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TRANSMISSION INTENSITY AND *PLASMODIUM FALCIPARUM* DIVERSITY ON THE NORTHWESTERN BORDER OF THAILAND

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Abstract. Genetic analysis of the number of *Plasmodium falciparum* genotypes per infected person in regions of holoendemic and hyperendemic malaria suggest that in areas of lower transmission intensity, significantly fewer parasite genotypes per infected person should be found. A predominance of single clone infections in the human population could generate the controversial clonal population structure proposed for *P. falciparum* by Tibayrenc and others. Characterization of *P. falciparum* from individuals on the Thai-Burmese border, an area of hypoendemic transmission, revealed a higher number of genotypes per infected person than that predicted. Possible reasons for this observation are discussed, with particular attention paid to human migration and multidrug resistance.

Plasmodium falciparum malaria exhibits great diversity in both phenotypic and genotypic characteristics on a worldwide scale.[1, 2] Many cross-sectional studies have revealed the extent of multiple infections of *P. falciparum* genotypes in human hosts from regions of differing endemicity and transmission characteristics.[1–6] Molecular epidemiology studies in Ifakara, Tanzania[7] and Madang, Papua New Guinea[8] have recently drawn attention to the relationship between the mean number of parasite genotypes in the infected population, transmission intensity, and the genetic structure of *P. falciparum* populations. The 10-fold lower levels of transmission intensity in Papua New Guinea[9] compared with Tanzania,[10] as measured by the entomologic inoculation rate (EIR), was associated with measurable differences, using the same polymerase chain reaction (PCR) techniques, in cross-sectional measures of the number of genotypes per infected person and parasite mating patterns as determined by oocyst heterozygosity. There was a lower number of genotypes in infected humans from Papua New Guinea with significantly higher levels of inbreeding in *P. falciparum* populations. As is evident from these two studies, the genetic structure of *P. falciparum* populations is neither clonal nor panmictic, but a function of the transmission intensity. Such variability in parasite mating patterns may have important consequences for the global spread of multigenic phenotypes such as vector competence, parasite virulence, and anti-malarial drug resistance. While the number of genotypes per infected person appears to correlate with transmission intensity, the five-fold difference in transmission between coastal and inland regions of Papua New Guinea results in only a small difference in the number of genotypes per infected person,[8] compared with the much larger difference observed in Tanzania. Such a discrepancy suggests that the relationship between transmission intensity and number of genotypes per infected person may be nonlinear.

Observations from Papua New Guinea and Tanzania predict that in areas of lower transmission intensity, significantly fewer parasite genotypes per infected person would be found with the concomitant effect on parasite mating patterns in the vector. Indeed, a predominance of single clone infections in the human population could generate the controversial clonal population structure proposed for *P. falciparum* by Tibayrenc and others.[11, 12] This paper aims to confirm the relationship between transmission intensity and the number of multiple infections per human host by detailing the extent of *P. falciparum* diversity in the Shoklo camp on the Thai-Burmese border, an area of very low transmission intensity, where individuals are believed to receive less than one infection per year[13] and thus are expected to harbor only one parasite genotype at one time. This is the first report of parasite diversity in this region of Thailand, although characterization of culture-adapted isolates taken from other regions of Thailand have demonstrated a high degree of diversity in enzymes, antigens, and drug sensitivity[14, 15] and have shown clonal diversity even within a single isolate.[16, 17]

MATERIALS AND METHODS

Study site. The project was carried out in association with the Shoklo Malaria Research Unit in the Shoklo camp for displaced persons of the Karen ethnic minority, situated near Mae Sod on the Thai-Burmese border. This camp has a population of approximately 5,000 inhabitants. Malaria is the main health problem in the camp, with a coexistence of both *P. falciparum* and *P. vivax* and very low levels of *P. malariae*. *Plasmodium falciparum* in this area is the most drug-resistant in the world and even mefloquine sensitivity is decreasing.[18] Malaria transmission is seasonal and unstable, with two peaks in May–July and November–January.[13, 19] The annual incidence of *P. falciparum* malaria is approximately 40%.[13] Incidence rates increase with age to a peak in individuals between the ages of 20 and 29 years, most probably because of their tendency to travel frequently in the forests surrounding the camp where there appears to be a higher risk of acquiring *P. falciparum*.[13] The incidence of severe malaria, however, decreases with age. Prevalence rates rarely exceed 10%, even during peak transmission;[13] 84–93% of infections are symptomatic cases and attack rates are estimated at about one per year even for the most infected age group.[13] The predominant mosquito vectors are *Anopheles maculatus* and *An. minimus*, with relatively few *An. dirus*. These mosquitoes are principally forest fringe dwelling, and the summer peak of malaria transmission coincides with the expansion of the vector population during the rainy season.

Patients were enrolled into the study after they or their

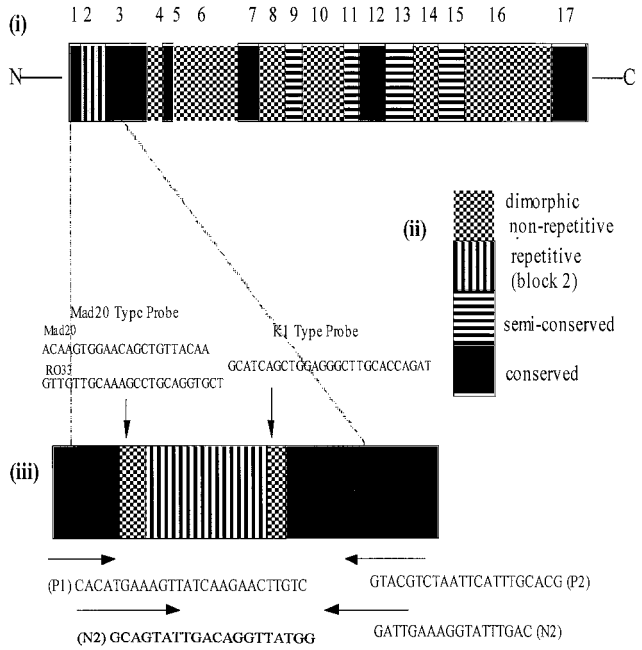


FIGURE 1. Schematic diagram of the Merozoite surface protein-1 gene after Ranford-Cartwright and others.[23] **(i)** The gene can be conveniently divided into blocks (1-17) according to the conserved and repetitive nature of the sequence. The N and C terminal ends of the gene are indicated. **(ii)** The conserved and repetitive nature of the sequence depicted in **(i)**. **(iii)** The region of amplification surrounds block 2, which contains tandem repeats and is flanked by a dimorphic sequence against which DNA probes will hybridize. Two pairs of primers are used in consecutive rounds of polymerase chain reaction amplification.

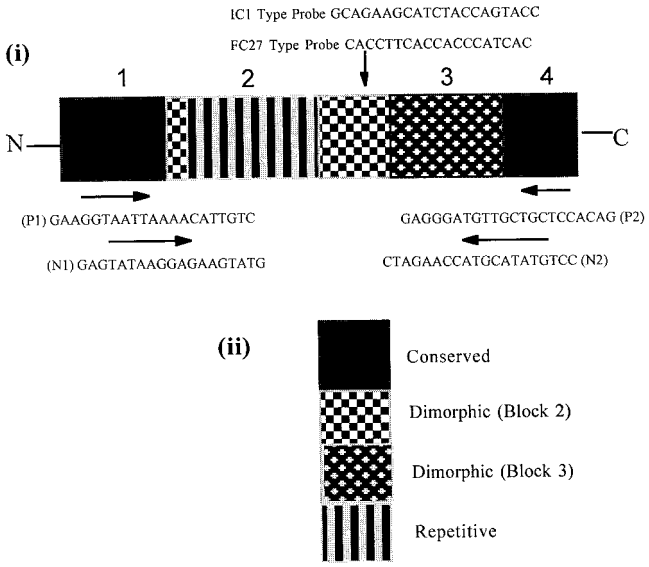


FIGURE 2. Schematic diagram of the merozoite surface protein-2 gene after Ranford-Cartwright and others.[23] **(i)** The gene can be conveniently divided into blocks (1–4) according to the conserved and repetitive nature of the sequence. The region of amplification surrounds block 2, which contains tandem repeats and is flanked by a dimorphic sequence against which DNA probes will hybridize. Two pairs of primers are used in consecutive rounds of polymerase chain reaction amplification. The N and C terminal ends of the gene are indicated. **(ii)** The conserved and repetitive nature of the sequence depicted in **(i)**.

accompanying relatives gave fully informed consent. The project was approved by the ethical committees of the Faculty of Tropical Medicine, Mahidol University (Bangkok, Thailand), and the Karen Refugee Committee (Mae Sod, Thailand).

Parasite sampling. Previous studies in areas of more intense transmission have shown that the number of *P. falciparum* genotypes per infected person decreases with age, which most probably reflects the development of immunity.[6] The ratio of asymptomatic/symptomatic malaria in this area has been reported as similar in all age groups.[13] Samples were taken from each age group to account for any such age-dependent variability in the number of genotypes per infected person. Because this study occurred during the recent SPf66 vaccine trial,[20] the majority of 2–15-year old individuals were receiving either SPf66 vaccine or a placebo (hepatitis B vaccine). Even though the SPf66 vaccine had no effect on the incidence of malaria, we analyzed the effect of treatment (placebo or vaccine) on the number of genotypes per infected person. Blood samples were spotted onto Whatman (Maidstone, United Kingdom) paper from 56 *P. falciparum* slide-positive persons attending health centers during March–July 1994. The range of parasite densities (1–2,000 infected red blood cells per 500 white blood cells were comparable with those observed in Papua New Guinea (1–500 infected red blood cells per 200 white blood cells), where similar genotyping studies were conducted (Paul REL, 1996, *The Genetic Diversity of Plasmodium falciparum*, Ph.D Thesis, Oxford University, Oxford, United Kingdom).

Extraction of DNA. Parasite DNA was extracted from the blots using a modification of the Chelex extraction method.[21, 22] The blood spot was soaked for 10–20 min in a 1.5-ml Eppendorf (BDH Merk, Poole, United Kingdom) tube containing 1 ml of phosphate buffer solution (8 g of NaCl, 0.2 g of KCl, 1.15 g of NaH_2PO_4, 0.24 g of K_2HPO_4 per liter, pH 7.4)/1% saponin, which increases DNA yield by aiding the release of hemoglobin from the paper. The tube was centrifuged for 2 min at 13,000 rpm in a microcentrifuge and the supernatant was discarded. One milliliter of 1× phosphate buffer solution was added to the tube, centrifuged for 2 min at 13,000 rpm, and the supernatant was discarded. One hundred fifty microliters of Molecular Biology Grade water and 50 μl of a 20% suspension of Chelex 100 resin (Bio-Rad, Hemel Hempstead, United Kingdom) was added. The tube was placed in a boiling water bath for 8 min and then centrifuged for 1 min at 13,000 rpm. The supernatant, which contained the DNA, was carefully removed to avoid transfer of the Chelex resin.

Amplification by PCR and in situ hybridization of three *P. falciparum* polymorphic loci. Three polymorphic loci, merozoite surface proteins 1 and 2 (MSP-1 and MSP-2) and the glutamate-rich protein (GLURP), were used for the genetic analysis of the parasite population. Regions of MSP-1 (Figure 1) and MSP-2 (Figure 2), which vary in repeat number and in adjacent sequence type, and a region of GLURP (Figure 3), which varies only in repeat number, were amplified using the PCR following the method of Ranford-Cartwright and others[23] and Paul and others.[8] The PCR

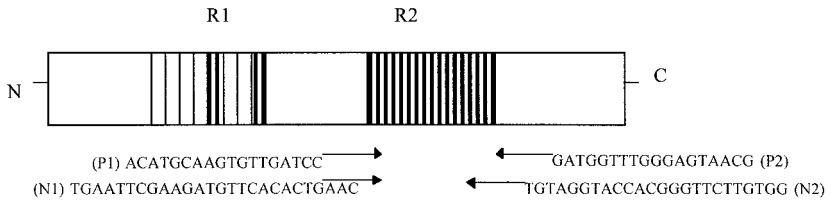


FIGURE 3. Schematic diagram of the glutamate-rich protein gene after Borre and others[43] depicting the region of amplification around the repetitive region R2. Also shown is R1, a region of repeats of varying lengths. The N and C terminal ends of the gene are indicated. Two pairs of primers are used in consecutive rounds of polymerase chain reaction amplification. Primer details are provided in the report by Paul and others.[8]

products were sized against standards of known molecular weight (VI pBR328 *Bgl* I/*Hinf;* Boehringer, Lewes, United Kingdom) on 1.8% agarose gels. The PCR products of MSP-1 and MSP-2 were transferred by Southern transfer onto nylon membranes (Boehringer) and probed with allele-specific probes using the nonradioactive chemiluminescent detection methods described in the studies of Babiker and others[7] and Paul and others.[8] The PCR detection of parasite DNA extracted using this Chelex method has been shown to be equally sensitive compared with other DNA extraction methods, requiring a minimum of 40 parasites or a parasite count of one infected red blood cell per 200 white blood cells.

Genetic and statistical analysis. The potential number of genotypes per infected person will depend on the amount of polymorphism in the transmission population. Previous data suggest that while the overall number of genotypes in the transmission population relates to the transmission intensity, it is not a limiting factor in the number of genotypes per infected person, i.e., there are more alleles in any study population than that found within any individual. Allele frequency distributions for all three loci are calculated for this region of Thailand to ensure that there is sufficient polymorphism to allow a comparison of genotypes per infected person with regions of higher transmission intensity. Furthermore, to exclude any confounding effect of vaccine treatment, age, and sex specific to Thailand, the association of multiple genotype infections with these factors was analyzed with GLIM 377[24] software.

Analysis of multilocus genotypes has often been used to investigate parasite mating patterns[11, 12] and provides information on the structure of the parasite population, which can vary regionally despite equivalent allelic diversity. The GENEPOP[25] (version 1.2) was used to analyze patterns of linkage between loci.

RESULTS

Patterns of parasite diversity for MSP-1, MSP-2, and GLURP were described for *P. falciparum*-positive cases of clinical malaria from the Shoklo Camp. From the 56 samples, there was a 95% (n = 53) PCR success rate with MSP-2, 86% (n = 48) with MSP-1, and 77% (n = 43) with GLURP. These rates are similar to those previously achieved with field samples.[7, 8] All PCR-positive samples probed successfully for MSP-2 and 92% (n = 44) probed successfully for MSP-1.

Despite the very low transmission intensity in Shoklo, the diversity of alleles of MSP-1 (nine alleles), MSP-2 (12 alleles), and GLURP (seven alleles) was comparable with that previously described for Papua New Guinea (MSP-1: nine alleles, MSP-2: 14 alleles, and GLURP: seven alleles),[8] but lower than that observed in Tanzania (MSP-1: 17 alleles, MSP-2: 23 alleles)[7] using the same genotyping methods and a similar sample size (Figures 4–7). The allele frequencies for MSP-1 and MSP-2 did not exceed 25% for any single allele, although for GLURP, one allele (820–879 basepairs) was particularly dominant (42%) (Figure 4).

The comparable nature of the parasite diversity, at least to Papua New Guinea, and the allelic frequencies at these three loci justified a comparison of the number of genotypes per infected person. A high number of double infections were observed for MSP-2 (57%) (Figure 5); 25% of the infections were double (n = 11) for MSP-1 (Figure 6) and only 9% were double for GLURP (Figure 7). The mean ± SE number of genotypes per infected person was found to be 1.67 ± 0.06), which is considerably larger than expected with such low malaria transmission. A study from Senegal[6] found a significant effect of age on the number of genotypes per infected person and a recent epidemiologic study in Shoklo suggests a peak incidence of *P. falciparum* in men in their third decade.[13] Using all three loci and taking the conservative estimate of genotype number, 68% (28 of 41) of those less than 15 years old compared with 77% (10 of 13) of those more than 15 years old had two parasite genotypes. Statistical analysis revealed no clear association between the number of genotypes per infected person with age or sex (GLIM Poisson, age $\chi^2_{0.05,\ 2} = 0.21$; sex $\chi^2_{0.05,\ 1} = 0.11$), although the sample size was small and biased toward those less than 15 years old as a result of the nature of self-presentations to health centers. There was no effect of vaccine treatment on genotype number ($\chi^2_{1,\ 0.05} = 0.875$).

Linkage analyses, which examine nonrandom associations between alleles from different loci, provide information on the genetic structure of the parasite population. Despite the unexpectedly high number of genotypes per infected person in Shoklo and the consequent potential for recombination, linkage analysis can still provide an insight into the mating structure of the parasite population. Because of the high degree of multiple infections detected with MSP-2, it was not possible to perform linkage analysis between all three loci. Linkage analysis was carried out for MSP-1 and GLURP using MSP-1 sequence categories (K1, MAD20, and RO33) and the seven alleles of GLURP. No linkage was detected (GENEPOP, Fisher exact $P = 0.17$, SE = 0.007, n = 31). The absence of linkage disequilibrium found between the above loci in the Shoklo parasite population is consistent with observations made in Papua New Guinea and Tanzania where out-crossing has been demonstrated, although as

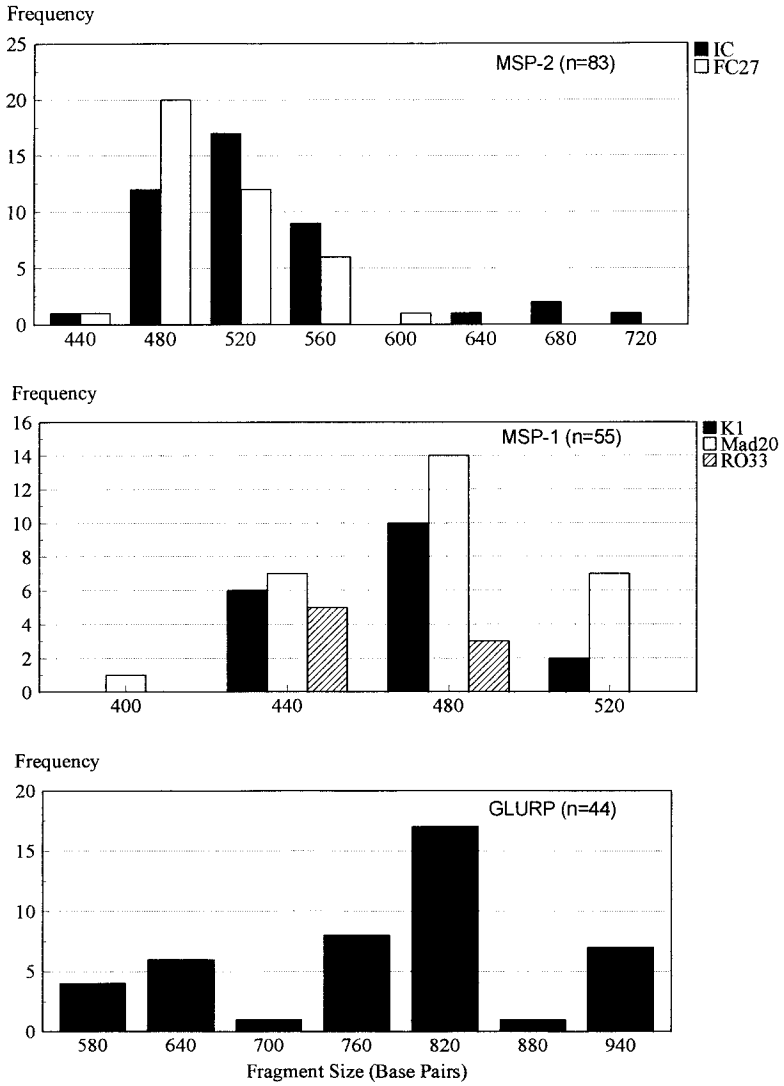


FIGURE 4. Allele frequency distribution of three *Plasmodium falciparum* polymorphic loci (merozoite surface protein-2 [MSP-2], MSP-1, and glutamate-rich protein [GLURP]) in 56 humans from Thailand. Alleles of MSP-1 and MSP-2 are characterized by fragment size and by sequence type, using digoxigenin DNA in situ hybridization. The GLURP alleles are characterized by fragment size only. n refers to the total number of alleles of each locus found.

pointed out by Tibayrenc and Lal,[26] the sample size is too small to prove significantly the occurrence of linkage equilibrium. Of the multiple infections with MSP-2 genotypes, three genotype pairs (480 IC/FC, 520 IC/FC, and 560 IC/FC) accounted for 83% (n = 25) of all MSP-2 double infections (n = 30). The MSP-1 alleles associated with these MSP-2 double genotypes suggest that they are not the same parasites.

DISCUSSION

The hypothesis tested in this study was that there was a predictable relationship between transmission intensity and the mean number of genotypes per infected person as measured at a point in time. Comparison of relevant cross-sectional data from the literature[4–8, 27, 28] suggests that such a relationship between transmission intensity and the mean number of genotypes per infected person does exist, but that it appears to be nonlinear. The 10-fold higher EIRs in Senegal[6] or Tanzania[10] compared with Papua New Guinea[9] are associated with a concomitant increase in the mean number of genotypes per infected person, but the much lower, albeit seasonal, EIR in The Gambia[27] results in a similar mean genotype number to that in Papua New Guinea.[5, 8, 28] This study examined an area of very low transmission that is a focus of multidrug resistance and found that overall diversity in the parasite population was less than that in regions of higher transmission intensity. There was a greater number of genotypes per infected person than predicted on the basis of the EIR. Although this result contradicts the proposed relationship, the deviation from the prediction may be the result of particular demographic or parasitologic features of the region.

In Shoklo, a region of hypoendemic malaria, the number of infective bites received per year was estimated from the number of independent symptomatic attacks received; i.e.,

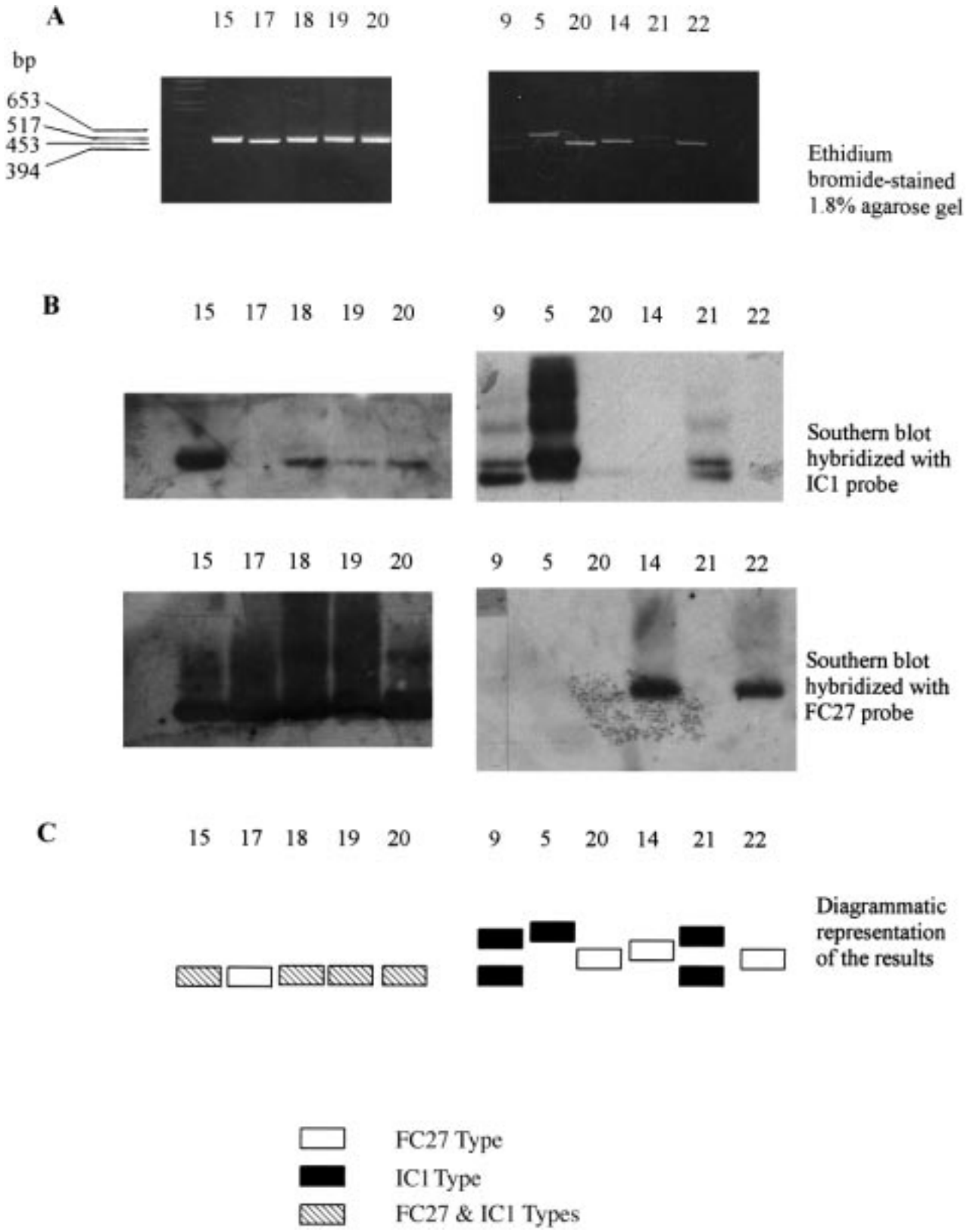
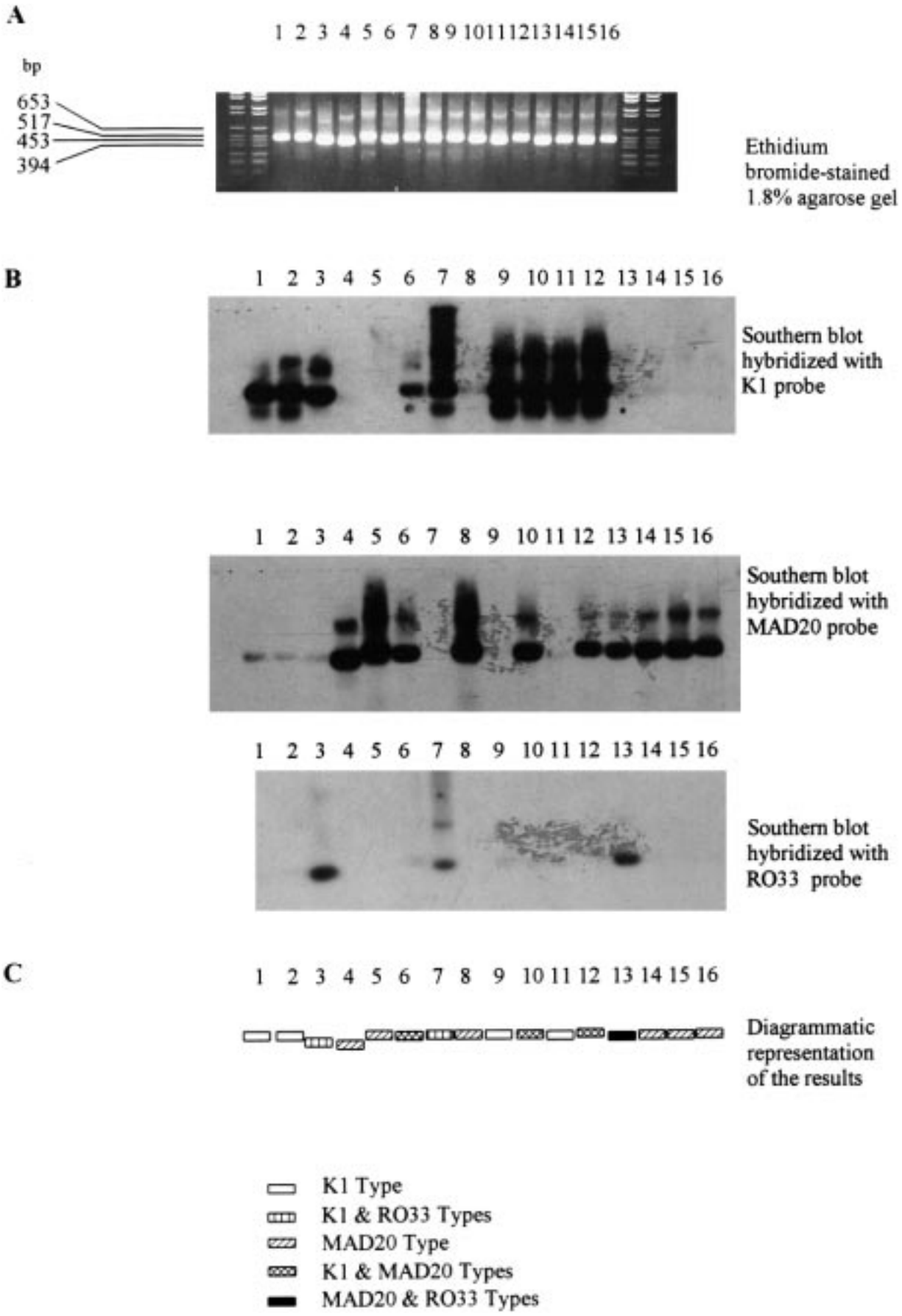


FIGURE 5. Polymerase chain reaction (PCR) typing of merozoite surface protein 2 (MSP-2) (repeats) alleles in a sample of self-presentations in the Shoklo camp on the Thai-Burmese border during July 1994. **A,** analysis of the PCR products by ethidium bromide staining of the agarose gel. **B,** results of hybridization with family-specific probes (ICI and FC27). **C,** schematic representation of the alleles detected in the various blood samples; codes for each family are as indicated. Numbers represent patient code numbers. bp = basepairs.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
bp
653
517
453
394
Ethidium bromide-stained 1.8% agarose gel
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Southern blot hybridized with K1 probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Southern blot hybridized with MAD20 probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Southern blot hybridized with RO33 probe
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Diagrammatic representation of the results
K1 Type
K1 & RO33 Types
MAD20 Type
K1 & MAD20 Types
MAD20 & RO33 Types

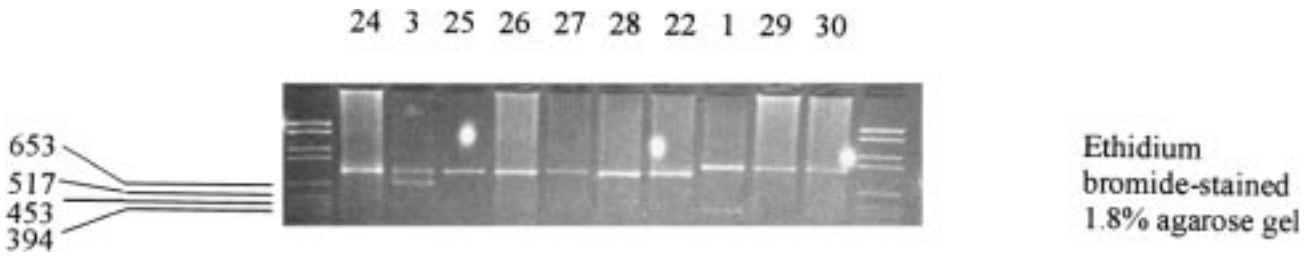


FIGURE 7. Polymerase chain reaction (PCR) typing of glutamate-rich protein (repeats) alleles in a sample of self-presentations in the Shoklo camp on the Thai-Burmese border during July 1994. Analysis of the PCR products by ethidium bromide staining of the agarose gel. Numbers represent patient code numbers. Values on the left are in basepairs.

because immunity is acquired so slowly in such areas of low transmission, almost every infective bite (90% for all age groups) is believed to cause a symptomatic response.[13] If an individual receives less than one infective bite a year, to be infected with multiple parasite genotypes there are two possibilities: 1) individuals infected with multiple genotypes in another region of more intense transmission generate multiple parasite genotypes in mosquitoes that then inject a single inoculum containing multiple genotypes; 2) the duration of a single infection must exceed one year and so overlap with new infections. The low incidence levels suggest that the generation of a multiple genotype inoculum as a result of an interrupted mosquito feed is of negligible importance. Thus, the observed high numbers of multiple genotype infections either reflect patterns of migration or result from factors influencing the duration of infection.

The intensity of transmission is known to vary substantially over short geographic distances in much of Southeast Asia.[29, 30] The elevated numbers of genotypes per infected person may result from migration between areas of more intense transmission, for example, the surrounding forests and even Burma, and the study area.[13, 19] This would artificially increase the number of genotypes per infected person in Shoklo and even produce a transmission cycle of multiple genotype inoculations, resulting in multiply infected nonimmigrants. The demographic nature of Shoklo, which is a Karen refugee camp, ensures some immigration of individuals exposed to malaria from outside the camp. Although the risk of malaria in this area is increased with travel and being a young adult male,[13] less than 14% of clinical cases of malaria are attributable to migration outside the camp.[13] Despite this and from the limited analysis performed here, the absence of any discernible increase in mean parasite genotype number associated with age, young adult males could provide the reservoir of multiple genotype inocula.

Information concerning the duration of infection is difficult to obtain in endemic areas because of the occurrence of sequential infections, the difficulties of repeated sampling, and the need to treat symptomatic infections with antimalarial drugs. In this region, since virtually every case is symptomatic, drug treatment precludes study of the duration of infection. Induced infection experiments in neurosyphilitic patients produced infections that lasted a year.[31] It has been recently reported that *P. falciparum* can last for more than two years in a semi-immune individual.[32] Whether such persistence of asymptomatic subpatent infections occurs in Shoklo is unclear. Approximately 90% of the infections become symptomatic, but with no detectable differences across age groups,[13] suggesting that unlike regions of higher transmission, adults are not a particular reservoir of asymptomatic patent infections. Pregnant women tend to suffer from more severe disease and parasites are known to sequester in the placenta from where they recrudesce.[33, 34] Such individuals offer another potential source for extended duration of infection and thus more are prone to multiple infections.

The higher than expected number of genotypes per infected person in Shoklo could be therefore explained by speculating that infections persist at subpatent levels longer in areas of low compared to high transmission intensity to maximize gametocyte transmission success. Clearly, in areas of marked seasonality such as Sudan, parasites must persist in individuals in the asymptomatic state for periods up to 10 months to allow continued transmission at the start of the next wet season.[35] If the observations in Shoklo are explained in terms of long duration of infection, the question then arises as to why there are comparatively few infections (1–5) detected at a point in time in young children experiencing 300 infective bites per year in, for example, Senegal[6] or Tanzania.[7] Presumably, high rates of infection will lead to the development of both nonspecific immunity and cross-strain immunity that will shorten the duration of infection and thus the number of overlapping genotypes detected at a point in time. Superinfection data from induced *P. falciparum* infections in humans and *Saimiri* monkeys have shown that immunity is predominantly strain-specific and weakly cross-strain specific.[36, 37] Induced infection experiments in rodent models also support this conclusion.[38–40] Such an explanation is highly speculative and further data on both the duration

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FIGURE 6. Polymerase chain reaction (PCR) typing of merozoite surface protein 1 (MSP-1) (block 2) alleles in a sample of self-presentations in the Shoklo camp on the Thai-Burmese border during July 1994. **A,** analysis of the PCR products by ethidium bromide staining of the agarose gel. **B,** results of hybridization with family-specific probes (K1, MAD20, and RO33). **C,** schematic representation of the alleles detected in the various blood samples; codes for each family are as indicated. Numbers represent patient code numbers. bp = basepairs.

of infection and gametocyte rates across transmission intensities are required.

Another explanation for the Shoklo parasite diversity patterns within humans concerns the influence that antimalarial drug resistance may have on the parasite population dynamics within a human host. Multidrug resistance, a feature of the region, could induce a carrier state in humans by effectively increasing the duration of infection. Infections are often suppressed and not cleared by drugs and eventually recrudesce as asymptomatic infections. Recrudescence of parasites has been shown to occur up to 42 days[41] and is believed to occur up to 63 days following treatment.[42] Extension of the duration of a single infection as a result of treatment and subsequent recrudescence will increase the probability of coinfection with a second parasite genotype.

Irrespective of the actual origin of multiple genotype infections, such a high proportion of polyclonal infections could easily be maintained by the simultaneous transmission and inoculation of multiple genotypes within a single blood feed. Recycling of paired genotypes assumes no within host competition and equal growth and gametocyte production rates. The variety of possible causes for the unexpectedly large number of genotypes per infected person demonstrates the difficulties of interpreting the relationship between transmission intensity and the genetic structure of the parasite population. Our results highlight the limitations of extrapolating conclusions about the population genetics of *P. falciparum* from one region to another based on global malaria paradigms that so often emerge, particularly in limited regions of Africa, with limited knowledge of local epidemiologic conditions. We have made the generalization that the genetic structure of *P. falciparum* populations is a function of transmission intensity in a given endemic area but this relationship may be nonlinear. The observed unexpectedly high number of genotypes per infected person in the human population in this area of low endemicity precludes the use of transmission intensity alone as a predictor of parasite mating patterns. Understanding the causes of such unexpected results is essential for the development of a robust relationship from which predictions on control measures and parasite behavior can be made and so warrants further study.

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