Distinct polymorphism of DBLα domain of the *var* gene in laboratory-cultured and clinical field isolates of *Plasmodium falciparum* malaria parasites

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***Plasmodiasis* (malaria) is a major health problem in sub Saharan Africa and southern Asia. The deadly infectious disease kills more than a million people a year. The most fatal form of the disease is caused by *Plasmodium falciparum* parasites. *P. falciparum* harbours multi-copy *var* genes. The *var* genes encode PfEMP-1 protein (*P. falciparum* erythrocyte membrane protein) which is deposited on the parasite-infected erythrocyte membrane. This protein plays a crucial role in the sequestration of P-RBC to an array of receptors on the endothelial cells of post-capillary venules. The phenomenon is implicated in the immunopathology of the disease. We have analysed the Duffy binding-like domain (DBLα) of *var* genes by PCR in cultured parasites from India (*FAN5HS*, *PUNE1*, *FMN17*), Thailand (*SOHS*, *MP14*), the Netherlands (3D7), and uncultured clinical isolates from malaria patients (Pune, India). The analysis shows extensive size polymorphism in the *var* gene repertoire when analysed using different combinations of primers of the DBLα domain. Though all the parasites used in the study have amplified multiple bands with the three sets of DBLα primers, it also shows distinct polymorphism between the Indian, Thailand and the Netherlands parasites, as well as between laboratory cultured and uncultured clinical field parasite isolates. The importance of these studies and the possible use of *var* gene polymorphism in the epidemiology of malaria to understand pathogenesis is discussed.**

Keywords: Epidemiology, malaria parasites, size polymorphism, *var* genes.

MALARIA has been a major health problem, especially in tropical and subtropical regions of the world. Worldwide prevalence of the disease is estimated to be 300–500 million clinical cases and it is estimated to kill more than one million children annually in Africa[1]. There are 1.6 million cases and 500 deaths due to malaria every year in India[2]. The parasite causes mild, severe and life-threatening cerebral malaria (CM) form of the disease, including clinical asymptomatic infection. The *var* genes of *Plasmodium falciparum* malaria parasite encode PfEMP-1 protein which plays a crucial role as adhesion ligands to host endothelial receptors. This act triggers an immunopathological reaction, which leads to severity of the disease[3]. PfEMP-1 is implicated in severe complications like CM and placenta-associated malaria complications[4,5]. PfEMP-1 is highly variable between parasite isolates and there are reports of antigenic switching, resulting in distinct cytoadherence and agglutination phenotypes and variation during long-term chronic infection in the host[6,7].

The current use of MSP (merozoite surface protein) markers is only helpful in analysing genotypes of the parasite isolates[8]. However, this is not sufficient to understand parasite causing severity of the disease in epidemiological studies. It is important to follow up and document the epidemiology and spread of virulent malarial parasites. Parasite genetic diversity can be used to detect subtle changes in pathogen virulence. Thus, it is necessary to investigate the extent of diversity in the parasite populations from specific geographical locations or regions, and monitor the spread during primary infection and recurrence. There is a need to develop strategies to analyse the factors that are associated with disease severity and spread of virulence parasite strains in malaria endemic regions.

The DBLα domain of the *var* gene has been a target for investigations of the gene repertoire to study diversity in laboratory strains and wild *P. falciparum* isolates[9,10]. The flanking region of the DBLα domain is highly conserved and the middle region shows extreme diversity. We have used PCR typing to investigate the nature of genetic diversity with respect to the *var* gene repertoire of laboratory-cultured parasites of different geographical origin and clinical field isolates (blood samples from malaria patients) using various combinations of DBLα primers. The results show certain PCR amplified products of para-

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sites which clearly distinguish parasites from different locations and *in vitro* cultured parasites with clinical field isolates from malaria patients. The importance of these findings and their use in the epidemiological studies to understand the severity of the disease is discussed.

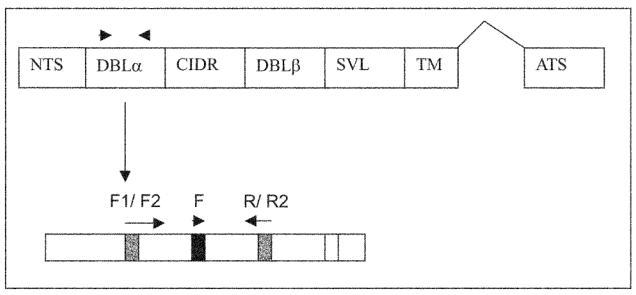
Materials and methods

Parasite culture

Erythrocytic stages of *P. falciparum* parasite lines (laboratory-cultured) from Thailand (*SOHS*, *MP14*), the Netherlands (*3D7*), and India (*FAN5HS*, *PUNE1*, *FMN17*) were cultured as described earlier[11]. The parasites were cultured in RPMI 1640 medium supplemented with 0.5% AlbuMAX II (Gibco-BRL). Parasite cultures were synchronized using sorbitol treatment[12]. Parasites (parasitaemia >15%, with >90% mature trophozoites and schizonts) were used for extraction of genomic DNA. Parasite pellets were digested with proteinase K in the presence of sodium dodecyl sulphate and DNA was isolated by phenol chloroform extraction and subsequent ethanol precipitation[13]. DNA was also isolated from uncultured *P. falciparum* parasite blood samples form malaria patients (Pune region) and used in the analysis of *var* gene polymorphism studies. All the four cases were suffering from severe malaria with 3–53% parasitaemia. The clinical field isolates were not cultured in the laboratory. Genotyping of the parasites was done with MSP-1 and MSP-2 primers.

PCR reaction

The position of DBLα primers in the *var* gene and primer sequences are shown in the Figure 1. We have used five primer sequences of DBLα in three combination sets



Primer Name	Sequence
DBLα- F (Forward)	CGACACCGGCGACATTATAAGAGG
DBLα- R (Reverse)	TCGCAGGTATTGTGGCACGTAGTC
DBLα- F1 (Forward)	CGAGGATCCGGWGCWGCWCCWTWYMG
DBLα- F2 (Forward)	CGAGGATCCCCATATAGACGATTACATSTA TG
DBLα- R2 (Reverse)	GCACTCGAGTTANARRTAYTGWGGWACRTARTC

Figure 1. (Top) Schematic location of DBLα domain in the *var* gene of *Plasmodium falciparum* malaria parasite and region of primers used in the study. (Bottom) Details of five sequences. The three primer sets are: DBLα–FR, DBLα–F1R2 and DBLα–F2R2. (W = A/T, Y = C/T, M = A/C, S = C/G, R = A/G.)

(DBLα–FR, DBLα–F1R2 and DBLα–F2R2) in the PCR reactions[14]. The PCR conditions for DBLα–FR primer combination were: 94°C for 2 min, 94°C for 1 min, 51°C for 1 min and 70°C for 2 min (30 cycles) and final extension at 70°C for 10 min. For DBLα–F1R2 and DBLα–F2R2 primer combinations, the PCR conditions were: 94°C for 5 min, 94°C for 30 s, 50°C for 30 s and 65° for 1 min (30 cycles) and final extension at 65°C for 10 min. The amplified PCR products were resolved on 5% non-denaturing polyacrylamide gel. Some of the PCR products were cloned in pGEM-T vector and sequenced (GenBank accession numbers: DQ 364441-364452, DQ 408208-408223; data not shown).

Results

The genomic DNA from all the parasites used in the study has amplified multiple bands which varied amongst the isolates with the three primer sets of DBLα used in the study. Details of PCR results are given in Figure 2 and Table 1. Some of the PCR products were common in different parasites, but they varied in different geographical isolates and also between cultured and uncultured field isolates.

DNA isolated from cultured parasites from Thailand (*SOHS* and *MP14*) showed a PCR product of 890 base pairs (bp) with the DBLα–FR primer set, which is absent in all Indian cultured and uncultured field parasite isolates as well as in cultured *3D7* parasites (Figure 2; Table 1). When DNA from Indian cultured parasites (*FAN5HS*, *PUNE1* and *FMN17*) was amplified with the DBLα–F2R2 primer set, a PCR product of 730 bp was observed, whereas this product was not amplified in the Thailand and the Netherlands (*3D7*) parasites and Indian field isolates (Figure 2; Table 1). The primer combination of DBLα–F1R2 and DBLα–F2R2 amplified 754 and 640 bp products respectively, in *3D7* parasites, but not in all Indian and Thailand parasites (Figure 2; Table 1). DNA of cultured parasites from India (*FAN5HS*, *PUNE1* and *FMN17*), Thailand (*SOHS* and *MP14*) and the Netherlands (*3D7*) amplified two PCR products (635 and 675 bp with DBLα–F1R2 primer set), but these bands were not amplified in all four uncultured field isolates from India (Figure 2; Table 1). Similarly, DBLα–F2R2 primer combination of the *var* gene amplified 670 bp in all cultured parasites lines but not in field isolates (Figure 2; Table 1). Thus there are distinct differences in the cultured parasites as well as in the field isolates with respect to size polymorphism.

Discussion

Malarial infection causes variable pathophysiological conditions ranging from a mild febrile to life-threatening severe anaemia or CM. Only 1–2% of *P. falciparum*

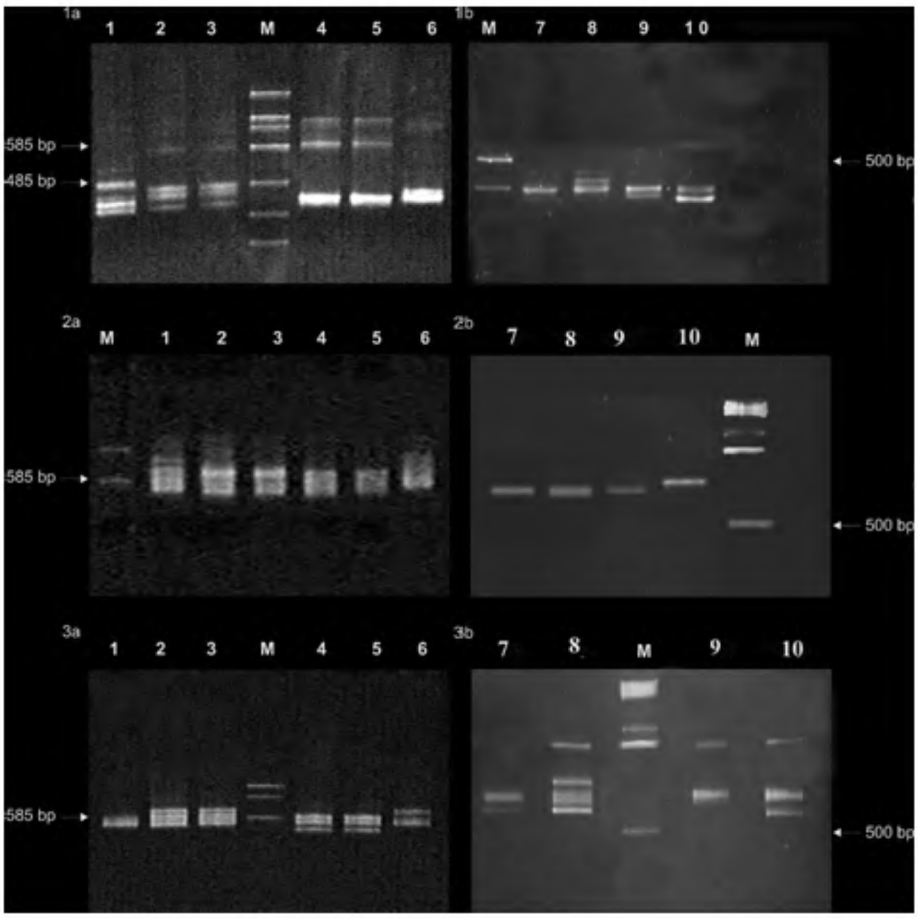


Figure 2. Polyacrylamide gel electrophoresis of PCR amplified products obtained with genomic DNA isolated from *in vitro* cultured parasites from India, Thailand, the Netherlands and clinical field isolates (not adopted to *in vitro* culture) from India. Lane 1, *3D7*; lane 2, *FAN5HS*; lane 3, *PUNE1*; lane 4, *SOHS*; lane 5, *MP14*; lane 6, *FMN17*; M, Molecular weight marker; lane 7, C; lane 8, D; lane 9, NS2, and lane 10, NS6. Indian: *FAN5HS*, *PUNE1*, *FMN17*; Thailand: *SOHS*, *MP14*; Indian field isolates: C, D, NS2, NS6. 1a–3a, Cultured parasites. 1b–3b, Field isolates amplified with DBLα–FR (1b), DBLα–F1R2 (2b) and DBLα–F2R2 (3b) primers.

malaria cases results in severe and complicated disease. Deaths occur primarily in young children and other immunocompromised individuals. Various reasons have been implicated in susceptibility to pathogenesis of malaria. One of the factors is sequestration of parasite-infected erythrocytes (P-RBCs) to different cells. PfEMP-1 protein, a *var* gene product of the parasite, which is present in the infected erythrocytes, plays an important role in the adhesion of the P-RBCs to an array of receptors on the endothelial cells of post-capillary venules of various organs, i.e. kidney, lung, brain and placenta of pregnant women. PfEMP1 interacts with molecules like CD36, ICAM-1, CSA, VACAM-1, E-selectin and PECAM-1 of various cells[15–19]. Such an adhesion facilitates escape of *P. falciparum* from spleen-mediated destruction and immune evasion, thus playing an important role in the genesis of disease severity. As the *var* gene (PfEMP-1) is implicated in virulence and immunopathology due to sequestration, the use of these primers for *var* gene polymorphism analysis along with standard genotyping MSP-1 and MSP-2 markers in malaria epidemiological studies will help in better understanding of the spread of the virulence strain of *P. falciparum*.

The *var* gene family has been reported to be diverse[20] and DBLα sequence of *var* gene varies greatly within an isolate and also between different isolates[21–25]. Our results show that particular geographical parasite isolates do amplify some PCR products of the same size, but vary with specific amplified products, as observed in the case of parasites from Thailand (*SOHS* and *MP14*) and India (*FAN5HS*, *PUNE1* and *FMN17*). Thus analysis of genomic DNA of these parasites with different combinations of *var* gene primers shows distinct size polymorphism. The present study shows some interesting findings. The three sets of DBLα primers amplify multiple PCR products in all parasites of laboratory adopted cultures as well as those directly taken from malaria patients (uncultured clinical field parasites).

Another observation from the present study is the absence of certain amplified PCR products (635 and 675 bp with DBLα–F1R2, and 670 bp with DBLα–F2R2 primer

Table 1. Comparative chart of amplified PCR products in *Plasmodium falciparum* parasites of different geographical regions with DBLα primers (results summarized from Figure 2)

Primer combination	Band size (bp)	Indian parasites (LCP)			The Netherlands parasites (LCP)	Thailand parasites (LCP)		Indian field isolates*			
		FAN5HS	*PUNE1*	*FMN17*	*3D7*	*SOHS*	*MP14*	*C*	*D*	*NS2*	*NS6*
DBLα–FR	355	+	+	–	+	+	+	–	–	–	+
	380	–	–	–	+	–	–	–	–	+	–
	400	+	+	+	–	+	+	+	+	+	+
	430	+	+	+	–	+	–	–	+	–	–
	455	–	–	–	+	–	–	–	+	–	–
	585	+	+	–	–	+	+	–	–	–	–
	890	**–**	**–**	**–**	**–**	**+**	**+**	**–**	**–**	**–**	**–**
DBLα–F1R2	585	+	+	+	+	+	+	+	+	+	–
	595	–	–	–	–	–	–	+	–	–	+
	610	–	–	–	–	–	–	–	+	+	–
	620	–	–	–	–	–	–	–	+	–	–
	635	**+**	**+**	**+**	**+**	**+**	**+**	**–**	**–**	**–**	**–**
	675	**+**	**+**	**+**	**+**	**+**	**+**	**–**	**–**	**–**	**–**
	720	+	+	–	+	+	+	–	–	–	–
	754	**–**	**–**	**–**	**+**	**–**	**–**	**–**	**–**	**–**	**–**
DBLα–F2R2	540	–	–	–	–	–	–	+	+	–	+
	585	+	+	–	–	+	+	+	+	+	+
	615	+	+	+	–	+	+	+	+	–	–
	640	**–**	**–**	**–**	**+**	**–**	**–**		**–**	**–**	**–**
	670	**+**	**+**	**+**	**+**	**+**	**+**	**–**	**–**	**–**	**–**
	700	+	+	–	+	+	+	–	–	–	–
	720	–	–	–	–	–	–	–	+	+	+
	730	**+**	**+**	**+**	**–**	**–**	**–**	**–**	**–**	**–**	**–**

PCR amplified products (+) with three different sets of DBLα primers in different geographical parasites are indicated. '+' indicates amplification of PCR products; '–' indicates absence of PCR products; LCP, Laboratory cultured parasites; *Not cultured in the laboratory (clinical field samples from malaria patients). The difference between parasites can be distinguished from '+' (bold) with parasites from other regions and also between LCPs and clinical field isolates (not cultured in the laboratory).

sets) in all four uncultured clinical field isolates from malaria patients compared to *in vitro* cultured parasites (irrespective of geographical region), which amplify the PCR products. The reason for such a striking difference in the amplification of the PCR products in laboratory-cultured parasites and its absence in the uncultured parasites is not clear at present. But we speculate that the changes in the parasites are due to *in vitro* culture adoption. However, it will be interesting to study such changes further.

Interestingly, these parasites show distinct geographical polymorphism. The four Indian isolates collected from severe malaria cases show different polymorphism patterns. Such variations could be due to a variety of reasons and may prove useful if they can be correlated to virulence (*var* gene polymorphism) and disease severity. This might help in devising better therapeutic strategies to minimize the morbidity induced by the virulent parasites.

The DBLα primer set used in the present study seems to be useful in detection of polymorphic characters in the clinical isolates of parasites from malaria patients. Studies using large samples with known clinical form of the disease (mild, severe, CM and clinical asymptomatic infection) will help in understanding the relationship between virulent strains of parasites and disease severity.

Molecular sequencing has provided an important insight into the *var* gene organization and polymorphism. Sequencing and SNP typing provide invaluable data, but have financial and technical limitations when applied on a massive scale in the developing and underdeveloped countries. Analysis of the parasite genomic DNA with *var* gene primers as described in the present study is useful in analysing the diversity of the *var* gene repertoire of isolates of *P. falciparum* at a preliminary level. Such an analysis can be used to trace the spread of virulent *Plasmodium* genotypes. It has epidemiological potential and can easily be adapted to study human malaria parasites in clinical field isolates. Changes in the overall genetic composition of the parasite population, particularly during multiplicity of infection observed in individuals, indicate transmission of various isolates. It can also offer recurrence and follow-up strategy for vaccination campaign.

Thus, we propose that the amplified fragment length analysis with DBLα primers can serve as a useful tool for rapid primary screening of outbreaks of malaria as well as for examination of the interrelations between different isolates, particularly in tropical countries where malaria is endemic. This can be used for quick assessment of parasite population (species-specific analysis) for malaria

control and intervention strategies. It also enables one to assess the composition of the parasite population. This analysis is useful in the quick detection of polymorphism of *var* genes in malaria parasites and will aid in designing effective control measures by providing insight into malaria epidemiology.

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