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Random mating of natural *Plasmodium* populations demonstrated from individual oocysts

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DNA amplified from individual *Plasmodium vivax* oocysts, produced by feeding mosquitoes directly on naturally infected humans in Thailand, was used to study cross-mating of 2 polymorphs of the circumsporozoite (CS) gene, VK 210 and VK 247. Alleles were detected in matched blood parasites, sporozoites, and individual oocysts with oligoprobes specific to characteristic repeat units. Oocysts developing from 3 cases in which mixed alleles were present in the blood parasites had genotype frequencies, including hybrids, consistent with the Hardy-Weinberg equilibrium. There was apparently no barrier to hybridization of the 2 alleles nor a bias, as has been found in some laboratory experiments, favoring hybrid formation. These are the first measurements of cross-mating frequencies directly from natural *Plasmodium* infections and the first observations of genetic hybridization in *P. vivax*.

Key words: Malaria; Oocyst; Polymerase chain reaction; Genetics; Circumsporozoite; *Plasmodium vivax*

Introduction

Although malaria parasites are dangerous to man because of their enormous asexual reproductive capacity, they must also undergo a single fertilization and meiosis in the gut of the mosquito vector before transmission can occur. This sexual union is the basis for *Plasmodium*'s wide adaptability and is therefore of great interest. The principal strategy for studying the products of mating has been experimental [1]: mosquitoes are infected with gametes from mixed clones bearing well-defined protein and nucleotide markers; the resulting sporozoites are inoculated into a suitable laboratory host; analysis is then done on blood parasites cloned from these vertebrate models. Such complex, manipulative procedures have limitations. In particular, involvement of model hosts and cloning following mosquito infection could artificially select certain phenotypes, as may have happened in experiments with *Plasmodium falciparum* and rodent parasites that indicated higher than expected numbers of hybrids had been produced [1].

We wished to test the cross-mating ability of 2 naturally occurring variants of the CS gene in *P. vivax* [2]. There have been indications from seasonal [2] and spatial distributions (R. A. Wirtz and M. H. Rodriguez, unpublished data) that the polymorph VK 247 may not freely combine with the predominant type, VK 210. To this end we have measured gene hybridization in individual oocysts from mosquitoes fed on volunteers naturally infected with both CS variants. We present evidence here that the

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Abbreviations: PCR, polymerase chain reaction.

ratios of hybridization occurring are consistent with random mating. This is the first report of plasmodial cross-fertilization analysis directly from natural infections and the first of any kind from *P. vivax*.

Materials and Methods

Individual oocysts of *P. vivax* were removed by collagenase digestion [3] from the gut epithelium of the laboratory reared mosquito *Anopheles dirus* 10–14 days after directly feeding on gametocytemic human volunteers who had been naturally infected at widely separated foci in western Thailand [2]; *A. dirus* is the principal vector in those areas. Infected guts were dissected from 5–10 mosquitoes chosen at random from groups of more than 50; at this stage oocysts are approximately 40 μm in diameter and contain about 4000 nearly mature haploid sporozoites [3], the form eventually infective to man. Oocysts for amplification were chosen randomly, separated into 2 μl PBS (0.01 M, pH 7.4), and then lysed by the addition of 8 μl lysing buffer (50 mM KCl 10 mM Tris 2.5 mM $MgCl_2$, pH 8.3) containing 0.5% Tween 20 and 0.8 μg fresh proteinase K. After 120 min incubation at 55 C, residual proteinase K activity was destroyed by incubation at 94 C for 10 min. DNA in a total reaction volume of 100 μl was amplified by polymerase chain reaction (PCR) [5] using reagents from a commercial kit according to manufacturer's protocol (GeneAmp, Perkin-Elmer Cetus, Norwalk, CT, USA). The oligonucleotide primers Pv5 (5′-GTCGGAATTCAATAAGCTGAAACAAC-C-3′) and Pv6 (5′-GAGCGGATCCACAGGT-TACACTGCAT-3′) are complementary to 2 highly conserved regions of the CS protein gene that bracket a domain consisting of either of 2 variant, tandem repeated sequences: VK 210 and VK 247 [2]. Fragments of about 700 bp were amplified. A temperature profile for 45 amplification cycles that worked well was to denature at 94 C for 1 min, anneal at 37 C for 2 min, and extend at 72 C for 2 min. Amplified products were transferred to a slot blot apparatus and hybridized with complementary ^{32}P end-labeled oligonucleotides derived from the repetitive sequences of VK 210 (5′-GGAGATAGAGCAGCTGGA-3′) and VK 247 (5′-ATTGCCAGCCCCATTTGC-3′). Filters were washed at 45 C, high stringency, and then exposed to radiographic film for 4–18 h at −70 C; 10^3 plasmids containing inserts of VK 210 or VK 247 were amplified as controls. In addition to oocysts, blood stage parasite DNA extracted from filter paper [5] and at least 10^3 pooled salivary gland sporozoites from each case were also amplified [2] and probed. CS phenotypes were determined by monoclonal antibody based ELISAs specific for VK 210 [6] and VK 247 (R. A. Wirtz, unpublished); standardized quantification of sporozoites using the ELISAs was matched to Neubauer chamber counts for each case to assure that only the 2 epitopes were present. Agarose gel electrophoresis visualized with ethidium bromide stain was done on 10% of the amplified product for a subsample of oocysts.

Results

Oocyst genomic DNA was first amplified from 2 cases judged to be infected with only the VK 210 or the VK 247 variants on the basis of results from oligoprobing the blood and sporozoites (Fig. 1). The success of oocyst amplification for case #1112, which was positive only for the VK 210 genotype, was 73% (11/15); for case #1095, which was VK 247 positive, it was 93% (14/15). Neither case showed mixing or heterozygosity of the CS genotypes. Oocysts shown in Fig. 2 are some of those developed in mosquitoes feeding on a case (#1195) infected with both variants; both parental and hybrid genotypes were clearly discernable in the oocysts. A tabulation of the number of selfed and hybrid oocysts developing from 3 mixed infections is presented in the Table; there is no indication from these data that the methods we employed favored a particular ratio.

The results of at least 2 of the 3 mixed cases are consistent with fertilization ratios expected

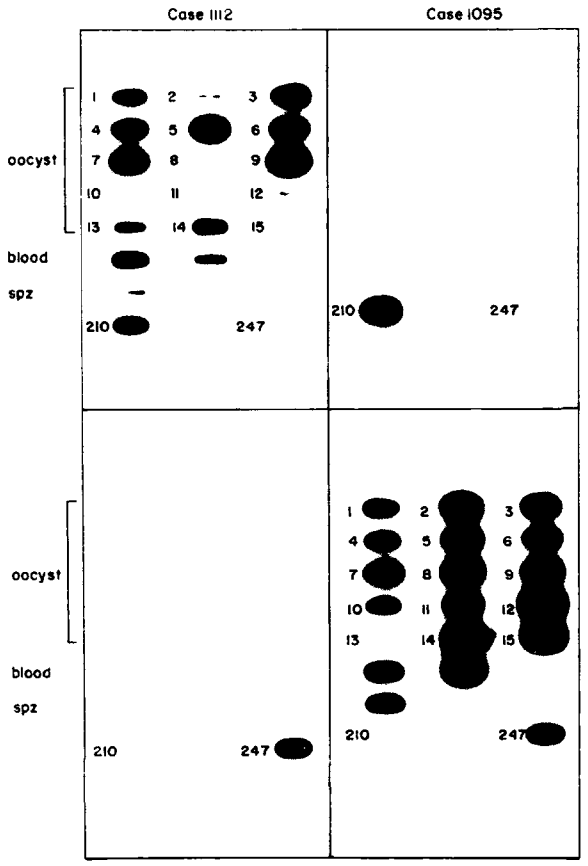

Fig. 1. Amplified DNA of oocysts, sporozoites, and blood parasites from 2 cases of *P. vivax* that have been hybridized with oligoprobes to 2 polymorphic circumsporozoite genes: VK 210 and VK 247. Slots 1–15 are individual oocysts; 'blood' are parasite DNA extracted from duplicate blood specimens; 'spz' are salivary gland sporozoites; 210 and 247 are plasmid controls. Slots 2 and 12 of #1112 were positive for VK 210, but relatively weak.

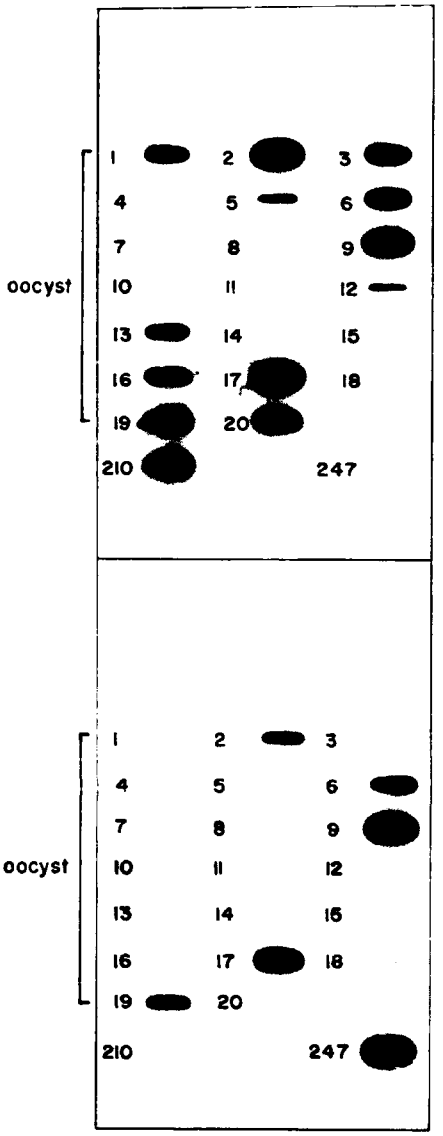

Fig. 2. Amplified DNA of 20 individual oocysts developed from *P. vivax* blood parasites positive for both VK 210 and VK 247 (case 1195) that have been hybridized with allele-specific probes. There are 5 hybrid, 7 VK 210 positive, and 8 negative oocysts shown; there are no VK 247 positive on this autoradiograph.

from the Hardy-Weinberg equilibrium ($p^2 : 2pq : q^2$), and therefore indicate random mating and simple Mendelian segregation. For case #1195, a VK 210:VK 247 allele ratio of about 2:1 can be deduced from the genotype frequencies. Using $p = 2q$ to solve $p^2 + 2pq + q^2 = 32$ yields expected zygote frequencies ($p^2 = 14.2$, $q^2 = 3.6$, $2pq = 14.3$) essentially the same as those observed ($\chi^2 = 0.21$, df = 2, $P_{null} = 0.90$). χ^2s calculated for allele ratios other than 2:1 (0.5:1, 1:1,...3.0:1, 3.5:1) were larger than 0.21. The oocyst genotype data for case #1089 suggest a VK 210:VK 247 allele ratio of about 1:8 ($q = 24^{1/2} = 4.9$; $p = 6/(2 \times 4.9) = 0.61$). Such a ratio ($p^2 = 0.37$, $q^2 = 23.7$, $2pq = 5.94$) would result in only one homozygous zygote of VK 210 in 81 oocysts; a proportion consistent with finding $p^2 = 0$ in a sample size of 30 ($\chi^2 = 0.37$, df = 2, $P_{null} = 0.84$). The ratio 1:8 was shown empirically, as before, to be a best fit. The data for case #1096 suggest

TABLE I

Genotype frequencies of *P. vivax* oocysts produced by blood infections positive for both VK 210 and VK 247

Case #	VK 210	VK 247	Hybrid	Total positive	% Amplified[a]
1195	15	4	13	32	67
1089	0	24	6	30	63
1096	11	0	0	11	48

All cases produced salivary gland sporozoites positive for both probes.
[a]Percentage of all oocysts that amplified.

that the allelic frequency for VK 247 was very low. This conjecture is supported by our failure to detect VK 247 CS protein, using a specific ELISA, on sporozoites produced by this case, although the amplified sporozoite DNA did hybridize both probes.

A case-dependent percentage of oocysts did not react with either probe. Evidence suggests that these oocysts failed to amplify. First, characteristic bands were not detected by ethidium bromide staining after electrophoresis of their amplified product. Second, even in cases where none of the oocysts reacted, DNA of sporozoites subsequently developing from these oocysts and recovered from the salivary glands were always successfully amplified and probed, indicating that lack of reaction was not due to the presence of as yet unrecognized target sequences. This conclusion is enforced by the finding that visual counts of sporozoites using a hemacytometer always matched the sum of quantifications using only the VK 210 and VK 247 specific ELISAs. The cause of selective amplification failure is not known.

Discussion

Despite the rather small sample size and the use of only one gene locus, we feel justified in drawing several tentative conclusions. We have demonstrated that the *P. vivax* CS variants VK 210 and VK 247 are alleles that hybridize in a natural vector, *A. dirus*, in predictable ratios. Each of the 3 cases was characterized by distinctly different gene frequencies. One can predict that gene ratios remain constant in each population of parasites until the timely infection of a human with more than one population (e.g., inoculation by 2 mosquitoes in the same night), such that infective gametocytes of both populations become simultaneously available to a vector. Reassortment will then quickly stabilize new frequencies. Contrary to evidence from earlier experiments with *P. falciparum* [1], we found no indication in the 3 natural mixed cases examined that cross-fertilization occurred in higher than predictable numbers, supporting the speculation [1] that biased hybrid ratios may have resulted from selection pressure inherent in prolonged laboratory manipulation. Neither does this data support the hypothesis that gamete fusion without meiosis may be occurring in Plasmodia [6]. The cosmopolitan range and sympatry of the 2 polymorphs [5,8] and the marked difference between them in the coding of the repeat domains [9] argue that divergence is evolutionally old. No evidence of intragenic recombination involving the repeat domains has yet been published, but there is obviously no mating barrier to its occurring.

There are advantages to using individual oocysts as surrogate diploid stages. Ranford-Cartwright et al. [10], who independently developed the same general method and recently published electrophoretic data on 10 oocysts, including several hybrids from cloned lines, have pointed out the potential for use on wild-caught mosquitoes. Additionally, the greater simplicity and speed of crossing experiments, especially when oocysts are enzymatically removed rather than surgically excised [3], is attractive. Theoretically, the population genetics of a given cross could be ascertained by examining oocysts from the gut of a single mosquito.

In most cases at least some oocysts did not

hybridize probes (Table I), even though probes reacted strongly with amplified blood parasite and sporozoite DNA. We have encountered some cases where none of as many as 50 oocysts gave a reaction and others where all were strong positives. Electrophoretic analysis suggests that DNA from negative oocysts was not amplified. There was no obvious correlation between such failures and genotype or parameters of infection. Possibly the use c^7dGTP during amplification [10] will improve results, but the data imply that the failure is not systematic. Virtually nothing is known of molecular events during sporozoite formation and the existence of variable (eg. age dependent) factors that may interfere with amplification is possible.

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