal calibration curve to determine the size of each PCR fragment. In comparison with PCR-RFLP, this technique increases throughput and avoids subjectivity in analyzing the readout.

We report analyses of *P. falciparum* infections in 100 individuals of all ages from the Kassena-Nankana District (KND) of Ghana sampled at 2-monthly intervals over 1 year. We compare the performance of genotyping by PCR-RFLP versus GeneScan for the analysis of the force of infection (clonal acquisition rate) and duration of individual infections based on the patterns of persistence of individual genotypes.

MATERIALS AND METHODS

Study site and population. The population of KND in northern Ghana is plagued by high infant and maternal mortality rates.[10] In KND, malaria transmission is seasonal, but even in the absence of any rain, widespread transmission of malaria continues. For a molecular epidemiologic survey of *P. falciparum* multiplicity of infection and infections dynamics among asymptomatic inhabitants of a holoendemic malarious area, a cluster sample of the KND population was drawn by selecting 16 index compounds at random from the 14,000 within the district. From each index compound, two people in each of the following age categories were selected: < 1, 1–2, 3–4, 5–9, 10–19, 20–39, 40–59, and 60+ years. Blood samples were collected on DNA ISOCode Stix (Schleicher & Schuell, Dassel, Germany) in intervals of 2 months, resulting in a total of six samples per participant (R1–R6). Blood samples for the first time-point (R1) were collected in June/July 2000, and genotyping results of R1 were presented previously.[11] In-

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formed consent was obtained from participants by signature or thumbprint in the presence of a witness. Ethical clearance for this study was obtained from the Ghana Health Service Ethics Committee. For this comparison of two genotyping methods, a subset of the 1,848 samples collected were used, amounting to 600 samples deriving from 100 individuals.

DNA isolation and genotyping. Whole blood (10 μL) was dotted onto ISOCode Stix. Irrespective of microscopy results, six samples from each of 100 individuals were screened for presence of *P. falciparum* by PCR. Processing of Stix, PCR conditions, and RFLP procedures have been described in detail.[3,12] In brief, primary and nested PCR were performed for RFLP analysis using primer pair S2/3 (5′-GAAGGTAATTAAAACATTGTC-3′/ 5′-GAGGGATGTTGCTGCTCCACAG-3′) and S1/4 (5′-GAGTATAAGGAGAAGTATG-3′/5′-CTAGAACCATGCATATGTCC-3′), respectively, followed by restriction digest with *Hin*fI and *Dde*I as described elsewhere.[3,12]

For GeneScan analysis, 2 μL of primary PCR product was amplified in nested PCR with the fluorescent-labeled family-specific primer M5 (FC27-specific: 5′-6-FAM-GCATTGCCAGAACTTGAA-3′) or N5 (3D7-specific: 5′-VIC-CTGAAGAGGTACTGGTAGA-3′) at a concentration of 100 and 200 nM, respectively. The non-fluorescent–labeled forward primer S_{Tail} (5′-CTTATAATATGAGTATAAGGAGAAA-3′) was modified at the 5′ end by adding a 7-bp tail to avoid non-template–directed addition of a single nucleotide to the 3′ end of a blunt-end double-stranded DNA ("plus-A-artefact").[13] The cycle conditions were 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 1 min at 50°C, and 1 min at 70°C, and a final elongation for 7 min at 70°C.

nPCR product (0.5 μL) was combined with 10 μL ROX-labeled size standard (diluted 1:10 with H_2O to minimize pipetting errors). Samples were dried and sent to the Genomics Core Laboratory of the MRC Clinical Science Center in London. Highly deionized formamide was added, and after denaturation, samples were analyzed on an ABI PRISM 3700 genetic analyzer.

Determination of detection limits. Genomic DNA was isolated from *P. falciparum in vitro* cultures (FC27 and 3D7 strains) as described previously.[14] DNA concentration was determined photometrically. Tenfold dilutions of genomic DNA were amplified in PCR reactions containing 1000, 100, 50, 10, 5, 1, and 0.1 genomes of either 3D7 or FC27 DNA or a mixture of both.

Data analysis. An in-house–generated computer program was used to process the output of the GeneScan analyzer. The main tasks of this program were as follows: 1) Cut-off determination: a sample-specific cut-off was used to separate real signals from noise and to allow for variability between GeneScan runs. The cut-off was established by GeneScan analysis of sequenced *msp2* reference alleles (cloned *msp2* fragments or single clone infections). The presence of a single template per reaction made it possible to determine background fluorescence levels to 300 arbitrary fluorescence units. A sample-specific cut-off was determined by multiplying the arithmetic mean of peak heights of the size standard signals per sample by a constant (the empirically chosen cut-off of 300 units divided by the mean peak height of size standard peaks of all samples). Because with our primers none of the *msp2* sequences available at GenBank would give rise to a PCR fragment of < 216 bp, peaks with a measured size of < 200 bp were not considered. 2) Elimination of bleeding and "plus-A-artifacts": spectral overlap of the fluorescent dyes labeling the family-specific primers caused bleeding in case of strong signals caused by PCR fragments present at very high concentrations (> 5,000 fluorescent units). Although the dyes emit light at different wavelengths, some overlap exists despite using a GeneScan software matrix file to remove spectral overlaps. *Taq* polymerase has terminal deoxynucleotidyl transferase activity to add an extra nucleotide, usually adenine ("plus-A"), at the 3′ end of PCR products. This results in two populations of amplified products with a size difference of one nucleotide. Despite the use of a tailed primer that supports "plus-A" addition, we observed in case of high intensity peaks also a small peak about one nucleotide before the actual peak. Our software eliminated peaks caused by bleeding and "plus-A-artifact." 3) Elimination of PCR artifacts: allele-specific PCR artifacts were detectable when particular alleles were present at a very high concentration. Such artifacts are likely caused by the intragenic repeats of *msp2* alleles, which can facilitate aberrant annealing of an incompletely synthesized strand to the repeat region. We determined allele-specific PCR artifacts by analyzing cloned *msp2* alleles at high DNA concentrations. Artifacts were omitted from subsequent analyses. 4) Genotype calling: For analysis of longitudinal sets of samples, a persisting genotype must be accurately identified in sequential blood samples. There were slight variations among repeated size determinations of identical fragments depending on the concentration of the amplified fragment. To allow for inaccuracies in size determination, peaks were assigned to size bins with a width of 2.4 bp. Because a coding region is genotyped, fragment sizes must differ by multiples of 3 bp.

Statistical analysis. For comparing the two genotyping methods, SAS statistical software Release 8.2 (SAS Institute, Cary, NC) was used. Infection dynamics were analyzed by calculating the frequency of gains, losses, and persistence of infecting clones. An infection present in survey at time t, but not seen in the subsequent survey $t + 1$ was considered as "loss" (+ −), whereas "gain" (− +) was noted when an infection was observed in round t but not in the previous round $t - 1$. Where infections were observed in consecutive surveys this was recorded as "persistence."

We analyzed the infection dynamics using methods that allow for imperfect detection using the method of Smith et al.[7] In further analysis, we fitted an immigration-death model to the full sequences of six observations.[8] This provided estimates of the rate of new infection, λ, clearance rate, μ, and detectability.

RESULTS

Limit of detection and evaluation of GeneScan. Serial dilutions of genomic DNA from parasite culture strains FC27 and 3D7 were prepared to determine the detection limit of GeneScan-based *msp2* genotyping. After primary PCR, nested PCR was performed either independently for each primer set (3D7 simplex PCR and FC27 simplex PCR) or with a combination of FC27- and 3D7-specific primers (duplex PCR). While both the FC27-and 3D7-specific simplex PCR detected one parasite genome per reaction, performing a duplex PCR with equal amounts of both templates reduced the

TABLE 1
Sensitivity of detection of *msp2* PCT fragments by agarose gel electrophoresis compared to GeneScan (GS)

Number of templates/reaction*	1000		100		50		10		5		1		0.1	
Detection technique	Gel	GS	Gel	GS	Gel	GS	Gel	GS	Gel	GS	Gel	GS	Gel	GS
Fc27 amplified in simplex PCR	+	+	+	+	nd	nd	+	+	+	+	+	+	−	−
3D7 amplified in simplex PCR	+	+	+	+	nd	nd	+	+	+	+	−	+	−	−
Fc27 amplified in duplex PCR	+	+	+	+	nd	nd	+	+	+	+	−	−	−	−
3D7 amplified in duplex PCR	+	+	+	+	nd	nd	+	+	+	+	−	−	−	−
Fc27 in presence of 1000 3D7 copies	+	+	+	+	+	+	−	+	−	−	−	−	−	−
3D7 in presence of 1000 Fc27 copies	+	+	+	+	+	+	+	+	+	+	−	−	−	−

* One template corresponds to one genome of a FC27 or 3D7 parasite from an *in vitro* culture.

detection limit to 5 genomes per reaction (Table 1). This sensitivity was considered sufficient and considerably reduced costs justified duplex PCR for field samples.

The effect of excess DNA of the alternative allelic family was assessed for duplex PCR. 3D7-specific amplification remained unaffected by the presence of 1000 FC27 genomes. However, FC27-specific amplification detected only 10 genomes in the presence of 1000 3D7 genomes. This difference is probably caused by the smaller amplicon size of 3D7 (267 bp) versus FC27 (358 bp; accession numbers M28891 and J03828).

Before application to field samples, the technique was validated on a panel of sequenced reference alleles (data not shown). The SD of repeated sizing of the same fragment was only about 0.06 and 0.17 nucleotides for FC27 and 3D7 DNA, respectively. For the most frequent allele in the Ghanaian field samples, an SD of 0.14 was calculated.

When comparing all *msp2* sequences submitted to GenBank until 2004, we detected two genotype pairs of the same allelic family sharing the same amplicon size (accession numbers U07001/U16842 and AY534506/AF010461). Thus, GeneScan cannot discriminate these alleles. This represents the limitation of our method.

Longitudinal genotyping in field samples from Ghana. From 100 individuals enrolled at baseline, 550 blood samples were collected during the 1-year follow-up in 2-monthly intervals; 78.2% were found to be positive for *P. falciparum* by PCR. For 99 individuals, GeneScan analysis was successfully performed, and 96 individuals were analyzed by PCR-RFLP, accounting for 1,405 and 1,084 observed clonal infections, respectively. The discrepancy is caused by differences in the resolution of these methods. Problems occurred in particular with the PCR-RFLP method when interpreting superimposed RFLP patterns.

A total of 164 different *msp2* alleles were distinguished by GeneScan analysis; 116 and 48 belonged to the 3D7 and FC27 allelic family, respectively. Frequencies of 3D7-type alleles were all < 3% (Figure 1a). Some FC27-alleles occurred at very high allelic frequencies, the most frequent one reaching 14% (Figure 1b).

Multiplicity of infection. Overall multiplicity of infection (MOI) assessed by GeneScan was higher than by PCR-RFLP with a mean multiplicity of 6.6 (95% CI: 5.6–7.6) in age group 5–9 years, whereas RFLP analysis only detected a mean of 5.0 (95% CI: 4.5–5.5) infections. The age trends were similar with the two methods, with average MOI increasing until the age of 5–9 and decreasing during adolescence and adulthood (Figure 2a). Mean multiplicity in each age group was analyzed separately for 3D7- and FC27-type alleles (Figure 2, b and c). 3D7-type infections were more frequent in all age groups than FC27-type infections.

Infection dynamics. To describe the longitudinal genotyping data, we determined the numbers of transitions between genotypes of consecutive samples (Table 2). A transition is either a loss of a genotype or a gain (depicted as patterns + −and − +, respectively). When comparing both genotyping methods, a similar age distribution of transitions (Figure 3, a and b) was found. For both methods, the gain of new infections was highest in children aged 5–9. The loss of infection was highest in people > 60 years of age, and the persistence of infecting clones was less frequent in older age groups.

The pattern of gains and losses of genotypes over the course of the 12 months of the study exhibited seasonal variation (Figure 4). The number of gains was highest in the first to second survey, reflecting the higher transmission rate during the wet season. Gains became gradually less frequent in the following surveys. Loss and persistence rates remained unchanged throughout the year. Comparison of both genotyping methods showed that new alleles (gain − +) were more frequently detected by GeneScan than by RLFP. The number of losses was slightly higher in samples analyzed with RFLP.

Statistical analysis and modeling. The above description of simple transitions does not take into account the imperfect

TABLE 2
Frequencies of transition by allelic family assessed by GeneScan and RFLP

Family	Number of intervals	Gain		Loss		Persistence	
		Number of transitions	Gains per interval	Number of transitions	Percent of possible losses	Number of transitions	Percent of possible losses
GeneScan							
3D7	418	820	1.96	891	80.3%	218	19.7%
FC27	418	360	0.86	370	59.4%	253	40.6%
RFLP							
3D7	406	600	1.477833	616	83.5%	122	16.5%
FC27	406	323	0.795567	314	48.2%	338	51.8%

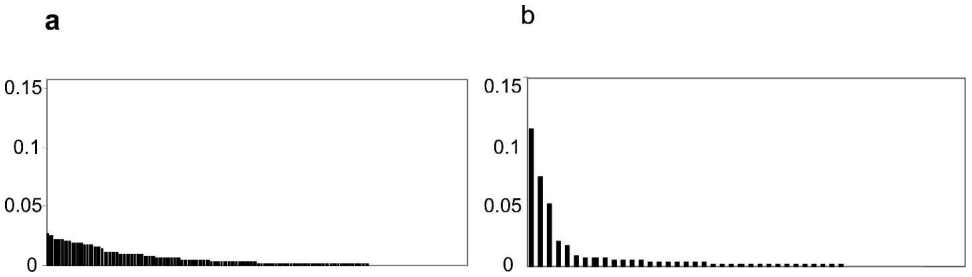

FIGURE 1. Frequencies of 164 different *msp2* genotypes detected by GeneScan in six 2-monthly surveys of 99 individuals from Ghana (total number of parasite clones detected $N = 1{,}405$). (a) Genotypes belonging to the 3D7 allelic family ($N = 116$). (b) FC27-type genotypes ($N = 48$).

detection of sequestered parasite clones. Statistical models can be applied to estimate the true presence and the actual persistence of genotypes.[1,8] While paired samples do not allow identification of the detectability (the detected proportion of all genotypes actually present), this can be estimated from sequences of three or more consecutive samples from the same individual. We analyzed our data according to an approach proposed by Sama and others,[8] making use of an immigration death model. Immigration refers to the acquisition of a new parasitic genotype with rate λ, and death refers to the clearance of a parasitic genotype with rate μ. The model assumes that corresponding to each observed process that is the detection or failure to detect a parasitic genotype is an underlying true process, which is hidden as a result of imperfect detection by the parasitological diagnostic tool (GeneScan or RFLP). For GeneScan, this approach estimates a detectability of 0.35 (95% CI: 0.31–0.39) that is an average of 35% of the parasite present in the host are detected in a finger-prick blood sample. For RFLP, the detectability was 0.47 (0.42–0.51). This estimation of the detectability was also done based on a simpler approach,[7] which led to comparable results (data not shown).

Based on the genotype acquisition and clearance rates λ and μ, respectively, we determined the average duration of an infection. We estimated a value of 19.6 (17.3–22.0) per year for λ and 1.7 (1.4–2.1) per year for μ, which corresponds to an average duration of infection of 210 (176–256) days when samples were analyzed by GeneScan. For RFLP analysis, λ and μ were found to be 16.3 (14.8–18.0) and 2.4 (2.1–2.7), respectively, which corresponds to an average duration of infection of 152 (133–177) days. Our comparison of both genotyping techniques and both statistical approaches showed that the turnover rate for 3D7- type alleles was almost twice as high as for the FC27-type alleles and that infections with FC27-type alleles persisted longer.

DISCUSSION

The *P. falciparum msp2* gene is highly polymorphic and therefore qualifies as an ideal marker gene for analysis of parasite dynamics. The PCR-RFLP genotyping technique has been applied in many descriptive and analytical studies.[15–18] GeneScan-based *msp2* genotyping overcomes some limitations of PCR-RFLP. The major advantage lies in the accuracy of identifying particular genotypes in complex mixtures and in different samples. Misclassification of the allelic family is prevented by using fluorescently labeled primers, allele frequencies can be determined precisely, and throughput is increased considerably. Alternative genotyping approaches were also based on discrimination of amplicon size, but genotypes represented discrete bins, spanning 20 nucleotides and comprising several alleles of similar size.[19,20] For some loci, this identifies only a few alleles, making it necessary to use statistical models to estimate allele frequencies.[21] Our new methodol-

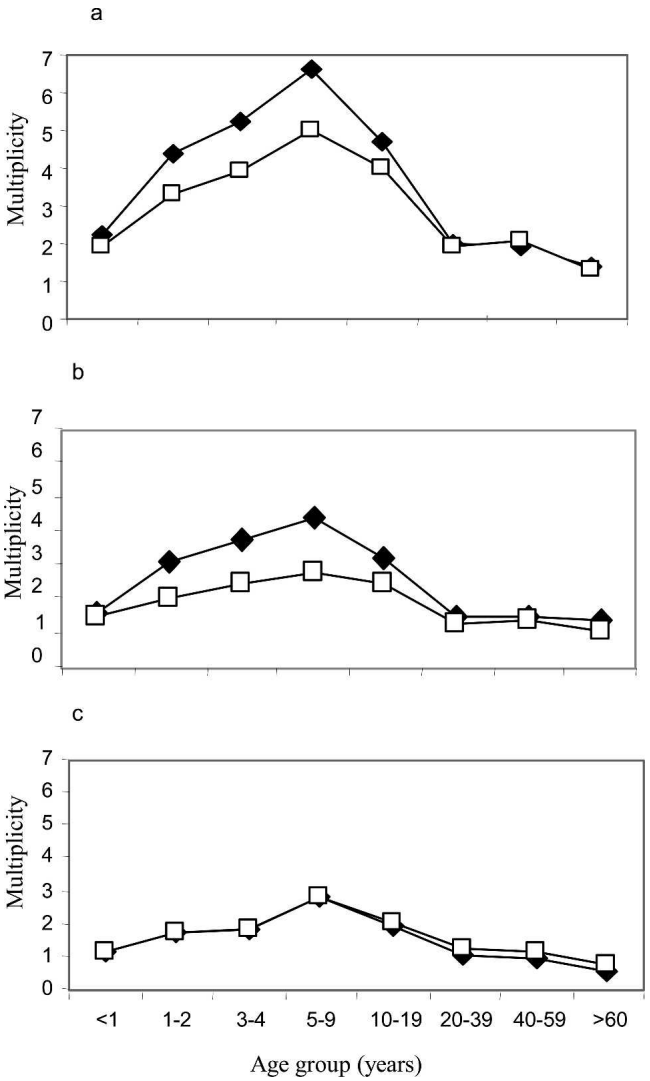

FIGURE 2. Age dependency of mean multiplicity. (a) Overall multiplicity assessed by GeneScan and PCR-RFLP. (b) 3D7-type *msp2* alleles. (c) FC27-type *msp2* alleles.

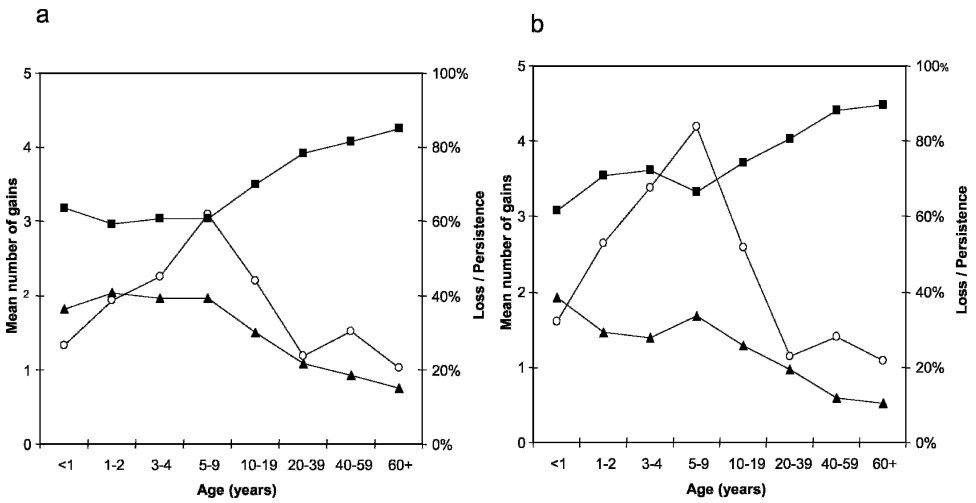

FIGURE 3. Number of newly acquired, lost, or persisting infections per person-interval by age group, determined with (a) RFLP and (b) GeneScan.

ogy produces precise genotype frequencies that are essential to estimate the rate of reinfection with the same genotype.

In our study, we investigated several parameters: MOI by age, different transition states by allelic family, and seasonal effects on these transitions. In both data sets, mean MOI was low among infants, increased steadily until 5–9 years, and started to decrease again during adulthood. This finding is consistent with the previously published cross-sectional results from the first survey.[11] The distribution of the three transition types (gain, loss, and persistence of clones) was similar in both methods, with acquisition of infections being most frequent within the first and second transition interval, reflecting the increase in transmission rate during the second half of the wet season. The number of transitions, however, was different between the two laboratory methods. By GeneScan, more genotypes were gained, whereas by PCR-RFLP, the clearance rate was higher. Both these findings might be caused by higher sensitivity of GeneScan. In particular, 3D7-type alleles are difficult to determine in polyacrylamide gels used for PCR-RFLP but easy to identify by using family-specific fluorescent primers in GeneScan analysis, resulting in a higher number of acquisitions. The higher percentage of losses by PCR-RFLP can also be explained by higher sensitivity of GeneScan, because the transition pattern + − − by RFLP analysis was found to be + − + by GeneScan.

Another parameter calculated from transition rates was the duration of infections. The turnover rate of 3D7 infections was higher in both data sets. This suggests that FC27 alleles are more stable over time and more resistant to elimination under selective pressure by the host's immune system than 3D7 alleles.

Sensitivity and detectability. The sensitivity of PCR-RFLP versus the GeneScan technique and their performance in a molecular epidemiologic field study can be compared by the total number of parasite clones detected by each technique. Compared with PCR-RFLP, we found an increase by 22.8% for GeneScan-based genotyping. This is also reflected by the parameter "mean MOI," which by GeneScan showed an increase of 1.6 infections at the peak of the age distribution. We found that the increased sensitivity in field samples was caused by higher precision in discriminating 3D7-type genotypes. This can be explained by the scrambled repeat structure of 3D7-type alleles, giving rise to only small differences in fragment sizes that can hardly be discriminated by PCR-

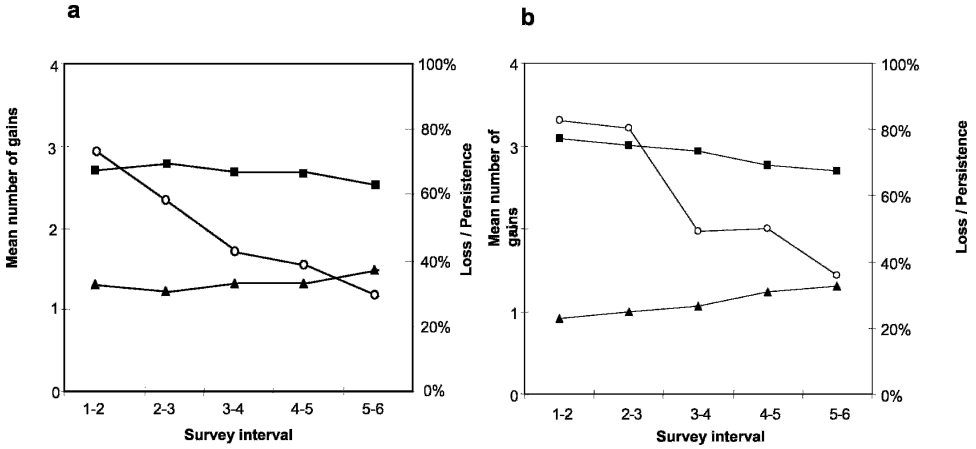

FIGURE 4. Frequencies of transition types by survey interval determined by (a) RFLP or (b) GeneScan.

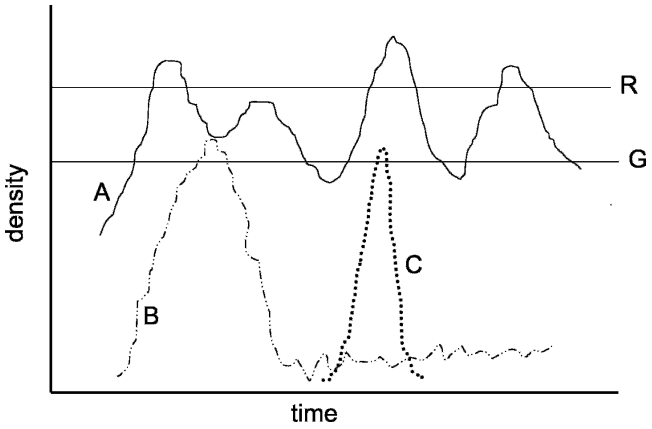

FIGURE 5. Illustration of detection limits of two methods, PCR-RFLP (R) and GeneScan (G), and the implication for detection of infections with three different density profiles, A, B, and C.

RFLP in gel electrophoresis. We conclude that the increase in detected clones by GeneScan typing was accounted for by problems of PCR-RFLP in resolving the complex 3D7-type patterns in multiple infections.

Even optimal sensitivity of *P. falciparum* detection does not reflect the whole parasite population in an infected individual. Sequestration of late stage parasites causes a 48-hour periodicity in detectability. Such fluctuations were observed when monitoring the daily dynamics of *P. falciparum* clones.[5] Thus, imperfect detection of some of the *P. falciparum* clones concurrently present in a host is a consequence of the parasite's life cycle, and the estimation of persistence is complicated even using molecular methods. Therefore, mathematical models need to be applied to estimate the molecular parameters based on such imperfect data.

Both the approaches we used[7,8] take into account the frequencies of transitions. A substantial frequency of the pattern {+ − +} indicates imperfect detection. The detectability is our estimate of that proportion of the duration of an infection during which the densities are high enough to be detected, averaged over all the infections known to be present. Paradoxically, we found a lower detectability by GeneScan (35%) than by PCR-RFLP (47%), despite the fact that GeneScan had detected more genotypes. We arrived at the same conclusion when the method of Smith et al.[7] was used. This can be explained by different detection limits. Figure 5 shows the density profile of three hypothetical infections. Curve A represents an infection that persists at detectable density and is detected in almost the same proportion by both techniques. Curve B represents an infection that occasionally reaches detectable density, but persists sub-patently for a long time. A proportion of such infections is detected by GeneScan, but not by RFLP. Curve C represents an infection that persists for a short time and is also more likely to be detected by GeneScan. It is likely that patterns B and C contribute to a reduced overall estimate of GeneScan detectability compared with RFLP, which never sees infections such as B or C.

Relevance of molecular parameters. The molecular parameters we have used to describe multiple *P. falciparum* infections longitudinally can give new insights in malaria epidemiology. The force of infection can be studied with respect to age, seasonal variation, or as an effect of an intervention. Also, the duration of infections in endemic areas can only be estimated by molecular means because of frequent superinfection. Effects of interventions on duration of infections can provide a valuable outcome measurement.

The potential of GeneScan-based *msp2* genotyping has recently been shown for the discrimination of recrudescent from new infections in drug efficacy studies.[22] We anticipate further applications of molecular parameters in a range of intervention studies against malaria.[23] In malaria vaccine trials, molecular monitoring has been applied to detect selective effects if the vaccine was polymorphic.[17,24] Molecular infection dynamics parameters could further contribute to describe the parasitological outcomes of vaccine trials and perhaps elucidate biologic effects of candidate vaccines.

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REFERENCES

1. Smith T, Vounatsou P, 2003. Estimation of infection and recovery rates for highly polymorphic parasites when detectability is imperfect, using hidden Markov models. *Stat Med 22:* 1709–1724.
2. Ntoumi F, Contamin H, Rogier C, Bonnefoy S, Trape JF, Mercereau-Puijalon O, 1995. Age-dependent carriage of multiple Plasmodium falciparum merozoite surface antigen-2 alleles in asymptomatic malaria infections. *Am J Trop Med Hyg 52:* 81–88.
3. Felger I, Irion A, Steiger S, Beck HP, 1999. Genotypes of merozoite surface protein 2 of Plasmodium falciparum in Tanzania. *Trans R Soc Trop Med Hyg 93 (suppl 1):* 3–9.
4. Smith T, Felger I, Tanner M, Beck HP, 1999. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. *Trans R Soc Trop Med Hyg 93 (suppl 1):* 59–64.
5. Farnert A, Snounou G, Rooth I, Bjorkman A, 1997. Daily dynamics of *Plasmodium falciparum* subpopulations in asymptomatic children in a holoendemic area. *Am J Trop Med Hyg 56:* 538–547.
6. Bruce MC, Donnelly CA, Packer M, Lagog M, Gibson N, Narara A, Walliker D, Alpers MP, Day KP, 2000. Age- and species-specific duration of infection in asymptomatic malaria infections in Papua New Guinea. *Parasitology 121:* 247–256.
7. Smith T, Felger I, Fraser-Hurt N, Beck HP, 1999. Effect of insecticide-treated bed nets on the dynamics of multiple *Plasmodium falciparum* infections. *Trans R Soc Trop Med Hyg 93 (suppl 1):* 53–57.
8. Sama W, Owusu-Agyei S, Felger I, Vounatsou P, Smith T, 2005. An immigration-death model to estimate the duration of malaria infection when detectability of the parasite is imperfect. *Stat Med 24*: 3269–3288.
9. Felger I, Tavul L, Kabintik S, Marshall V, Genton B, Alpers M, Beck HP, 1994. *Plasmodium falciparum:* extensive polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua New Guinea. *Exp Parasitol 79:* 106–116.

10. Binka FN, Morris SS, Ross DA, Arthur P, Aryeetey ME, 1994. Patterns of malaria morbidity and mortality in children in northern Ghana. *Trans R Soc Trop Med Hyg 88:* 381–385.
11. Owusu-Agyei S, Smith T, Beck HP, Amenga-Etego L, Felger I, 2002. Molecular epidemiology of *Plasmodium falciparum* infections among asymptomatic inhabitants of a holoendemic malarious area in northern Ghana. *Trop Med Int Health 7:* 421–428.
12. Felger I, Beck HP, 2002. Genotyping of *Plasmodium falciparum*. PCR-RFLP analysis. *Methods Mol Med 72:* 117–129.
13. Brownstein MJ, Carpten JD, Smith JR, 1996. Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. *Biotechniques 20:* 1004–1006.
14. Beck HP, 2002. Extraction and purification of Plasmodium parasite DNA. *Methods Mol Med 72:* 159–163.
15. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, Rare L, Baisor M, Lorry K, Brown GV, Pye D, Irving DO, Smith TA, Beck HP, Alpers MP, 2002. A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. *J Infect Dis 185:* 820–827.
16. Felger I, Smith T, Edoh D, Kitua A, Alonso P, Tanner M, Beck HP, 1999. Multiple *Plasmodium falciparum* infections in Tanzanian infants. *Trans R Soc Trop Med Hyg 93 (suppl 1):* 29–34.
17. Fraser-Hurt N, Felger I, Edoh D, Steiger S, Mashaka M, Masanja H, Smith T, Mbena F, Beck HP, 1999. Effect of insecticide-treated bed nets on haemoglobin values, prevalence and multiplicity of infection with *Plasmodium falciparum* in a randomized controlled trial in Tanzania. *Trans R Soc Trop Med Hyg 93 (suppl 1):* 47–51.
18. Irion A, Felger I, Abdulla S, Smith T, Mull R, Tanner M, Hatz C, Beck HP, 1998. Distinction of recrudescences from new infections by PCR-RFLP analysis in a comparative trial of CGP 56 697 and chloroquine in Tanzanian children. *Trop Med Int Health 3:* 490–497.
19. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol S, 1999. Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans R Soc Trop Med Hyg 93:* 369–374.
20. Ranford-Cartwright LC, Balfe P, Carter R, Walliker D, 1993. Frequency of cross-fertilization in the human malaria parasite *Plasmodium falciparum*. *Parasitology 107:* 11–18.
21. Hill WG, Babiker HA, 1995. Estimation of numbers of malaria clones in blood samples. *Proc Biol Sci 262:* 249–257.
22. Jafari S, Le Bras J, Bouchaud O, Durand R, 2004. *Plasmodium falciparum* clonal population dynamics during malaria treatment. *J Infect Dis 189:* 195–203.
23. Felger I, Genton B, Smith T, Tanner M, Beck HP, 2003. Molecular monitoring in malaria vaccine trials. *Trends Parasitol 19:* 60–63.
24. Bojang KA, Milligan PJ, Pinder M, Vigneron L, Alloueche A, Kester KE, Ballou WR, Conway DJ, Reece WH, Gothard P, Yamuah L, Delchambre M, Voss G, Greenwood BM, Hill A, McAdam KP, Tornieporth N, Cohen JD, Doherty T, 2001. Malaria Vaccine Trial Team. Efficacy of RTS,S/AS02 malaria vaccine against *Plasmodium falciparum* infection in semi-immune adult men in The Gambia: a randomized trial. *Lancet 358:* 1927–1934.