TABLE 1

Polymorphisms of *Plasmodium falciparum pfcrt* and *dhfr* genes in archival samples collected from Indochina and the Western Pacific between 1984 and 1998*

pfcrt

Date	Indochina				Western Pacific		
	Myanmar	Thailand	Thailand/Laos†	Laos	Indonesia	Papua New Guinea	The Philippines
1984		CVIET (2)					
1985							CVMNK (1) SVMNT (2)
1986					CVMNN (1)	SVMNT (3)	SVMNT (1)
1987						SVMNT (1)	
1988							
1989							
1990						SVMNT (1)	
1991		CVIET (1)			SVMNT (1)		SVMNT (1)
1992		CVIET (1)					SVMNT (1)
1993							
1994	CVIET (1)			CVIET (1)	SVMNT (1)		
1995						SVMNT (1)	
1996							
1997	CVIET (1)					SVMNT (1)	SVMNT (1)
1998			CVIET (1) CVIDT (1)	CVIDT (1)		SVMNT (1)	CVIET (1)‡ CVMNK (1)‡
Total (n = 29)	2	4	2	2	3	8	8

dhfr

Date	Indochina				Western Pacific		
	Myanmar	Thailand	Thailand/Laos†	Laos	Indonesia	Papua New Guinea	The Philippines
1984		CIRNI (2)					
1985							CNCSI (1) CNCNI (1) CNRNI (1)
1986					CNCNI (1)	CNCSI (2) CNCNI (1)	CNCSI (1)
1987						CNRNI (1)	
1988							
1989							
1990						CNCSI (1)	
1991		CIRNI (1)			CNRNI (1)		CNRNI (1)
1992		ND (1)					CNRNI (1)
1993							
1994	ND (1)			CNRNI (1)	CNRNI (1)		
1995						CNRNI (1)	
1996							
1997	CNCSI (1)					CNRNI (1)	CNRNI (1)
1998			CNRNI (2)	CNRNI (1)		CNCNI (1)	ND (1)
Total (n = 26)	1	3	2	2	3	8	7

* Values in parentheses indicate number of samples. Mutated residues are in **bold** and underlined. *pfcrt* = *P. falciparum* chloroquine resistance transporter; *dhfr* = dihydrofolate reductase; ND = not done.
† Persons visited both countries.
‡ Mixed infection of two distinct *pfcrt* genotypes.

the persistence of this resistant genotype in Thailand from at least 1979 to the present time. The CVIDT polymorphism, a variant of CVIET, was reported in Cambodia in 2001 and 2004,[30,31] and was also detected in two of our 1998 samples from Laos and/or Thailand. These findings suggest an earlier presence of this resistant genotype in central Indochina (Laos/Thailand/Cambodia) than previously reported. The Papua New Guinea form of CQ-resistant *pfcrt* genotype (SVMNT), was not detected in our limited samples from Indochina.

In the Western Pacific countries (Indonesia, Papua New Guinea, and the Philippines), most samples (n = 16) showed the CQ-resistant *pfcrt* polymorphism SVMNT. This genotype was detected in a sample from Indonesia from 1991. This date is much earlier than previous records from Indonesia collected in 1999 and 2002.[32,33] We identified another *pfcrt* genotype (CVMNN) from Indonesia in 1986 and it is reported that this CVMNN mutant exhibits resistance to CQ *in vitro*.[34] In Papua New Guinea, all samples (n = 8) isolated between 1986 and 1998 showed the SVMNT *pfcrt* genotype, which is consistent with the report by Mehlotra and others.[35] In contrast, in samples collected from Papua New Guinea between 1956 and 1965, all carried the wild-type *pfcrt* genotype.[36] Our results, together with these records, suggest that the SVMNT genotype, which appeared before 1982, persisted until 1998 (and probably until now). In samples from the Philippines, the SVMNT genotype was detected in samples from 1985, six years earlier than the first record of this type in 1991.[37] We also detected the wild-type (CVMNK) *pfcrt* genotype in samples from the Philippines in 1985 and 1998. A substantial parasite population showed the wild-type *pfcrt* genotype present in 1997,[29,38] which suggested the persistence and co-

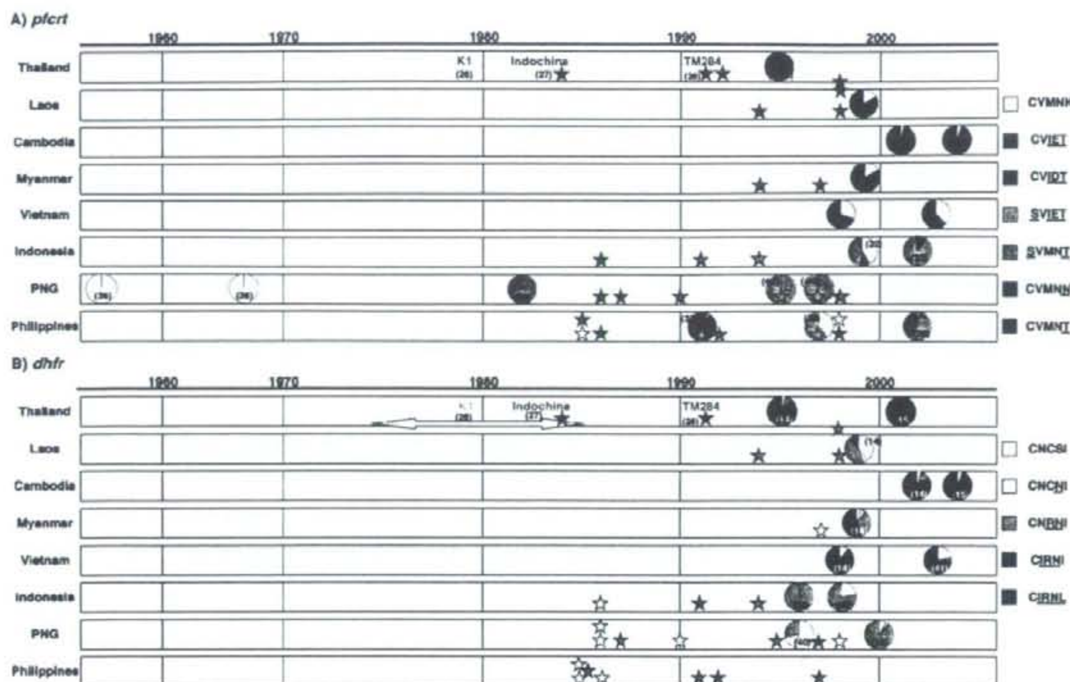

FIGURE 1. Time-line scheme of genetic evidence for *Plasmodium falciparum* resistance to chloroquine and pyrimethamine in Indochina and the Western Pacific. Genotypes of **A**, the *Plasmodium falciparum* chloroqunie resistance transporter (*pfcrt*) gene and **B**, the dihydrofolate reductase (*dhfr*) gene obtained in this study and previous reports are combined. Genotypes of *pfcrt* and *dhfr* identified in this study are indicated by a star and those from cultured parasites are shown with the name of parasite strain in matching colors. Frequencies of *pfcrt* and *dhfr* genotypes are shown in pie charts with reference number in parenthesis. The blue arrow in **B** indicates the period when sulfadoxine-pyrimethamine (SP) was used as a first-line treatment in Thailand.

prevalence of both CQ-resistant and CQ-sensitive parasite populations in the Philippines.

Polymorphism in *dhfr*. Of the 29 samples sequenced for *pfcrt*, 26 samples were successfully sequenced for three *dhfr* fragments, encompassing four polymorphic sites. Results are shown in Table 1 and Figure 1B. The triple mutant Pyr-resistant *dhfr* genotype CIRNI was present in samples from Thailand collected in 1984 (n = 2). This triple mutant *dhfr* is identical to the genotype of the Indochina III strain in 1984 (Figure 1).[27] Thus, the CIRNI genotype was already prevalent as early as 1984 in Thailand. In Laos, the double mutant Pyr-resistant *dhfr* genotype CNRNI was first reported in 1999.[14] In our study, this double mutant was detected in isolates from Laos from 1994 and 1998 (n = 4), which suggested the presence of this double mutant in Laos at least five years earlier than previously recorded. In Myanmar, one sample isolated in 1997 was wild-type (CNCSI). However in 1999, *dhfr* polymorphism reportedly consisted of 90% Pyr-resistant genotypes (CNRNI, CNRNL, and CIRNL).[14] It is not known whether a Pyr-resistant *dhfr* genotype was present in Myanmar before 1999.

In the Western Pacific, the triple mutant *dhfr* genotype was not found, but the wild-type, single, and double mutant *dhfr* genotypes were detected. Wild-type *dhfr* was present between 1985 and 1990 in Papua New Guinea (n = 3) and the Philippines (n = 2). The Pyr-resistant *dhfr* (single mutant) genotype CNCNI was detected in samples from Indonesia, Papua New Guinea, and the Philippines between 1985 and 1986, and also in one isolate from Papua New Guinea in 1998. The double mutant *dhfr* (CNRNI) was obtained from samples from Indonesia in 1991, Papua New Guinea in 1987, and the Philippines in 1985. These dates are much earlier than the first record of this double mutant type in 1996 from Indonesia and Papua New Guinea.[39,40] In addition, the presence of these Pyr-resistant *dhfr* mutant genotypes have not been previously reported in the Philippines.

DISCUSSION

The aim of this study was to obtain genetic evidence of *P. falciparum* drug resistance to CQ and Pyr in Indochina and the Western Pacific between 1984 and 1998, during which time reports of *pfcrt* and *dhfr* genotypes have been limited.[35,36] Our results obtained with archival samples present genetic evidence of resistance of this parasite to CQ and Pyr during this period. Most of our samples (96%, 28 of 29) had a CQ-resistant *pfcrt* genotype, and there was a clear geographic separation of two resistant genotypes: CVIET in Indochina and SVMNT in the Western Pacific (Figure 1). Ge-

netic resistance to Pyr was somewhat lower in frequency (77%, 20 of 26) than CQ resistance. The fact that Pyr was introduced in Indochina approximately 20 years later than CQ for treatment failure of *P. falciparum* malaria is consistent with the late spread of Pyr resistance in these areas.[1,4] Also, single, double, and triple mutants of *dhfr* were detected. This situation reflects the present distribution of *dhfr* polymorphism in these areas (Figure 1).

These results of genetic evidence for drug resistance are generally consistent with the history of clinical resistance in Indochina and the Western Pacific.[32,40–42] Thus, the present study has substantiated a widely distributed idea that treatment failures were ascribed to genetic resistance to these drugs in Indochina and the Western Pacific. We were unable to find an association of genetic resistance with clinical resistance in our samples because records of drug treatment were accessible to only two malaria patients: one who traveled to Thailand in 1992, and the other who traveled to Papua New Guinea in 1998 (Table 1). The first patient cured after receiving quinine, and the second patient, who had parasites of the CVIET type CQ-resistant *pfcrt* genotype and the CIRNI type Pyr-resistant *dhfr* genotype, died after being treated with quinine and SP.

Additionally, when combined with results of previous reports, our study has three interesting findings. First, a CQ-resistant *pfcrt* genotype (SVMNT) was co-prevalent with a CQ-sensitive genotype in the Philippines in 1998, which is consistent with a relatively high prevalence (30%) of the CQ-sensitive *pfcrt* genotype in 1997.[29] Treatment with CQ is still effective in more than half of *P. falciparum*-infected patients in this country.[43] Notably, the persistence of this CQ-sensitive *pfcrt* genotype is in sharp contrast to countries, such as Thailand, the Solomon Islands and Vanuatu,[6,44,45] where there is 100% prevalence of the CQ-resistant *pfcrt* genotype. Second, the CVIET *pfcrt* genotype was present in 1998 in the Philippines. Together with reports showing the same type in 1991 and 2002,[37,38] this finding suggests the persistence of this resistant type throughout the 1990s in the Philippines. This CQ-resistant form of *pfcrt* was also reported in Indonesia in 1999 and 2002.[32,33] It remains to be clarified whether the CVIET genotype originated independently in the Western Pacific or was imported from Indochina.[38] Third, the CVMNN *pfcrt* genotype was present in Indonesia. One field isolate of this type was reported from Indonesia in 2002.[33] Importantly, the CVMNN mutant, which was obtained from *in vitro* culture under CQ pressure, showed resistance to CQ.[34] The *pfcrt* mutant carrying N at residue 76, an amino acid change other than T that results in CQ resistance, can occur in field isolates.

The efficacy of Pyr in treatment of persons with *P. falciparum* infections and the prevalence of both wild-type and Pyr-resistant genotypes of *dhfr* currently vary in the countries of Indochina and the Western Pacific.[14,15,40–42] Together with these reports, our present finding of the three resistant *dhfr* genotypes (single, double, and triple mutants), as well as the wild-type genotype, between 1984 and 1998 may reflect different histories of the use of Pyr in these areas. Thailand is the only country that introduced SP as a first-line treatment in the mid 1970s in Asia.[5,6] However, *in vivo/in vitro* resistance to SP reached 100% in the 1980s.[7] As a result, the drug policy of Thailand was then switched to mefloquine as a first-line treatment in the mid 1980s.[6] We identified *dhfr* triple mutants in two Thai samples collected in 1984, which is consistent with the change of drug policy in the mid 1980s. We did not detect the *dhfr* quadruple mutant, which currently accounts for the highest population of *dhfr* mutants in Thailand,[15] in our archival samples. The quadruple mutant was first identified in samples collected from Thailand in 1995.[14] We report of *dhfr* polymorphism in the Philippines, which shows the wild-type and resistant-type *dhfr* genotypes (single and double mutants) in the 1980s and 1990s.

In conclusion, our analysis of archival samples shows genetic evidence for a wide distribution of *P. falciparum* resistance to CQ and Pyr in Indochina and the Western Pacific during the 1980s and 1990s. It also sheds light on the history of drug resistance in these areas, supporting previous records of clinical resistance during this period.

Received February 25, 2008. Accepted for publication July 8, 2008.

Financial support: This work was supported by a Grant-in-Aid from the Ministry of Health, Labor and Welfare (grant H17-Shinkou-ippan-019) of Japan.

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INFECTION AND IMMUNITY, Apr. 2008, p. 1702–1708
0019-9567/08/$08.00+0 doi:10.1128/IAI.01539-07
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Vol. 76, No. 4

Wheat Germ Cell-Free System-Based Production of Malaria Proteins for Discovery of Novel Vaccine Candidates▽†

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Received 20 November 2007/Returned for modification 22 December 2007/Accepted 1 February 2008

One of the major bottlenecks in malaria research has been the difficulty in recombinant protein expression. Here, we report the application of the wheat germ cell-free system for the successful production of malaria proteins. For proof of principle, the Pfs25, PfCSP, and PfAMA1 proteins were chosen. These genes contain very high A/T sequences and are also difficult to express as recombinant proteins. In our wheat germ cell-free system, native and codon-optimized versions of the Pfs25 genes produced equal amounts of proteins. PfCSP and PfAMA1 genes without any codon optimization were also expressed. The products were soluble, with yields between 50 and 200 μg/ml of the translation mixture, indicating that the cell-free system can be used to produce malaria proteins without any prior optimization of their biased codon usage. Biochemical and immunocytochemical analyses of antibodies raised in mice against each protein revealed that every antibody retained its high specificity to the parasite protein in question. The development of parasites in mosquitoes fed patient blood carrying *Plasmodium falciparum* gametocytes and supplemented with our mouse anti-Pfs25 sera was strongly inhibited, indicating that both Pfs25-3D7/WG and Pfs25-TBV/WG retained their immunogenicity. Lastly, we carried out a parallel expression assay of proteins of blood-stage *P. falciparum*. The PCR products of 124 *P. falciparum* genes chosen from the available database were used directly in a small-scale format of transcription and translation reactions. Autoradiogram testing revealed the production of 93 proteins. The application of this new cell-free system-based protocol for the discovery of malaria vaccine candidates will be discussed.

Plasmodium falciparum is the protozoan responsible for the widespread return of malaria to tropical countries, particularly in Africa. This reemergence is generally credited to two causes: the development of multidrug-resistant parasites and the development of insecticide-resistant mosquitoes (10). Through decades of work, scientists have learned that vaccination could be a potent curative, but efforts to develop a successful vaccine have not yet succeeded (25). One of the bottlenecks in vaccine development is at the malaria protein production step and is mainly due to the lack of a methodology to enable preparation of quality proteins in an efficient manner. *P. falciparum* genes have a very high A/T content (average, 76% per gene), and a number of them encode repeated stretches of amino acid sequences (8); these features have been proposed as the major factors limiting *P. falciparum* protein expression in cell-based systems. Moreover, the presence of glycosylation machinery in eukaryotic cell-based systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses (9, 21, 26). In fact, the three pioneering genome-wide studies on the production of *P. falciparum* proteins in cell-based systems faced serious problems. For instance, Aguiar et al. (1) were able to obtain expression in *Escherichia coli* cells of only 39 of 292 malaria genes cloned into the glutathione *S*-transferase (GST) fusion vector. Mehlin et al. carried out an even more challenging trial in which 1,000 genes encoding relatively small (<450 amino acids) malaria cytosolic proteins were expressed in *E. coli* (24). In that study only 30% of the genes were expressed and only 6.3% of the proteins were soluble, yielding 0.9 mg to 406 mg of protein per liter of culture medium. The other approach used an engineered *E. coli* strain with tRNAs genetically supplemented to allow reading of the high number of A/U codons in malaria mRNA (31). A significant improvement in protein solubility, up to 20.9%, was observed (38 out of 182 proteins tested were soluble). However, although the *E. coli* translation system is known to support folding of prokaryotic and small eukary-

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▽ Published ahead of print on 11 February 2008.

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otic proteins, the multidomain proteins common in eukaryotes tend to fold incorrectly in the *E. coli* system, resulting in the formation of inclusion bodies.

Through decades of laborious work, scientists have identified three leading vaccine candidates from the pool of *P. falciparum* proteins: Pfs25 (19), PfCSP (5, 12, 34), and PfAMA1 (6, 11). Pfs25, a zygote/ookinete surface protein, is a promising candidate as a transmission-blocking vaccine. This protein is composed of four tandem epidermal growth factor-like domains, containing three putative N-linked glycosylation sites beside a signal peptide for the attachment of a glycosylphosphatidylinositol moiety (GPI anchor) at the C terminus. These characteristics render Pfs25 very difficult to express (18, 20). PfCSP, with its biased codon usage and lopsided amino acid composition, allows for only a minute amount of protein to be expressed in *E. coli* cells (34). The other antigen candidate is the PfAMA1 gene, which codes for a type 1 integral membrane protein of merozoites and is also difficult to express. Only a synthetic and codon-optimized gene has produced a fairly large amount of PfAMA1 protein in *E. coli* cells. Furthermore, a series of labor-intensive and technically complex refolding processes of the aggregates were required to use the protein as an antigen (6). The fact that only a few vaccine candidates are currently available (23) is most likely the result of difficulties in expressing malarial antigens in high quantity with their correct conformation.

We previously developed a wheat germ cell-free protein synthesis system for practical use in protein production. The system is especially powerful when used for the production of eukaryotic proteins because of its eukaryotic nature. We established two wheat germ cell-free protein protocols for practical use. The first can be used to produce a small amount of protein from a large number of cDNAs, in parallel, for the examination of product qualities and for the genome-wide biochemical annotation of gene products. In this approach, the templates for transcription are constructed using the split-PCR approach (29). The solution resulting from transcription is then directly used as the mRNA source in the small-scale bilayer translation system (28). The second protocol enables the production of large quantities of proteins. In this case, suitable gene products are first selected using the small-scale parallel production method and subsequent functional screening. Genes of interest are then cloned into the pEU plasmid (29), and the mRNA is transcribed. In the translational step, the protein production employs either the bilayer or the discontinuous batch translation method. The bilayer method has acceptable performance for the production of hundreds of micrograms of protein. Since 150 mg of a control protein in a reaction volume of 50 ml was produced in 5 h with the latter reaction method, the cell-free method can be scaled up (27). The system has been acknowledged in the fields of structural and functional genomics of eukaryotes (7, 32) and has proved advantageous due to its capacity to yield high-quality proteins. Taken together, the system seems to be powerful when used for the production of malaria parasite proteins, as no glycosylation takes place during the standard reaction. However, to date, there is no Good Manufacturing Practice facility for production of recombinant proteins for clinical studies using the wheat germ cell-free system in the world. In the present study, we first tested the versatility of the wheat germ cell-free system using as control models the leading vaccine candidate genes from *P. falciparum*. In addition, a series of experiments was conducted to prove the value of the system for the parallel expression of malaria proteins. The results presented here suggest that the wheat germ cell-free system may be useful as an additional protein production method in the field of *P. falciparum* research.

MATERIALS AND METHODS

Genomic cloning and construction of genes encoding fragments of Pfs25, PfCSP, and PfAMA1. The nucleotide sequences for the signal peptide and the GPI anchor were excluded from the expression constructs for genes encoding the PfCSP and Pfs25 proteins. The truncated versions of the PfCSP and Pfs25-3D7 genes were amplified by PCR from the genomic DNA of the *P. falciparum* 3D7 strain and subcloned into pEU3 (a vector carrying the C-terminal His_6 tag) (29) at the EcoRV site. The gene encoding Pfs25-TBV was a generous gift from Anthony W. Stowers (NIAID, NIH, Rockville, MD) (35). Pfs25-TBV, a synthetic version of the Pfs25 gene, was codon optimized for expression in the yeast *Saccharomyces cerevisiae*, and the replacement of Asn with Gln at three N-glycosylation sites was performed (20). DNA encoding full-length PfAMA1 protein was amplified from the genomic DNA of *P. falciparum* 3D7 and cloned into pEU-E01-GST (a vector with an N-terminal GST tag followed by a tobacco etch virus protease cleavage site) between the XhoI and BamHI sites. These pEU plasmid vectors are the expression vectors designed specifically for the wheat germ cell-free system (16). The inserted nucleotide sequences were confirmed using the ABI PRISM 310 Genetic Analyzer and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

Parallel construction of the DNA template from the parasite RNA. We selected 124 genes annotated as dominantly expressed in the blood stages of *P. falciparum* based on the microarray data integrated in the PlasmoDB database (http://www.plasmodb.org) (see Table S1 in the supplemental material). Extracted total RNA from *P. falciparum* 3D7 asexual blood-stage parasites was reverse transcribed into cDNA by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), and PCR amplification was performed using *LA Taq* DNA polymerase (Takara Bio, Otsu, Japan). The 5′ primers were designed as 46-mers: 16-mer nucleotide sequences (5′-CCACCCACCACCACCA) as the S1 tag sequence followed by a 30-mer of unique sequence covering each 5′ open reading frame containing the start codon. For the 3′ primers, 30-mer nucleotide sequences covering each unique sequence upstream from the termination codon were prepared. The PCR products were then cloned into the pCR2.1 plasmid using a TOPO TA cloning kit (Invitrogen), and their sequences at both ends were confirmed. Translation templates were prepared by in vitro transcription from PCR products amplified by the split-primer PCR method described earlier (29).

Production and purification of the Pfs25-3D7/WG, Pfs25-TBV/WG, PfCSP/WG, and PfAMA1/WG proteins. We employed the wheat germ cell-free protein expression system for protein production using the bilayer translation reaction method described previously (28). Briefly, 250 μl of transcription mixture containing 25 μg of the plasmid DNA, 80 mM HEPES-KOH, pH 7.8, 16 mM magnesium acetate, 2 mM spermidine, 10 mM dithiothreitol, 2.5 mM each of nucleoside triphosphates, 250 U of SP6 RNA polymerase (Promega, Madison, WI), and 250 U of RNasin (Promega) was incubated for 6 h at 37°C. After the incubation, the transcription solution containing transcribed mRNA was mixed with 250 μl of wheat germ extract (60 A_{260} units) supplemented with 2 μl of 20-mg/ml creatine kinase in a single well of a six-well plate. The 5.5-ml substrate mix (30 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM dithiothreitol, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate) from the ENDEXT Wheat Germ Expression S kit (CFS Co., Ltd., Matsuyama, Japan) was then added on top of the translation mix and incubated at 26°C for 12 h. After incubation, the reaction mixture was centrifuged at 21,900 × *g* for 20 min. Recovered supernatants were passed through Amicon Ultra centrifugal filter units (10-kDa molecular mass cutoff) (Millipore, Billerica, MA) to replace the translation buffer with phosphate-buffered saline. The samples containing the synthesized Pfs25-3D7/WG, Pfs25-TBV/WG, and PfCSP/WG proteins were purified using the Ni-nitrilotriacetic acid agarose column (Qiagen, Valencia, CA). The PfAMA1/WG protein was purified by passing the supernatant through the glutathione-Sepharose 4B column (GE Healthcare Bio-Sciences, Piscataway, NJ), followed by tobacco etch virus protease (Invitrogen) cleavage to remove the

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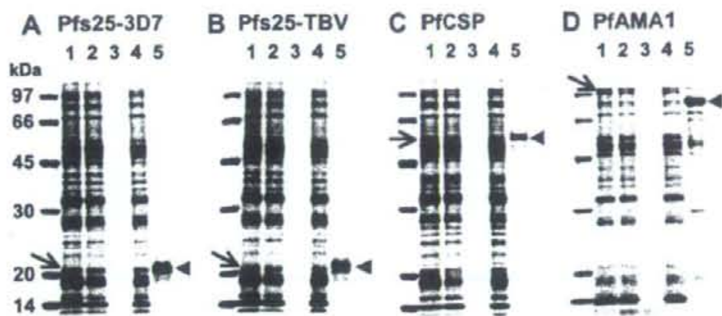

FIG. 1. SDS-PAGE analysis of the proteins expressed in the wheat germ cell-free system. Pfs25-3D7/WG (A), Pfs25-TBV/WG (B), PfCSP/WG (C), and PfAMA1/WG (D) were separated on SDS-12.5% polyacrylamide gels under reducing conditions and stained with Coomassie brilliant blue. The samples in each gel were as follows: total reaction mixture (lane 1), supernatant and precipitated fractions after brief centrifugation (lanes 2 and 3, respectively), and unbound and affinity-purified proteins (lanes 4 and 5, respectively). Products and purified proteins are indicated by arrows and arrowheads, respectively.

GST tag. Concentrations of affinity-purified proteins were determined using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (22), and the bands were visualized with Coomassie brilliant blue. Purified protein samples were stored in aliquots at −80°C until further use. For parallel protein synthesis from 124 malaria genes, the transcription and translation reactions were performed by a method similar to that described above. The 125-μl substrate mixture was overlaid on top of the 25-μl translation mixture containing transcribed mRNA in the presence of U-[^{14}C]Leu (11.1 kBq; 15 GBq/mmol of Leu). The reaction was performed in 96-well plates. Proteins were separated by SDS-PAGE and identified by autoradiography using an imaging analyzer (BAS-2500; Fujifilm, Tokyo, Japan). The solubility of each product was expressed as the percentage of trichloroacetic acid-insoluble radioactivity (counted using a liquid scintillation counter [LSC-6100; Aloka, Mitaka, Japan]) in a supernatant fraction recovered from centrifugation at 21,900 × *g* for 20 min compared to that of the total reaction mixture. The amount of target protein was estimated using the following formula where count is the radioactivity of the protein produced; Leu is the number of Leu residues in the protein, used to estimate the moles of Leu incorporated; MW is molecular weight; and ratio is the ratio of intensity of a specific protein band to the total intensity of bands on the autoradiogram: protein concentration = count/Leu × MW × ratio.

Preparation of antiserum. Groups of female BALB/c mice (five mice in each group) were subcutaneously immunized three times in the 1st, 3rd, and 5th weeks with 10 μg of affinity-purified proteins emulsified in Freund's adjuvant. As the control, a group of mice was administered GST in Freund's adjuvant, using the same protocol as described above. Antiserum preparation was as described elsewhere (2).

Preparation of *P. falciparum* asexual blood-stage parasites, ookinetes, and sporozoites. A mature schizont-rich fraction was obtained from cultured *P. falciparum* strain 3D7 (30). Parasite pellets were kept at −80°C until extract preparation.

To obtain ookinetes and sporozoites of *P. falciparum*, we used parasites derived from patient blood. The use of all human materials in this study was reviewed and approved by the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army. Peripheral blood was collected with heparinized syringes under written informed consent from patients who came to the malaria clinics in the Mae Sod district, Thailand. Infection with *P. falciparum* was confirmed by the microscopic observation of Giemsa-stained thick and thin blood smears. The gametocytemic patient blood was divided into two parts. One was used to grow zygotes/ookinetes in vitro for both Western blotting and immunocytochemical analyses, and the other half was subjected to propagation of sporozoites in mosquitoes for two further analyses, as described elsewhere (33). Western blot analysis and immunocytochemistry were performed as described previously (3, 17).

Transmission-blocking assays. We collected 20 ml of peripheral blood from a volunteer patient. Blood was divided into aliquots (300 μl/tube) and briefly centrifuged, and plasma was discarded. Mouse immune sera against both Pfs25-3D7/WG and Pfs25-TBV/WG were serially diluted with heat-inactivated normal human serum prepared from malaria-naive donors. Next, 180 μl of each diluted solution was added to the *P. falciparum*-infected blood cells and incubated for 15 min at room temperature. The mixture was placed in a membrane feeding apparatus kept at 37°C to allow *Anopheles dirus* A mosquitoes to feed on the blood in each apparatus for 30 min. Fully engorged mosquitoes were maintained for a week in the insectary. Oocysts that developed within the midgut were counted from 20 randomly selected mosquitoes. The Kruskal-Wallis test was applied to examine the differences in oocyst counts per mosquito between immunized groups and the control group fed on mouse serum raised against GST. Probability values (P) of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

We were able to successfully express the Pfs25/WGs, PfCSP/WG, and PfAMA1/WG proteins using the wheat germ cell-free system. Expression of the Pfs25 (Pfs25-3D7/WG) protein from a gene with a native nucleotide composition was shown by subsequent SDS-PAGE analysis (Fig. 1A) to be comparable in amount to that of Pfs25-TBV/WG (Fig. 1B) expressed from the codon-optimized synthetic gene. On the SDS-polyacrylamide gels, two protein bands appeared at 20 kDa, the expected mobility of the Pfs25 truncated form, lacking the signal peptide and the GPI anchor. Almost all of the Pfs25-3D7/WG protein from the biased DNA was recovered in the supernatant fraction (Fig. 1A, lane 2) and was easily purified as a single dominant band along with other nonspecific faint bands by affinity chromatography (Fig. 1A, lane 5). The amount of purified Pfs25-3D7/WG was 35 μg per 6.0 ml of the reaction mixture, while that obtained from the codon-optimized gene was comparable, at 30 μg protein per reaction mixture. These results demonstrate that the wheat germ cell-free system that we employed produced equal amounts of proteins with and without prior optimization of their biased codon usage in the DNA. Similarly, the amounts of the other two proteins, PfCSP/WG (Fig. 1C), and PfAMA1/WG (Fig. 1D), produced from a gene with a native nucleotide composition were 26 and 102 μg per reaction, respectively.

Immunological characterization of the protein products. To determine the creation of conformation-dependent epitopes in Pfs25 and AMA1, we examined and confirmed the reactivity of

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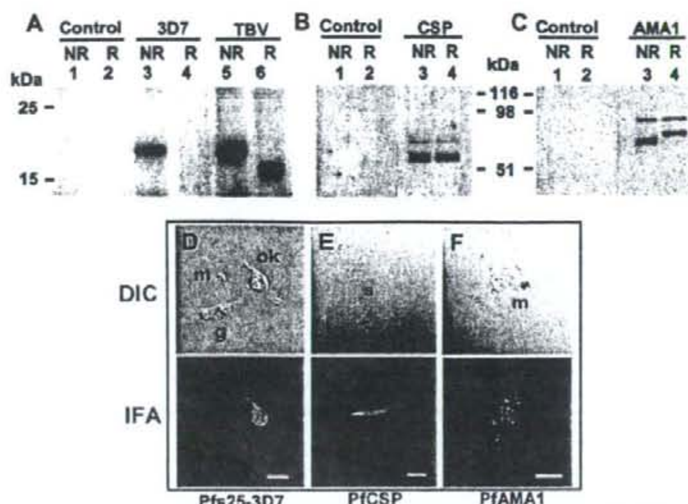

FIG. 2. Western blot and immunocytochemical analyses using antisera against Pfs25-3D7/WG, Pfs25-TBV/WG, PfCSP/WG, and PfAMA1/WG. Extracts prepared from *Plasmodium falciparum* zygotes/ookinetes (A), sporozoites (B), and schizonts (C) were separated on SDS-12.5% polyacrylamide gels under nonreducing (NR; lanes 1, 3, and 5) and reducing (R; lanes 2, 4, and 6) conditions. (A) Proteins on polyvinylidene fluoride membranes were immunostained either with mouse anti-Pfs25-3D7/WG serum (lanes 3 and 4) or mouse anti-Pfs25-TBV/WG serum (lanes 5 and 6) or with the negative-control serum (lanes 1 and 2). (B) The membrane was immunostained with either mouse anti-PfCSP/WG serum (lanes 3 and 4) or the control serum (lanes 1 and 2). (C) The membrane was immunostained with either mouse anti-PfAMA1/WG serum (lanes 3 and 4) or the control serum (lanes 1 and 2). (D to F) Samples prepared from *Plasmodium falciparum* immature ookinete (D), sporozoite (E), and schizont (F) were immunostained with the antiserum indicated at the bottom of the panel. Upper panels represent images obtained by differential interference contrast (DIC) microscopy, and lower panels represent immunostained images (immunofluorescence assay [IFA]) visualized with goat anti-mouse immunoglobulin G-fluorescein conjugate. These images have been taken by confocal scanning laser microscopy (LSM5 PASCAL; Carl Zeiss MicroImaging, Thornwood, NY). g, gametocyte; ok, immature ookinete; m, merozoite; s, sporozoite. Bars, 5 μm.

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anti-Pfs25 conformation-specific monoclonal antibody 4B7 (a generous gift from Carole A. Long [NIAID, NIH, Rockville, MD]) against Pfs25/WGs and the reactivity of anti-PfAMA1 3D7 conformation-specific monoclonal antibody 1E9 (a generous gift from Carole A. Long) against PfAMA1/WG by Western blotting under nonreducing conditions (data not shown). To evaluate the immunogenicity of each protein prepared, we then raised mouse antisera and determined their reactivity to the parasite-derived native proteins. Extract from approximately 5×10^5 zygotes/ookinetes per lane was separated by SDS-PAGE, and Western blot analysis was performed. Specific bands with the expected mobility of native Pfs25 protein were detected under nonreducing conditions using antisera against Pfs25-3D7/WG and Pfs25-TBV/WG. Anti-Pfs25-3D7/WG serum did not show any reactivity under reducing conditions (Fig. 2A). These results suggest that the Pfs25-3D7/WG protein prepared here retained a conformation similar to that of the native protein. The identity of the faint band detected at the lower position with anti-Pfs25-TBV/WG under reducing conditions is unclear at present (Fig. 2A). Similar experiments were performed using anti-PfCSP/WG and anti-PfAMA1/WG sera to study extracts from respective stages of the parasite. The analyses clearly showed specific reactivity of each antiserum to PfCSP and PfAMA1 proteins (Fig. 2B and C). Anti-PfCSP serum reacted to three protein bands in the sporozoite extract under both reducing and nonreducing conditions (Fig. 2B). The upper and lower bands appeared to correspond to precursor and mature forms, respectively, as reported earlier by Coppi et al. (4). Anti-PfAMA1 serum gave two signals, with the upper and lower bands corresponding to mature and processed forms, respectively (15). The signal shift of the two bands upon introduction of a reducing reagent was most likely due to the high content of disulfide bonds within the protein (14). These results are consistent with previously reported findings (13).

Immunocytochemical staining was performed against immature ookinetes obtained by in vitro short-term culture using anti-Pfs25-3D7/WG. As shown in Fig. 2D (differential interference contrast and immunofluorescence assay), the antiserum specifically stained the surface of the immature ookinete but not the gametocyte and the merozoite. Antiserum against Pfs25-TBV/WG yielded similar results (data not shown). These findings were consistent with our previous report in which Pfs25-TBV prepared from yeast cells was used to raise antiserum (2). These findings also verified that Pfs25 prepared using our protocols from a gene with an A/T-rich native nucleotide composition can yield a protein of sufficient quality to raise a specific antibody. Experiments using anti-PfCSP/WG and anti-PfAMA1/WG on the target stages of the parasite showed typical staining patterns. The entire surface of the slender sporozoite was stained by anti-PfCSP/WG serum (Fig. 2E), and the anti-PfAMA1/WG serum clearly visualized punc-

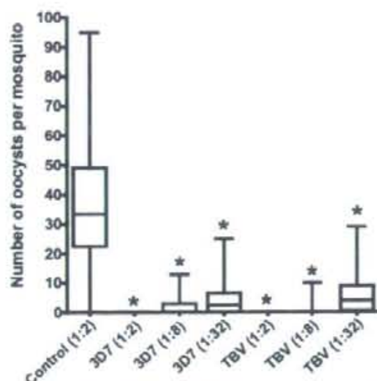

FIG. 3. Transmission-blocking efficacy of antibodies against *Plasmodium falciparum* parasites. The median numbers of oocysts per mosquito ($n = 20$) with quartiles (box plot) and ranges (lines on both top and bottom of the box) were compared among groups of mosquitoes fed on either anti-Pfs25 sera serially diluted or control mouse serum. Dilution of test immune serum is shown as 1:2 to 1:32. Statistical analysis was performed using the Kruskal-Wallis test for comparison of oocyst numbers between the test immune sera and control serum. Asterisks indicate statistically significant differences compared to the control group ($P < 0.05$).

tate localization of PfAMA1 at the apical end of merozoites (Fig. 2F).

Efficacy evaluation of the proteins as vaccine antigens. In view of a practical application of the system for discovery of malaria vaccine candidates, we evaluated the quality of antigens produced by performing a parasite growth inhibition assay using the antibodies raised against those antigens. We focused on Pfs25-3D7/WG and Pfs25-TBV/WG. Pfs25-TBV is currently the sole transmission-blocking vaccine candidate under clinical trial (23). A transmission-blocking assay was performed using both anti-Pf25-3D7/WG and anti-Pfs25-TBV/WG. A mixture containing *P. falciparum*-gametocyte infected erythrocytes and one of the antisera was fed to mosquitoes. The number of developed oocysts in the mosquitoes was then later counted. Both antisera at twofold dilution completely inhibited oocyst development, as we have seen no mosquito harboring oocysts (Fig. 3). The number of oocysts was inversely proportional to the concentration of antiserum added, findings consistent with previous experiments, in which Pfs25-TBV prepared from yeast was used to raise antiserum (2). It is important at this moment to stress the difference between this study and other studies: our proteins were produced from a non-codon-optimized gene in a cell-free system, while in other studies a codon-optimized engineered Pfs25-TBV gene was transformed into yeast cells (20). The results presented here strongly indicate the value of the wheat germ cell-free system for the production of malaria proteins that require complicated procedures in other systems.

Parallel syntheses of *P. falciparum* proteins. Although cell-based expression systems have been widely used in this field, they are limited mainly in their ability for efficient production of *P. falciparum* protein, primarily because of the complexity of the genome. In order to evaluate the capability of our cell-free system for parallel expression from the parasite genes, we selected 124 genes (see Table S1 in the supplemental material) encoding asexual blood-stage parasite proteins, based on the PlasmoDB database. Autoradiography demonstrated that 93 of the 124 genes yielded protein products. The average yield of expressed protein estimated for each full-size product was 1.9 μg per 150 μl of reaction mixture, an amount sufficient for preliminary antigen discovery studies using hyperimmune serum. Average protein solubility was 65% (see Table S1 in the supplemental material). There was significant inverse correlation between yield and molecular size of the protein; the greater the size, the lower the protein yield. There was also weak but significant inverse correlation between the protein yield and the relative frequency of low-complexity regions. In addition, solubility was inversely correlated with the pI value (Table 1). These observations have already been documented in earlier studies (24, 31). Surprisingly, we did not see any correlation between yield and A/T content, pI value, or the existence of a transmembrane domain (data not shown). We then analyzed the statistical difference in molecular weights, pI values, A/T contents, and relative frequencies of low-complexity regions between the expressed and nonexpressed groups of molecules, using the Mann-Whitney U test. The molecular weights in the nonexpressed group were significantly higher than those of the expressed group ($P < 0.0001$). In contrast, pI values, A/T contents, and the relative frequencies of low-complexity regions did not differ significantly (see Table S1 in the supplemental material). We currently have no explanation for why 25% of the tested genes failed to produce proteins in our system. One possible explanation is the sequence errors most likely present in the PCR products that were used as templates for transcription and subsequent translation. Such templates would cause mistranslation of the protein by frameshift.

In summary, the ability of the wheat germ cell-free protein synthesis system to produce *P. falciparum* proteins was examined. We found that (i) without the need for codon optimization, the cell-free system is able to produce a sufficient amount of high-quality proteins of the leading malaria vaccine candidates, Pfs25, PfCSP, and PfAMA1; (ii) biochemical, immunocytochemical, and biological analyses demonstrated that the prepared proteins could be directly used for immunization after a simple affinity purification step; and (iii) the system proved suitable for use as a parallel

TABLE 1. Correlation of expression or solubility and characteristics[a]

Parameter	Correlation coefficient (*P* value)	
	Protein concn	% Solubility
Mol wt	−0.3177 (0.0019)[b]	−0.1221 (0.2436)
pI	−0.1214 (0.2464)	−0.3519 (0.0005)[b]
% A/T	−0.1505 (0.1498)	
Low complexity[c]	−0.2276 (0.0283)[b]	
% Solubility	−0.0494 (0.6385)	

[a] Spearman's correlation coefficients by rank were calculated among the 93 proteins expressed. The probability values of the statistical significance are shown in parentheses.

[b] $P < 0.05$ was considered to indicate a statistically significant correlation.

[c] Relative frequency of low-complexity regions per molecular weight.

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way to produce parasite proteins. We believe that the wheat germ cell-free protein synthesis system may be a key tool for decoding genetic information above and beyond malaria vaccine research.

ACKNOWLEDGMENTS

We thank Jeeraphat Sirichaisinthop and the staff of the Vector Borne Disease Training Center, Pra Budhabat, Saraburi, Thailand, for assistance in setting up the field sites and the staff of the Department of Entomology, AFRIMS, Bangkok, Thailand, as well as Hiroko Suzuki, Limei Yin, Kana Kato, and Aya Tamai for their technical assistance. We are grateful to Ivona Kozieradzki for critical reading of the manuscript and valuable comments.

This work was supported in part by Grants-in-Aid for Scientific Research (18390129 and 19406009) and Scientific Research on Priority Areas (19041053) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a Grant-in-Aid from the Ministry of Health, Labor and Welfare (H17-Sinkou-ippan-019) of Japan.

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Editor: W. A. Petri, Jr.

Downloaded from iai.asm.org by on March 18, 2008

Journal of Antimicrobial Chemotherapy (2009) **63**, 252–255
doi:10.1093/jac/dkn482
Advance Access publication 25 November 2008

JAC

Indigenous evolution of *Plasmodium falciparum* pyrimethamine resistance multiple times in Africa

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Received 16 July 2008; returned 18 September 2008; revised 7 October 2008; accepted 31 October 2008

Objectives: Resistance to pyrimethamine in *Plasmodium falciparum* is conferred by mutations in the gene encoding dihydrofolate reductase (*DHFR*). It is known that *DHFR* double mutants have evolved independently in multiple geographic areas, whereas the triple mutant prevalent in Africa appears to have originated in south-east Asia. In this study, we investigated whether other triple mutants may have evolved independently in Africa.

Methods: We determined the *DHFR* genotypes and haplotypes of five microsatellite loci flanking the *DHFR* locus between 4.49 kb upstream and 1.48 kb downstream of 159 isolates collected from three African countries (Republic of Congo, Ghana and Kenya).

Results: The CIRNI type of *DHFR* triple mutant (with mutations underlined at amino acid positions 51, 59 and 108) was predominant in the Republic of Congo (82%) and Ghana (81%) and was the second most prevalent in Kenya (27%), where the CICNI type of *DHFR* double mutant was dominant. Three distinct microsatellite haplotypes were identified in the *DHFR* triple mutant. One haplotype was identical to that originating in south-east Asia. The other two haplotypes occurred in Ghana and Kenya, which were unique, previously undescribed and identical to those of the two *DHFR* double mutants found in the same locations.

Conclusions: This study presents strong evidence for the unique, multiple independent evolution of pyrimethamine resistance in Africa. Indigenous evolution of the triple mutant from the double mutant appears to have occurred in a step-wise manner in Kenya and Ghana or in nearby countries in east and west Africa.

Keywords: malaria, *dhfr*, microsatellite

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Introduction

The spread of *Plasmodium falciparum* resistance to commonly used antimalarial drugs is a major public health problem in malaria-endemic regions. The antifolate drug pyrimethamine/sulfadoxine inhibits two enzymes in the parasite's folate synthesis pathway. Amino acid changes at positions 50, 51, 59, 108 and 164 in one of these enzymes, dihydrofolate reductase (DHFR), are strongly associated with pyrimethamine resistance.[1,2] At present, isolates harbouring four mutations (CIRNL, with mutated amino acids underlined) show the highest degree of resistance to pyrimethamine. There are several *DHFR* genotypes in malaria-endemic regions,[3] but the number of times the resistant genotype has emerged in independent parasite lineages is thought to be considerably limited.[4–7] In south-east Asia, an analysis of microsatellite markers flanking *DHFR* indicated that all resistant parasites bearing two or more mutations share a single lineage.[4] In contrast, in Africa, multiple indigenous lineages have been reported for *DHFR* double mutants.[5,8] Nevertheless, it is currently accepted that these indigenous double mutants have not yet produced a triple mutant. Rather, nearly all lineages showing the *DHFR* triple mutant found in Africa have been ascribed to the migration of triple-mutant parasites from south-east Asia.[6–8] However, given the occurrence of multiple lineages of *DHFR* double mutants in Africa, it is likely that triple mutants may have evolved independently within the continent. In this study, we determined the *DHFR* genotype and flanking microsatellite haplotypes of *P. falciparum* isolates from the Republic of Congo, Ghana and Kenya. We discovered two previously undescribed lineages of the *DHFR* triple mutant, from Ghana and Kenya, showing the independent evolution of *DHFR* triple mutants within Africa.

Materials and methods

Study site and patients

Blood samples were collected from symptomatic individuals from Pointe-Noire, Brazzaville and Gamboma in the Republic of Congo in 2006 and from asymptomatic individuals at three villages near Winneba in Ghana in 2004 and at two villages in Kisii District in Kenya in 1998. In all regions, surveys were necessarily conducted prior to the official introduction of pyrimethamine/sulfadoxine treatment for malaria and so were not feasible with current populations. These studies were approved by the administrative authority of the Ministry for Research and Ministry for Health in the Republic of Congo, the Ministry of Health/Ghana Health Service and the Ministries of Health and Education in Kenya. In all surveys, each patient was informed about the study prior to sampling, consent was obtained (in the case of children, parents/guardians gave informed consent) and medical follow-up was provided if needed.

DHFR genotyping and microsatellite haplotyping

Parasite DNA was purified using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) for samples from Ghana and Kenya and the EZ1 BioRobot™ (QIAGEN) for samples from the Republic of Congo. The first 184 codons of *DHFR*, encompassing all known polymorphic loci were amplified by nested PCR. Amplified products were directly sequenced with a BigDye terminator v1.1 cycle sequencing kit in an ABI 377 DNA Sequencer (Applied Biosystems, CA, USA) as described previously.[9] In order to determine the evolutionary history of pyrimethamine-resistant *DHFR* genotypes, we identified individual haplotypes based on the association of five microsatellite markers closely linked to the gene (0.1, 3.87 and 4.49 kb upstream, and 0.52 and 1.48 kb downstream of *DHFR*).[4] Briefly, variation in the number of TA repeats in microsatellite loci was measured by semi-nested PCR using fluorescent end-labelled primers, followed by electrophoresis on an ABI 377 sequencer and analysed with the Genescan software (Applied Biosystems).

The microsatellite haplotype observed in south-east Asia, harbouring an association of 200-194-176-106-203 bp at microsatellite loci 4.49, 3.87 and 0.1 kb upstream and 0.52 and 1.48 kb downstream of *DHFR* was designated the SEA haplotype.[10] Microsatellite haplotypes unique to the Republic of Congo, Ghana and Kenya are described in the Results section. Haplotypes showing minor variations at one or two loci were included in the respective major haplotypes.

Isolates showing mixed *DHFR* genotypes were excluded from the analysis. Samples with more than one allele detected at any microsatellite locus were also considered as mixed allele infections and excluded. By these criteria, 131 (45%) of 290 samples were excluded from the analysis.

Results

DHFR genotypes

We identified five distinct *DHFR* genotypes in 159 isolates: one triple mutant (CIRNI), two forms of double mutant (CICNI and CNRNI), one single mutant (CNCNI) and the wild-type (CNCSI) (Figure 1). The triple mutant was found in all three African countries with high prevalence in the Republic of Congo and Ghana (81–82%), whereas the two double mutants were distributed differently, the CICNI type in the Republic of Congo and Kenya and the CNRNI type in Ghana.

Microsatellite haplotypes

DHFR double-mutant isolates carried microsatellite haplotypes that were unique to each country (Figure 2a). In Kenya, the majority of isolates were of a microsatellite haplotype consisting

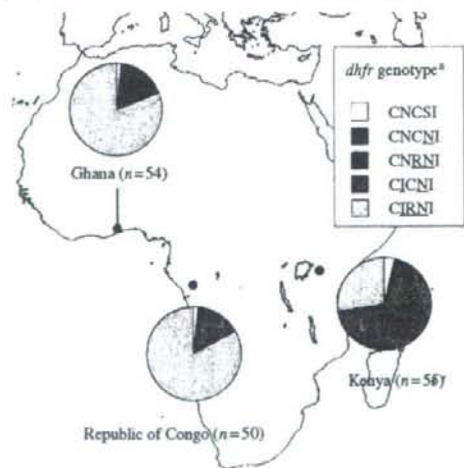

Figure 1. Frequency of *DHFR* genotypes in *P. falciparum* isolates from Kenya, the Republic of Congo and Ghana. ^a Genotype is expressed as an association of amino acids at positions 50, 51, 59, 108 and 164. Underlined amino acids indicate mutated residues.

(a)

Country	Size of microsatellite markers (bp) at indicated position					*n*	Haplotype
	−4.49 kb	−3.87 kb	−0.1 kb	0.52 kb	1.48 kb		
Kenya (CICNI)	204	190	156	96	203	33	KEN haplotype
	196	190	156	96	203	2	
	208	190	156	96	203	1	
	204	190	156	96	211	1	
	202	186	156	96	203	1	
Republic of Congo (CICNI)	198	194	170	98	197	5	CON haplotype
	206	194	170	98	197	1	
	198	190	170	96	197	1	
Ghana (CNRNI)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	8	GHA haplotype
	202	186	[illegible]	[illegible]	[illegible]	1	

(b)

Country	Size of microsatellite markers (bp) at indicated position					*n*	Haplotype
	−4.49 kb	−3.87 kb	−0.1 kb	0.52 kb	1.48 kb		
Kenya	200	194	176	106	203	4	SEA haplotype
	204	194	176	106	203	1	
	204	190	176	106	203	1	
	200	190	156	96	203	2	KEN haplotype
	204	190	156	96	203	7	
Republic of Congo	200	194	176	106	203	36	SEA haplotype
	202	194	176	106	203	1	
	200	194	176	106	197	1	
	204	194	176	96	203	1	KEN haplotype
	200	194	156	96	203	2	
Ghana	200	194	176	106	203	29	SEA haplotype
	[illegible]	194	176	106	203	1	
	200	194	176	106	[illegible]	1	
	200	206	[illegible]	[illegible]	[illegible]	1	[illegible]
	202	186	[illegible]	[illegible]	[illegible]	2	
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	9	
	204	190	156	96	203	1	KEN haplotype

Figure 2. Microsatellite haplotypes in *P. falciparum* isolates having *DHFR* double (a) and triple (b) mutations in Kenya, the Republic of Congo and Ghana. KEN haplotype, CON haplotype, GHA haplotype and SEA haplotype are unique associations at five microsatellite loci of alleles. Haplotypes showing minor variations at one or two loci (unboxed) were included in the respective major haplotypes. These alleles are likely to be generated by a TA repeat mutation and/or recombination events between unlike haplotypes. (b) A variant belonging to the SEA haplotype from the Republic of Congo had an allele at 1.48 kb that was seen in the *DHFR* double mutant in the same country.

of an association of 204-190-156-96-203 bp (KEN haplotype), and the rest had minor variations from the predominant haplotype. Similarly, all isolates from the Republic of Congo were of one predominant haplotype (198-194-170-98-197 bp; CON haplotype), as were those from Ghana (198-204-168-104-205 bp; GHA haplotype). No SEA haplotype was observed.

We identified three microsatellite haplotypes associated with the *DHFR* triple mutant (Figure 2b). In Kenya, both the KEN haplotype and SEA haplotype were observed. Chimeric haplotypes between the SEA and KEN haplotypes were observed, probably generated by recombination between the two haplotypes. In the Republic of Congo, the SEA haplotype was the most prevalent (93%) and the KEN haplotype was also found at low prevalence (7%). All three samples of the KEN haplotype were chimeric haplotypes, having alleles of the SEA haplotype at two loci. In Ghana, the SEA haplotype was most dominant (70%), followed by the GHA haplotype (27%) and the KEN haplotype (3%). Overall, among the three *DHFR* triple-mutant lineages, the SEA haplotype was the most prevalent (75%), and the prevalence of the KEN (13%) and GHA (12%) haplotypes was low but significant.

Discussion

The present study clearly shows that there are three lineages of the CIRNI pyrimethamine-resistant *DHFR* triple mutant in the Republic of Congo, Ghana and Kenya. One of the three lineages

is identical to the SEA lineage and the other two lineages, the KEN lineage and GHA lineage, are unique to Africa. The SEA lineage was the most prevalent of the *DHFR* triple mutants, but the prevalence of the two African lineages was significant. Importantly, microsatellite haplotypes of the two African lineages were also found from parasites with *DHFR* double mutants, while no double mutants shared the SEA microsatellite haplotype. These findings present strong evidence for the indigenous evolution of the *DHFR* triple mutant from the double mutant in Africa.

Previous reports have not identified indigenously generated African triple *DHFR* mutants. These include two studies analysing 24 triple mutants from South Africa,[6] and 204 from 11 sub-Saharan African countries.[7] In these countries, all triple mutants except one showed an identical or very similar microsatellite haplotype to the SEA haplotype. This suggests that the *DHFR* triple mutant currently predominant in Africa was imported from SEA, rather than indigenously generated. In the present study, the SEA lineage was the most prevalent in all countries considered, but two other indigenous lineages were also observed.

The co-prevalence of the SEA haplotype and the KEN/GHA haplotypes in Africa suggests that the indigenous *DHFR* triple mutant may have evolved earlier than or contemporaneously with the migration of the SEA type from south-east Asia—an initial triple mutant, either indigenously evolved or imported, would quickly spread out through the continent, as observed in south-east Asia, where only a single lineage of *DHFR* triple or quadruple mutant is presently prevalent.[4,10] This scenario is based on the assumption that pyrimethamine pressure was continuously and extensively present. However, in Africa, the history of pyrimethamine administration differs greatly country-by-country. Indeed, we observed the *DHFR* wild-type, a single mutant and double mutants in three African countries, a situation in sharp contrast to that in south-east Asia where all *P. falciparum* populations show a single lineage of *DHFR* triple/quadruple mutant.[4,10] Dispersal of drug resistance obviously depends on many factors, including movements of infected people, the intensity of malaria transmission, the level of host population immunity and the history and extent of drug usage. Thus, the strength of any selective sweep by pyrimethamine differs depending on area, making it difficult to infer which occurred earlier; the migration of the *DHFR* triple mutant from south-east Asia into Africa or the indigenous evolution of the triple mutant in Africa.

This study also presents strong evidence for the indigenous evolution of *DHFR* double mutants in Africa. All three countries studied have a unique, country-specific lineage of a *DHFR* double mutant, suggesting that the generation of *DHFR* double mutants is not a rare event in Africa, a situation that differs from south-east Asia and Melanesia, where the generation of double mutants has been very rare.[10] As the prevalence of *P. falciparum* is much greater in many parts of Africa than in most of south-east Asia and Melanesia, the pool of potential drug-resistant mutant parasites available for selection by drug pressure is much larger. We believe that this situation may explain the generation and selection of multiple independently originating drug-resistant mutants in Africa.

Acknowledgements

We would like to thank: all those who participated in the epidemiological studies for their kind cooperation; Hideaki Eto, Hikota Osawa and Ayako Nishiyama for their assistance in the field; David Conway, Osami Kanagawa, Ohara Osamu, Kiyoshi Kita and Toshihiro Horii for advice and helpful discussion; and Toshiyuki Hayakawa and Kikuno Reiko for advice regarding evolutionary analysis.

Funding

This study was supported by the Takeda Science Foundation, a Grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (17590380, 18073013, 18GS03140013), and a Grant-in-aid from the Ministry of Health, Labour and Welfare of Japan (H20-Shinkou-ippan-013).

Transparency declarations

None to declare.

T. M. designed the study, conducted the survey, analysed the data and wrote the report. K. T. participated in the design, analysed the data and reviewed the report. N. T. carried out molecular genetic analysis. R. C. participated in the survey in the Republic of Congo and reviewed the report. M. N. organized and participated in the survey in the Republic of Congo. M. D. organized and participated in the survey in Ghana. W. S. A. organized and participated in the survey in Kenya. A. K. organized and participated in the survey in Kenya and Ghana. T. K. participated in the study design, organized the survey in Kenya and Ghana and reviewed the report.

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Malaria Journal

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Failure to detect *Plasmodium vivax* in West and Central Africa by PCR species typing

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Published: 11 September 2008

Received: 28 March 2008

Accepted: 11 September 2008

Malaria Journal 2008, **7**:174 doi:10.1186/1475-2875-7-174

This article is available from: http://www.malariajournal.com/content/7/1/174

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Abstract

Background: *Plasmodium vivax* is estimated to affect 75 million people annually. It is reportedly absent, however, from west and central Africa due to the high prevalence of the Duffy negative phenotype in the indigenous populations. Despite this, non-African travellers consistently return to their own countries with *P. vivax* malaria after visiting this region. An attempt was made, therefore, to detect the presence of *P. vivax* parasites in blood samples collected from the indigenous populations of west and central Africa.

Methods: Parasite species typing (for all four human malaria parasites) was carried out by PCR on 2,588 blood samples collected from individuals from nine African malaria-endemic countries.

Results: Most infections (98.5%) were *Plasmodium falciparum*, *Plasmodium malariae* was identified in 8.5% of all infections, and *Plasmodium ovale* in 3.9%. The prevalence of both parasites varied greatly by country. Only one case of *P. vivax* was detected from Sao Tome, an island off the west coast of Africa, confirming the scarcity of this parasite in Africa.

Conclusion: The prevalence of *P. vivax* in local populations in sub-Saharan Africa is very low, despite the frequent identification of this parasite in non-African travellers.

Background

Plasmodium vivax has the widest geographic range of the four parasites responsible for malaria in man. Historically, its range has extended as far north as Finland and northern China, and as far south as northern Australia and South Africa [1]. Concerted malaria control initiatives in countries in temperate zones have today confined *P. vivax* mainly to the tropics, where its range overlaps that of the most important malaria parasite in terms of public health, *Plasmodium falciparum*. Thus, the two parasites co-exist in large parts of the tropical and semi-tropical world, except, strikingly, in large parts of western and central Africa, where *P. vivax* appears to be almost completely absent [2]. This situation is apparently caused by the high prevalence of the Duffy negative phenotype in the local populations, which confers complete protection against *P. vivax* malaria [3]. The Duffy antigen/receptor for chemokines (DARC) is a transmembrane glycoprotein that is present on epithelial cells [4], endothelial cells [5], and erythrocytes. It is utilized by *P. vivax* parasites as the receptor for attachment to the red cell surface [6]. Duffy negative individuals are homozygous for a DARC allele, FY^*B^{null}, which carries a single nucleotide mutation which impairs promoter activity by disrupting a binding site for the h-GATA-1 erythroid transcription factor [7]. This results in the loss of DARC expression on erythrocytes, but does not affect expression in epithelial or endothelial cells. Individuals who are homozygous for this allele thus express no DARC protein on the red cell surface and are completely protected from the erythrocytic cycle of *P. vivax*. The Duffy negative phenotype occurs in over 95% of the population of west and central Africa, but is extremely rare outside Africa and the Arabian peninsula [8].

Present day prevalence of *P. vivax* in Africa

Although *P. vivax* is known to be present in parts of northern, eastern and southern Africa, with some areas reporting a prevalence of around 20% of all malaria infections [9], it is extremely rare in west and central Africa. In fact, there are very few cases of *P. vivax* in the indigenous population at all, with the exception of the island of Sao Tome, which is known to harbour all four human malaria parasites [10]. It is possible that the lack of "local" *P. vivax* reported from these areas is due to the fact that its perceived absence precludes its identification. So ingrained may be the notion of the absence of *P. vivax* from west and central Africa that many surveys of parasite species composition from these areas do not include assays for the identification of the parasite [11], and many microscopists automatically designate any parasite associated with Schüffner's dotted erythrocytes as *P. ovale* [12]. There are, however, sporadic reports of its presence. One report from Equatorial Guinea describes the discovery of four cases of *P. vivax* in children of mixed race parentage [13], and another describes a mild infection of *P. vivax* in a Duffy negative woman from the Democratic Republic of Congo [14]. These rare accounts are supported by more extensive reports detailing *P. vivax* infections in non-African travellers returning from these areas. For example, an analysis of 618 imported *P. vivax* cases diagnosed in European clinics between 1999 and 2003, found that 17% of travellers had contracted the parasite in west and central Africa [15]. Furthermore, between 1995 and 1998 there were 73 reports of *P. vivax* imported into France from this region [16]. Imported malaria surveys from the USA report a similar pattern, with data from 2004 revealing that 65% of *P. vivax* imported into the USA from Africa in that year (n = 67) originated in countries in west and central regions [17]. As some of these reports [15,17] rely on microscopy for identification of parasite species, it may be argued that confusion between *P. ovale* and *P. vivax* may lead to a degree of misdiagnosis. However, there are an increasing number of reports that identify parasite species with molecular techniques [16,18], which are much less prone to misidentification of species. Given these data, it seems certain that transmission of *P. vivax* does occur in west and central Africa. However, it remains unclear how transmission is maintained in populations where the Duffy negative phenotype is almost at fixation.

In 1985, Van Ros described the presence of *P. vivax* in a Duffy negative individual [14] from the Democratic Republic of Congo. Recently, two new reports have also described this intriguing situation. One, from western Kenya, describes the presence of *P. vivax* circumsporozoite protein in 0.65% of mosquitoes from an area of high Duffy negativity [19]. More recently, Cavasini *et al* [20] reported clear evidence of *P. vivax* infections in two Duffy negative individuals in Brazil. It has been proposed that the parasite may be in the process of evolving mechanisms which allow the infection of Duffy negative individuals [21]. Such findings highlight the need for a clear investigation of the prevalence and population dynamics of *P. vivax* in west and central Africa, using accurate molecular species typing methods.

In order to assess the current prevalence of *P. vivax* in west and central Africa, PCR species typing of 2,588 samples from nine different countries throughout the continent was carried out.

Methods

Blood sample collection and parasite DNA extraction

A total of 2,588 blood samples collated from various surveys undertaken in nine African countries (Figure 1) between 1998 and 2006 were analysed by PCR for the presence of each of the four human malaria parasites, *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. The original sample collections were predominantly carried out as part of unrelated investigations and the methodology of indi-

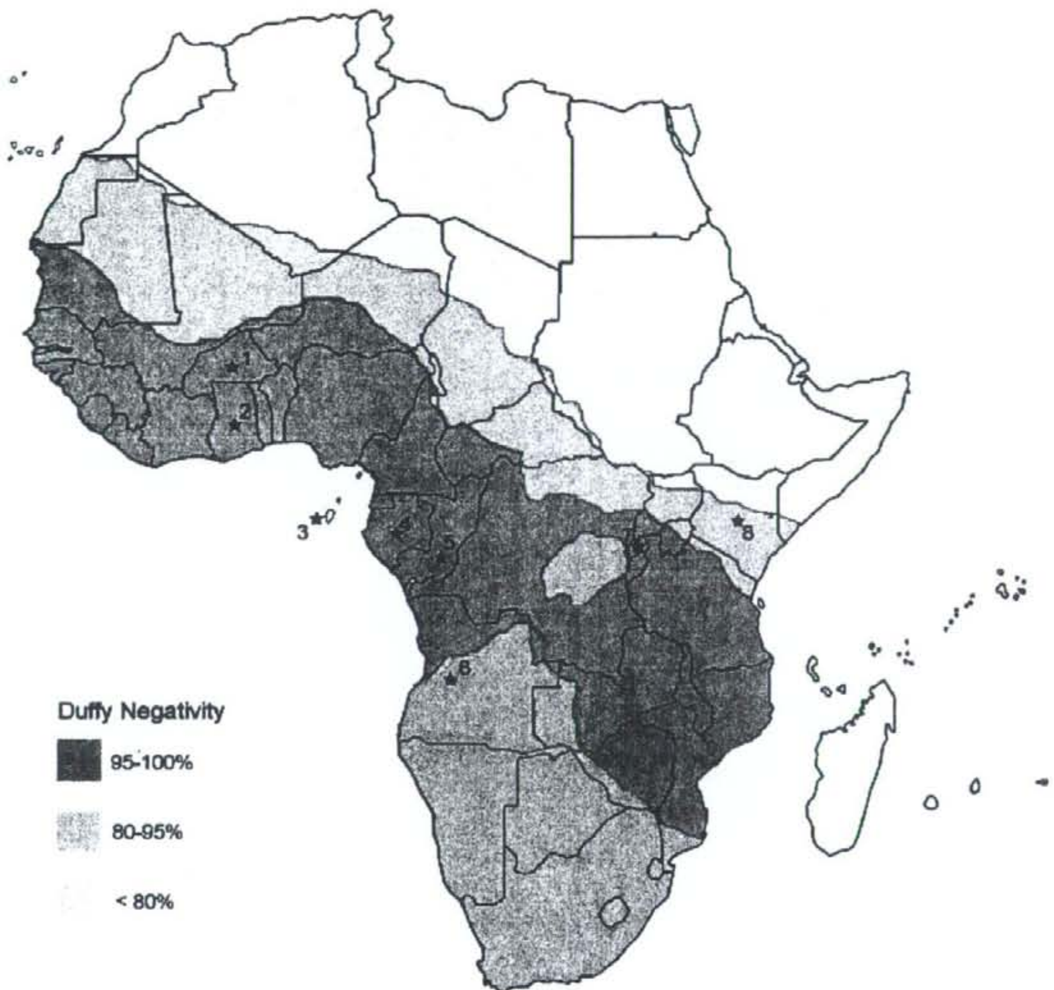

Figure 1

vidual collection varies. Table 1 outlines the details of these collections. In all cases, ethical clearance for sampling was obtained from the relevant ethical committees (for samples collected specifically for this study, details are given in the following paragraphs).

Samples from Burkina Faso

In 2002, samples were collected from asymptomatic children under 10 years old by Active Case Detection (ACD) in two villages (Bassy and Zanga) 60 Km east of Ouagadougou, the capital of Burkina Faso at the end of the transmission season [22]. DNA was extracted by the use of TRIZOL® reagent [22]. 108 *P. falciparum* positive samples (by PCR), were used in this study.

Table 1: Details of sample collections.

Collection area	Number	Sampling year	Sampling method (ACD/PCD)*	Age	Reference
Burkina Faso[1]	108	2002	ACD	< 10	22
Republic of Congo	851	2005–2006	PCD (clinic)	all ages	This study
Gabon	206	2005	PCD (clinic)	all ages	This study
Ghana[1]	352	2004	ACD	< 15	This study
Kenya	722	1998	ACD	All ages	This study
Sao Tome[1,2]	70	2004	PCD (clinic)	All ages	23
Angola[1,2]	90	2003–2004	PCD (clinic)	1–5	24
Mozambique[1,2]	90	2004	PCD (clinic)	1–5	25
Rwanda[1,2]	99	2003	PCD (clinic)	All ages	26
Total	2588				

[1] Only *P. falciparum* positive samples were available for analysis
[2] Only *P. falciparum* single species infections (diagnosed by microscopy) were analysed
*ACD; active case detection, PCD; passive case detection

Samples from Angola, Mozambique, Rwanda, and the Democratic Republic of Sao Tome & Principe (DRSTP)

Blood samples were collected by Passive Case Detection (PCD) at clinics in the following countries: DRSTP, February 2004, at the Centro Policlínico de Saúde de Água Grande, São Tomé (all ages)[23]; Angola, 2003–2004 at the Hospital Pediatrico de Luanda (1–5 year olds)[24]; Mozambique from July to October 2004 at the Hospital Central de Maputo (1–5 year olds)[25]; Rwanda, November to December 2003, at the Rukara Health Centre (1–60 years old)[26]. Only samples identified by thick and thin smear microscopy as *P. falciparum* single species infections were available for analysis. Blood samples were spotted onto Whatman® n°4 (n°3 in the case of Rwanda) filter paper and parasite genomic DNA was obtained by boiling in Chelex-100 [27] and subsequent ethanol precipitation.

Samples from Gabon

206 samples were collected specifically for this study. Samples were collected in and around Lambarene, Departement du Moyen Ogooué at five different locations (Hôpital Albert Schweitzer, Hôpital General de Lambaréné, Dispensaire d'Isaac, Adouma and PK48, a village 48 kilometres from Lambaréné). 100–200 µl of venous blood was applied to Whatman® FTA® Classic Filter paper cards (Whatman®, USA) and left to air dry. Whatman® FTA® filter cards deactivate viral DNA/RNA and preserve human and parasite DNA for downstream analyses. DNA extractions were carried out according to the manufacturer's instructions. Briefly, a disc of 1.2 mm in diameter was punched from the centre of each dried blood spotted card and washed three times with Whatman® FTA® Purification Reagent, and twice with TE buffer. This treated disc was then used directly in subsequent PCR analyses. Ethical clearance for sampling in Gabon was obtained from the ethical committee of the International Foundation of the Albert-Schweitzer Hospital and Edinburgh University. Prior to sampling each patient was informed about the study, consent was obtained (in the case of children parents/guardians gave informed consent) and medical follow-up was provided if needed.

Samples from the Republic of Congo

359 samples were collected by passive case detection from two health centres (Madibou and Tenrikyo) within Brazzaville, the capital of the Republic of Congo, in 2005. No age restrictions were applied to sampled individuals. These samples were collected on Whatman® FTA® filter paper, and processed as previously described. A further 492 samples were collected in 2006 by PCD from three separate locations within the Republic of Congo, 150 from Pointe-Noire, on the west coast of the country, a further 201 from the Tenrikyo health centre in Brazzaville, and 141 from Gamboma, a town in the east. These samples were collected on Whatman® 31ETCHR filter paper, and DNA extraction was performed using the EZ1 BioRobot™ (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Ethical approval for this collection was obtained from the ethical committee at Osaka University, and sampling was authorized by the administrative authority of the Ministry for Research and Ministry for Health in the Republic of Congo. Informed consent was obtained from individual patients, and antimalarial treatment was provided when appropriate.

Samples from Kenya

722 samples were collected in 1998 by active case detection in the Kisii district of Kenya. All age groups were sampled. Blood was collected by finger-prick on Whatman® 31ETCHR filter paper, and DNA extracted by boiling in Chelex-100 [27] and subsequent ethanol precipitation. Ethical clearance for this collection was obtained from the Ministries of Health and Education in Kenya.