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Sympatric *Plasmodium falciparum* isolates from Venezuela have structured *var* gene repertoires

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Abstract

Background: The human malaria parasite *Plasmodium falciparum* expresses adhesins belonging to the erythrocyte membrane protein I (PfEMP1) family on the surface of the infected host erythrocyte. These antigens elicit a strain-specific antibody response that is associated with protection from disease. During clonal expansion of blood-stage parasites, the surface phenotype of the infected erythrocyte changes because of transcriptional switching among the 40 to 50 members of the highly polymorphic *var* multi-gene family which encode PfEMP1 variants. Studies to date have compared *var* repertoires of natural isolates from various geographical locations but have not addressed any within-population structure that may exist among repertoires.

Methods: Distinct parasite genotypes from a single population co-circulating among a defined group of hosts were selected. PCR products encoding the DBL- α domain of PfEMP-1 were cloned and sequenced from each of three isolates. Repertoire similarity was statistically evaluated using combinatorial analysis. The chromosomal location of shared sequences was inferred from similarity to *dbl- α* of known location in the 3D7 genome.

Results: Sympatric parasites were found to share few *var* gene sequences, even when alleles at other polymorphic loci were shared. A number of the sequences shared by at least two of the isolates studied were found to be related to 3D7 genomic sequences with non-telomeric chromosomal locations, or atypical domain structures, which may represent globally conserved loci.

Conclusion: The parasite population studied is structured, with minimal overlap in PfEMP1 repertoires. The *var* gene family accumulates diversity more rapidly than other antigen genes examined. This may be facilitated by ectopic recombination among the sub-telomeric regions of *P. falciparum* chromosomes.

Background

Human erythrocytes infected with certain life-cycle stages of the malaria parasite *Plasmodium falciparum* express on their surface a family of parasite-encoded adhesins known

collectively as PfEMP1, encoded by 40 to 50 members of the *var* multi-gene family [1]. The extra-cellular portion of PfEMP1 proteins is made up of multiple domains [1] which mediate cytoadherence to a wide range of receptors

in host tissues, permitting sequestration out of the peripheral circulation. One class of adhesive domain is named "Duffy-binding like" (DBL) because of similarity to the ligand that binds the Duffy antigen on the surface of the host reticulocyte/erythrocyte during invasion in certain species of *Plasmodium* (see references in [2]). Each *var* gene potentially encodes between one and six DBL domains. The amino-terminal DBL- α is the most common extra-cellular domain, occurring in all but a handful of PfEMP1 polypeptide sequences predicted to date [2,3]. *var* sequence similarity does not necessarily indicate shared geographic origin, and substantial *dbl- α* sequence polymorphism is found at the genomic level among parasite isolates both within and among endemic areas [6,7].

PfEMP1 elicits an antibody response which develops rapidly in the course of an infection but is of finite duration [8,9]. These strain-specific agglutinating antibodies are the only humoral immune responses to *P. falciparum* strongly associated with protection from malaria disease in humans [10,11]. The parasite evades the host immune response against PfEMP1 by clonal switching of transcription among *var* genes encoding the variants on the erythrocyte surface. This process of antigenic variation extends the duration of infection with any particular *P. falciparum* genotype, as a small proportion of each new generation of progeny expresses novel surface phenotypes not recognised by concurrent anti-PfEMP1 antibodies.

It has been suggested that such protective, variant-specific host immune responses may impose a strain structure upon *P. falciparum*, as observed in some populations of prokaryotic pathogens such as *Neisseria meningitidis* [11]. Such strain complexes would be characterised by little or no overlap in expressed PfEMP1 repertoire or Varotype [2]. Studies of *var* gene repertoires to date have not adequately tested this hypothesis for several reasons. Firstly, most have examined diverse collections of clinical isolates [4–7] rather than parasite genotypes co-circulating among the same hosts. Secondly, the multi-clonal infections characteristic of most malaria-endemic areas frequently prevent resolution of individual *var* gene repertoires from single isolates [2,6]. Thirdly, as circulating antibodies specific for the infected erythrocyte surface may not persist for long after a particular infection is resolved [8], any immunity-induced strain structure is likely to be transient. Thus any attempt to test the effect of immune selection on *P. falciparum* strain structure by the definition of *var* repertoires [2,11] requires examination of monoclonal parasite isolates circulating among a defined human host population at one particular time.

We have been investigating genetic diversity in the *P. falciparum* population circulating among the Yekwana and Yanomami peoples of the Padamo Basin, Amazon region,

Venezuela [12]. Malaria is meso-endemic, with a level of transmission comparable to that in Sudan. Genetic diversity among the parasite population is low, there is significant linkage disequilibrium among antigen loci, and a dominant clone accounted for the bulk of transmission in each of two seasons examined [12]. We selected three monoclonal isolates previously characterised at a number of polymorphic loci, and determined the sequence of between 14 and 32 *dbl- α* sequences from each. The three *dbl- α* repertoires were then examined for shared identical sequences. We demonstrate that these three isolates have minimal overlap and discuss these findings in terms of the contribution of both ectopic recombination and immune selection to the generation of PfEMP1 diversity in *P. falciparum*.

Methods

Study subjects

In a cross-sectional survey of all members of nine communities in the Padamo River basin, Amazon region, Venezuela between November 1995 and February 1996 (n = 708), 43 (6%) individuals were slide-positive for *P. falciparum*. The genotype of 41 of these was determined at the single-locus genes (*msp-1*, *msp-2*, *glurp*) and the multi-locus gene family (*rif*) [12]. 40 of the isolates were deemed monoclonal in that they carried only one allele of each of the single-copy loci tested.

Samples and nucleic acid extraction

Isolates were collected and stored as described [12]. DNA extractions were performed as previously described [12].

Amplification and purification of PCR products, cloning in plasmids and sequencing

We amplified *dbl- α* sequences from purified isolate DNA using the α -AF and α -BR primers of Taylor *et al.* [4]. The DNA bands (approximately 400 \pm 50 bp) representing amplified *dbl- α* sequences were excised from agarose gels, purified, cloned into plasmids and sequenced as described previously [12]. Sequence data were viewed and annotated using Chromas software <http://www.technelysium.com.au>. Sixty-one sequences described have been deposited in the EMBL Nucleotide Sequence Database and have the accession numbers AJ536694 to AJ536754.

Data analysis

Sequence data were edited, translated and compared using a variety of bioinformatic tools available at the Baylor College of Medicine <http://www.hgsc.bcm.tmc.edu/SearchLauncher> and the ExpAsy site <http://www.expasy.ch/tools/dna.html>. Predicted *dbl- α* amino acid sequences were compared with those derived from the annotated genomic sequence of the laboratory clone 3D7 using BLAST software at the PlasmoDB site, Release 4.0 <http://www.plasmodb.org>.

Table 1: Genotype of three *P. falciparum* isolates described in this study, and by Tami et al. (2002).

Isolate	GLURP	MSP1*	MSP2	RRM type	Relative frequency among 40 isolates**
#1	1000 bp	K1 232 bp	IC 535 bp	Ia	62.5%
#2	1000 bp	K1 232 bp	IC 535 bp	Ib	12.5%
#3	883 bp	MAD20 155 bp	IC 523 bp	II	5%

*K1 and MAD20 represent distinct allelic families of MSP1 sequences; IC represents a distinct allelic family of MSP2 sequences [20]. **Of the 41 isolates obtained in 1996, 40 were mono-clonal [12].

Repertoire overlap was investigated in a pair-wise fashion using combinatorial probabilities. Assuming 58 *dbl-α* copies per genome as in laboratory clone 3D7 [3], we tested the null hypothesis that the two isolates being compared have *dbl-α* repertoires drawn randomly from a single pool, size $N = 58$. If a sequences were obtained from isolate A, we have the proportion $p = a/58$ of the full *dbl-α* repertoire of isolate A. Then if we have b sequences from isolate B, of which r are identical to sequences in isolate A, the probability of this outcome (ie r successes from b trials) if the null hypothesis is true, is derived directly from the binomial distribution by:

$$P(r) = \frac{b!}{r!(b-r)!} \times p^r(1-p)^{b-r} \text{ (as described in [13])}.$$

Results

Similarity between var gene repertoires

Three monoclonal isolates that had been collected within six weeks of each other were selected. Isolate genotypes at three polymorphic loci and the rif / rrm fingerprint type of each are shown in Table 1, together with their relative frequencies. These 3 multi-locus genotypes together accounted for 80% of the 40 isolates collected in 1996. The reference isolate (#1; from a male of 13 years) represents the most common genotype [12]. Isolate #2 (from a male of 40 years living 20 km away) is identical at the single-copy polymorphic loci *mSP-1*, *mSP-2* and *glurp* to isolate #1, but differs in rif fingerprint [12]. Isolate #3 (from a female of 36 years living near the host of isolate #1) differs from both #1 and #2 at *mSP-1*, *mSP-2*, *glurp* and rif.

130 plasmids containing amplified *dbl-α* sequences encoding the DBL-1α domain of PfEMP1 from isolate #1 were sequenced. 32 distinct *dbl-α* sequences were obtained, 55% of the estimated full complement of genes [2,3]. Similarly, 15 distinct *dbl-α* sequences were determined from isolate #2 and 14 from isolate #3. As previously reported by Taylor *et al.* (4), binomial probability analysis revealed a low level of measurable primer bias (data not shown), but this is unlikely to have affected our findings.

The three isolates share few *dbl-α* sequences, and so have distinct *var* repertoires with little overlap (Figure 1). Estimates of the probability that the isolates have identical *var* repertoires were obtained from pair-wise comparisons as follows:

Isolate #1 and isolate #2: (4 successes from 15 trials) $P = 0.0273$

Isolate #1 and isolate #3: (5 successes from 14 trials) $P = 0.0748$

Are var sequences common to sympatric isolates globally conserved?

Unrelated *P. falciparum* parasites may be expected to have some *var* loci in common, as approximately 40% of loci are located in central chromosome regions [3] and, therefore, are less likely to diverge through sub-telomeric recombination [2,14]. In addition, recent work has demonstrated functional conservation of genes encoding placental-binding PfEMP1, and this conservation may be preserved by either chromosomal location or orientation with respect to the telomere [15]. Such functional conservation may also hold for other sub-classes of PfEMP1. Of the 51 distinct *dbl-α* sequences described herein, only eight were found in more than one of our three sympatric isolates. These eight sequences were used to search the annotated genome of *P. falciparum* clone 3D7 for similar *var* genes of known chromosomal location.

The closest 3D7 BLAST match for three of these eight sequences belonged to the Type 1 *var* class (which includes 38 of the 59 described 3D7 loci), but two of those were in central chromosome locations (Table 2). Of the remaining five matches, three are in the group of sub-telomeric *var* genes oriented for transcription towards the telomere, which is expected to hinder recombination at these loci [3,14]. Therefore, of the loci common to at least two of our sympatric isolates, five show a similarity to 3D7 *var* loci in chromosomal sites where ectopic recombination is not favoured.

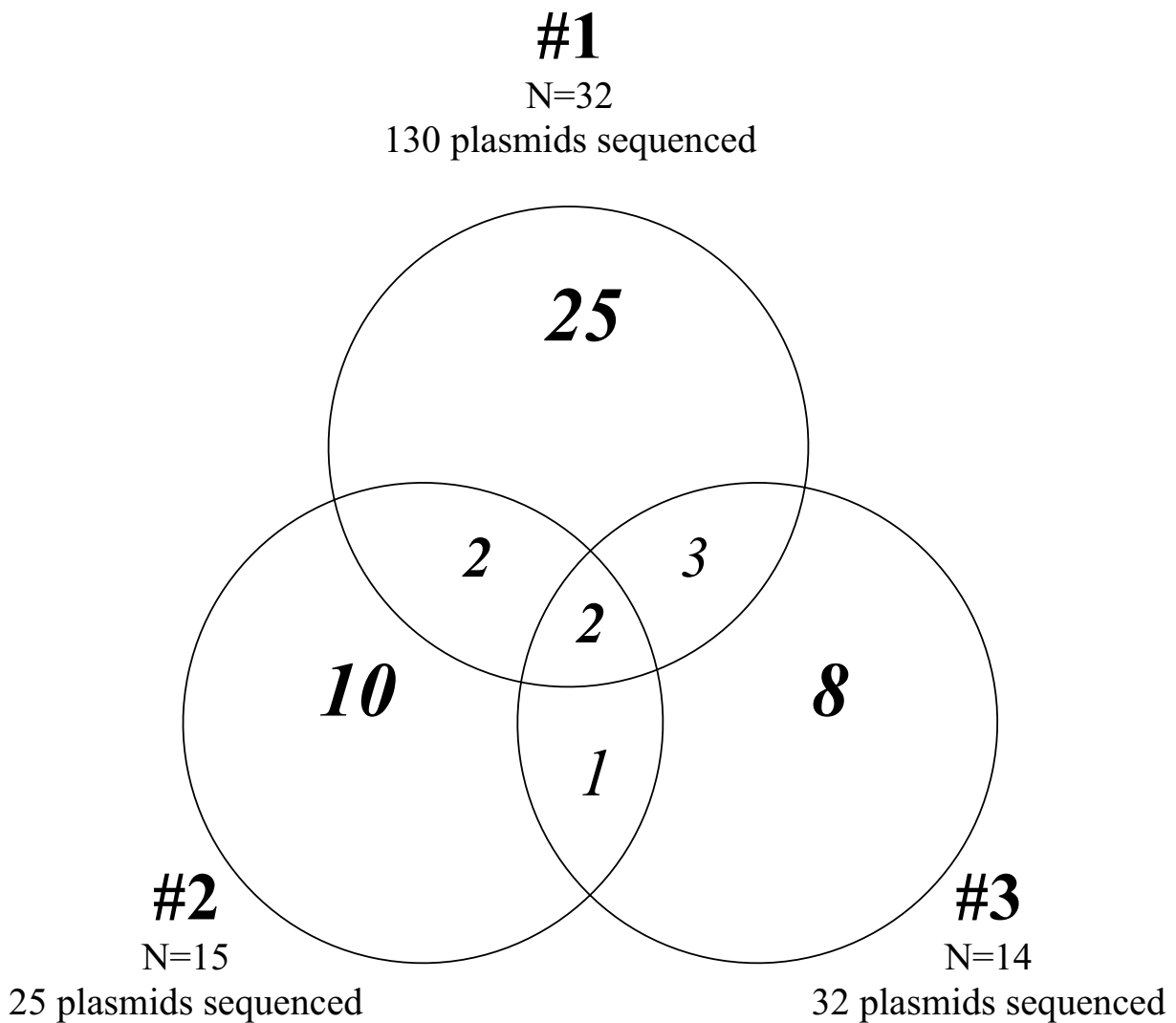


Figure 1
Overlap in *dbl-α* repertoires among 3 sympatric parasite isolates. Venn diagram showing the number of var *dbl-α* sequences that overlap among the three isolates. N refers to the number of unique *dbl-α* sequences among the total number of sequenced plasmids from each isolate.

Discussion

The divergence in *var* repertoires we have observed fits with theoretical predictions that immune selection of polymorphic immunodominant antigens such as PfEMP1

can structure a parasite population into discrete strains defined by their antigenic phenotypes [2,11]. The small amount of overlap observed among the three examined isolates is not inconsistent with this, as it is unlikely that

Table 2: 3D7 loci with sequence similarity to var loci found in at least two of the three isolates described in this study. Sequences from isolates 1 or 2 which were found in at least one other isolate were compared to sequences in the genome of clone 3D7 using BLAST software at the PlasmoDb site. The number of times each sequence was identified in each isolate is shown in columns two to four. The 3D7 locus with the most similarity (i.e. lowest probability) is shown in each case.

DBL sequence	Isolate			3D7 Locus	Probability	var class	Orientation
	#1	#2	#3				
#1-01	3	-	1	Chr 6: MAL6P1.316	$5.0 \times e^{-42}$	Atypical Type 2	Away from telomere
#1-02	2	-	1	Chr 6: MAL6P1.316	$7.0 \times e^{-38}$	Atypical Type 2	Away from telomere
#1-05	3	1	-	Chr 9: PFI1820	$2.1 \times e^{-37}$	Atypical Type 3	Towards telomere
#1-08	7	5	3	Chr 11: PFI1_0521	$3.0 \times e^{-48}$	Atypical Type 9	Towards telomere
#1-09	2	-	6	Chr 12: PFL0020	$5.4 \times e^{-38}$	Type 1	Away from telomere
#1-14	1	2	-	Chr 8: PF08_0106	$2.2 \times e^{-37}$	Type 1	Central cluster
#1-22	19	1	3	Chr 4: PFD1000 / PFD0995	$7.1/7.4 \times e^{-42}$	Type 1	Duplicated; central cluster
#2-01	-	3	8	Chr 8: PF08_0141	$4.6 \times e^{-46}$	Atypical Type 2	Towards telomere

the entire *var* genotype actively contributes to the Varo-type, or repertoire of PfEMP1 phenotypes expressed in the course of a single infection [2]. As a result, immune selection would not act upon all members of the genomic *var* repertoire. In addition, genomic data indicate that numerous *var* gene fragments and pseudogenes are scattered around the telomeric regions of *P. falciparum* chromosomes [3]. These may be among the sequences we have identified and can account for a degree of overlap in *var* repertoires at the genomic level. Further, we have shown that five of the eight shared *var* loci have similarity to 3D7 *var* genes located in the central chromosomal regions or in reverse orientation with respect to the telomere, positions in which ectopic homologous recombination may not be favoured [3,14]. These may, therefore, be "globally conserved" *var* genes.

We have shown that isolates #1 and #2, which between them represent 75% of the parasite population studied, are identical at all single-copy polymorphic loci examined [12], but differ in their repertoires of two multi-gene families: *rif* (*rrm*) [12] and *var*. In fact, the *var* repertoire of isolate #2 is as different from that of isolate #1 as is that of the third isolate examined, which has no single-copy polymorphic loci in common with either. Therefore, these sub-telomeric loci accumulate diversity more rapidly than other loci in this parasite population. This is the first such demonstration in natural isolates and supports the hypothesis that ectopic recombination among sub-telomeres in *P. falciparum* is a crucial source of diversity among multicopy genes such as *var* and *rif* which are located there [5,14]. In the parasite population studied self-fertilisation is predicted to be a common event [12], and so ectopic re-

combination during meiosis is a likely major source of variation among the progeny. The contribution, if any, of mitotic recombination events remains unclear [5,14].

The *dbl-α* and *rif* 3' UTR sequences we have examined, while sharing a sub-telomeric chromosomal location, may accumulate diversity by different mechanisms. It is known that novel *dbl-α* sequences are generated by intra-genic recombination [5], presumably facilitated by substantial tracts of homologous sequence to be found around each sub-telomeric *var* locus, which permit heterologous meiotic chromosome exchange [14]. This mechanism will also contribute to recombination among *rif* loci on heterologous chromosomes. However, in addition, the *rif* 3' UTR RRM sequence we have used for "fingerprinting" contains a six base-pair repeat sequence which varies in number, giving the size polymorphisms we have measured [12]. Thus, new RRM configurations can arise by slippage in the number of repeats at particular loci, as is seen in other polymorphic *P. falciparum* genes such as *msp1* and *msp2*. Such slippage is probably exacerbated by ectopic recombination, as *rif* loci with different numbers of repeats pair and undergo exchange.

Conclusions

We have shown that sympatric parasites share few *var* gene sequences, even when alleles at other polymorphic loci are identical, consistent with immune selection acting against overlap in PfEMP1 repertoires. Demonstration that these distinct repertoires are widespread in the parasite population and stable over time, particularly in the Padamo population where self-fertilisation is predicted to be common, would provide stronger support for this in-

terpretation. In parasite populations where outcrossing is the norm, genetic recombination is expected to exert a strong reshuffling effect, and stable inheritance of *var* repertoires by self-fertilisation will be less likely. Whether immune selection working against repertoire overlap can maintain a structured parasite population under such conditions remains an open question.

Authors' contributions

AT collected the samples, participated in design of the study, carried out cloning and sequencing of PCR products and contributed significantly to writing the manuscript. RO carried out cloning and sequencing of PCR products, performed clustal alignments and collated the sequence data. GATT participated in the design of the study, provided logistic support and contributed significantly to writing the manuscript. CJS conceived the study, participated in its design and coordination, carried out cloning and sequencing of PCR products and drafted the manuscript. All authors read and approved the manuscript.

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