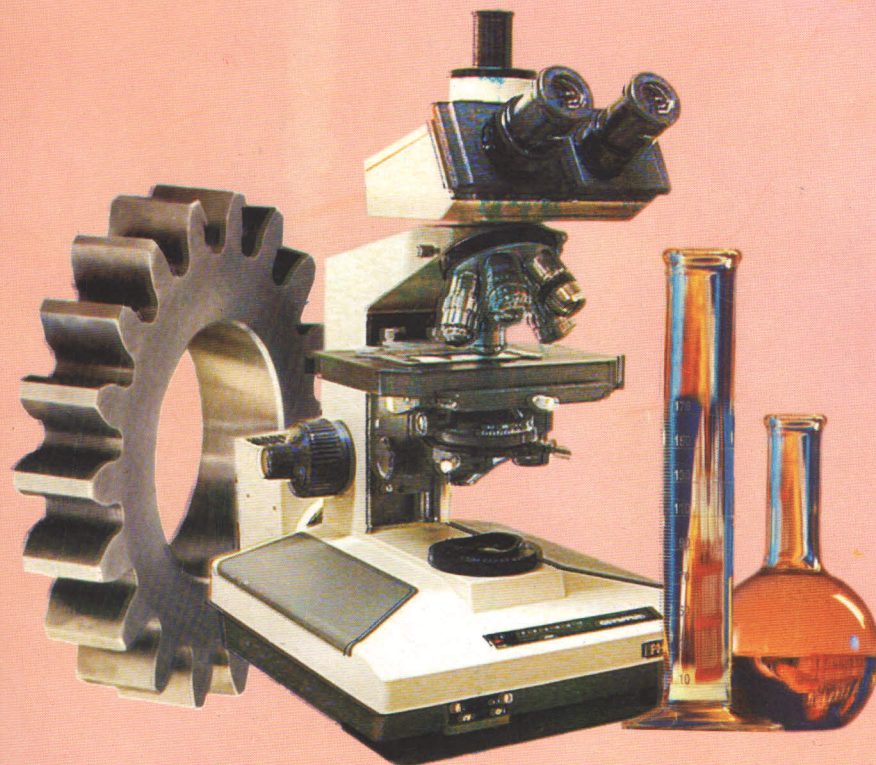


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In silico characterization of some hypothetical proteins in the proteome of *Plasmodium falciparum*

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ABSTRACT

The *Plasmodium falciparum* genome sequencing project was undertaken between 1996 and 2003. This effort produced a reference genome sequence with the *Plasmodium falciparum* clone 3D7 of this human pathogen. This reference genome now provides a basis on which to conduct comparative proteomics studies in order to identify new potential drug and vaccine targets and to better understand the biology of the causative organism of human malaria.

Large amounts of genomic sequence data from other *Plasmodium* species (*Plasmodium vivax*, *Plasmodium yoelii*, *Plasmodium berghei*, *Plasmodium knowlesi* and *Plasmodium gallinaceum*) are also available in various databases, notably the PlasmoDB database (<http://plasmodb.org>). During the initial annotation of these genomes in 2002, several protein sequences (about 2,800) could not be identified by matching them with known proteins in existing databases. These were subsequently classified as “hypothetical proteins”.

We have used available genome tools to study further these hypothetical proteins with the aim of finding out which of them could be useful as drug targets, vaccine candidates or of diagnostic value.

Keywords: Malaria; *Plasmodium falciparum*; Proteomics; Hypothetical proteins; Diagnostic tools.

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Introduction

It is now more than a century since the British physician and entomologist, Sir Ronald Ross, identified mosquitoes as the vectors for the parasites that cause the deadly disease, malaria. The experiments that conclusively proved that malaria is transmitted by mosquitoes were carried out by Sir Ross in 1895 when he worked out the life cycle of the malaria parasite in the *Anopheles* mosquito. For this discovery he was awarded the 1902 Nobel prize in Physiology or Medicine.

In spite of the enormous research and control efforts on malaria, it is still the most prevalent and most devastating parasitic disease in the tropics. It is one of the five leading causes of death in children under five years old. It has been estimated that over 1 million children die every year of malaria (Gething et al., 2011; Hay & Snow, 2006; WHO, 2010).

Various strategies have been adopted to eradicate malaria. In the years following World War II, efforts were made at controlling malaria by targeting the mosquito vectors of the malaria parasites. This involved the use of the residual (long-acting) insecticides such as dichloro-diphenyl-trichloroethane (DDT) and benzene hexachloride (BHC), an action which resulted in a dramatic decrease in malaria infection in many countries. Simultaneously, a series of new drugs were introduced for the prevention and treatment of malaria. It was thought, at that time, that malaria would be completely eradicated by 1960. However, in the late 1960s and early 1970s the malaria control efforts suffered a setback mainly as a result of the transfer of control to the general health services, insufficient funds and/or skilled personnel. Furthermore, mosquitoes became resistant to insecticides and the parasites became resistant to antimalarial drugs, notably chloroquine.

In recent years, there has been a resurgence of malaria (Sleigh et al., 1998; Wellems, 2002). Over 40% of the world's population is now at risk from the disease as the malaria parasite moves back into areas from which it had once been eradicated. As a result, new drugs are being introduced to combat the disease and an alternative approach, through the development of vaccines to stimulate the immune response, has been considered (Carucci, 2002; Chiodini et al., 1998; Gillon, 1998; Hoffman & Carucci, 2000).

Africa has abundant medicinal plants which need to be investigated and developed to acceptable drugs. One antimalarial drug that has gained prominence in recent time is artemisinin (known in China as qinghaosu) (Ittarat et al., 1998; Navaratnam et al., 1998). It has been used in Chinese herbal medicine as a crude extract from the plant *Artemisia annua* for over 2000 years. Its active ingredient, artemisinin, was only isolated and characterized in 1972. It was shown to possess antimalarial activity and a sesquiterpene lactone structure. Africa also has several antimalarial herbal medicines which are classified under the generic name Agbo iba. Many of them could be more potent than artemisinin but sufficient scientific

work has not been done to highlight the active ingredients in these medicinal plants. At best, the work has been left to herbalists whose main job is to dispense crude extracts in the rural villages. Many of them have even moved into the metropolis and upgraded their concoctions into lotions and 'mixtures'.

There is an urgent need to devise means for the rapid diagnosis of malaria (Bruce et al., 200a,b; Coppel et al., 1998). The current practice of relying on microscopic evidence to confirm malaria infection may not be very useful in cases where the patient has high fever and needs urgent treatment. The ultimate goal is to develop biochemical tests that could be used in the laboratory to detect metabolites of the parasite in the blood or urine with ease as is currently done with glucose testing in cases of patients suffering from diabetes mellitus. In this respect, quite a number of malaria rapid diagnostic tests (RDTs) are becoming popular as a result of the recommendation by the WHO that parasitological confirmation of suspected malaria cases should be made prior to treatment (WHO 2010b). One of the most prominent of the RDTs is the water soluble protein produced by *P. falciparum* which has been named histidine-rich protein 2 (PfHRP2) (Desakorn et al., 1997; Biswas et al., 2005). There is need to develop more diagnostic techniques for the rapid detection of malaria parasites, based on proteins that are unique to the parasites. There is also the need to discover drug targets from the vast amount of proteomic information now available from the *Plasmodium* genome sequencing projects.

The first release of genome sequencing data from the *Plasmodium falciparum* clone 3D7 in 2002 contained several proteins which could not be classified because they had no similarities with proteins in existing databases. These were then referred to as “hypothetical proteins”. The latest release of annotated data (PlasmoDB Release 8.2, January 11, 2012) shows that the number of hypothetical proteins has been drastically reduced to less than 50. We report here the *in silico* characterization of some of these hypothetical proteins using genomic tools that are freely available on the internet as well as some that were developed in our laboratory.

Materials and Methods

Hypothetical proteins in *P. falciparum* 3D7

Information on *Plasmodium* genome sequencing project was obtained from the PlasmoDB website (<http://plasmodb.org>) and from GenePlot v3.0 *Plasmodium falciparum* data release 8.19 (Table 1). GenePlot is a scriptable application with built-in functions which include the ability to read and parse EMBL, GenBank and TIGR format XML files. Output formats include linked DHTML database and TIGR format XML. It is a tool which can provide a convenient interface for the user to organize and store information related to any DNA sequence.

GenePlot was designed to provide malaria researchers around the world with access to the genome sequence and annotations for the malaria parasite *P. falciparum*. Access to GenePlot is available online, or through a stand-alone CD-ROM that does not require internet connectivity.

The annotated protein database for *Plasmodium falciparum* 3D7 was obtained from the PlasmoDB Release 8.2 in FASTA format (<http://plasmodb.org>). From this database, thirty-seven hypothetical proteins were identified. These had not been classified or assigned any role since the sequences do not match those of proteins in existing databases. Out of these we have characterized sixteen proteins which could be of value for the rapid diagnosis of malaria. Four other proteins are also possible candidates for vaccine development. Table 2 provides information on the proteins examined and some of their characteristics.

Table 1: Release statistics from the *Plasmodium falciparum* 3D7 genome sequencing project

Chromosome	Sequencing Centre	No. of Nucleotides
1.	Sanger Institute, Cambridge	643,292
2.	TIGR/NMRC	947,102
3.	Sanger Institute, Cambridge	1,060,087
4.	Sanger Institute, Cambridge	1,204,112
5.	Sanger Institute, Cambridge	1,343,552
6.	Sanger Institute, Cambridge	1,377,956
7.	Sanger Institute, Cambridge	1,350,452
8.	Sanger Institute, Cambridge	1,323,195
9.	Sanger Institute, Cambridge	1,541,723
10.	TIGR/NMRC	1,694,445
11.	TIGR/NMRC	2,035,250
12.	Stanford University	2,271,477
13.	Sanger Institute, Cambridge	2,747,327
14.	TIGR/NMRC	3,291,006

Table 2: Annotated proteins currently classified as hypothetical proteins in the proteome of *Plasmodium falciparum* strain 3D7 (PlasmoDB Release 8.2).

S/No	Gene number	Gene Location in PlasmoDB	No. of amino acids in Protein
1.	PFB0056c	Pf3D7_02:60107-60319(-)	70
2.	PF10_0384	Pf3D7_10:1587151-1588617(-)	238
3.	PFA0645c	Pf3D7_01:511710-511955(-)	81
4.	PF10_0382	Pf3D7_10:1575471-1575947(+)	158
5.	MAL7P1.350	Pf3D7_07:1462229-1462441(+)	70
6.	PFF0475w	Pf3D7_06:408037-411998(+)	1257
7.	PF11_0026	Pf3D7_11:87041-87889(-)	282
8.	PF10_0044	Pf3D7_10:182245-187101(+)	1190
9.	MAL8P1.210	Pf3D7_08:1366967-1367167(+)	66
10.	PF14_0755	Pf3D7_14:3220861-3221511(+)	216
11.	PF10_0388	Pf3D7_10:1590145-1590972(+)	260
12.	PFB0973c	Pf3D7_02:873915-874109(-)	64
13.	MAL8P1.320	Pf3D7_08:1369451-1369762(+)	103
14.	PFI0060c	Pf3D7_09:64216-64428(-)	70
15.	PF11_0015	Pf3D7_11:60928-61769(-)	230
16.	PF08_0136	Pf3D7_08:105649-106012(-)	78
17.	PFC1075w	Pf3D7_03:1002622-1003470(+)	282
18.	PFB0951w	Pf3D7_02:851422-851960(+)	152
19.	PFA0035c	Pf3D7_01:56913-57116(-)	67
20.	MAL7P1.230	Pf3D7_07:92135-93136(-)	284
21.	MAL13P1.65	Pf3D7_13:555769-556044(-)	91
22.	PFB0976w	Pf3D7_02:874963-875163(+)	66
23.	MAL8P1.97	Pf3D7_08:637748-642578(-)	1567
24.	PFA0365c	Pf3D7_01:301369-302022(-)	155
25.	PFC0191c	Pf3D7_03:207808-208477(-)	87
26.	MAL8P1.211	Pf3D7_08:1367237-1367455(-)	72
27.	PFC0540w	Pf3D7_03:532720-533951(+)	273
28.	PFB0972w	Pf3D7_02:873129-873317(+)	62
29.	PF14_0019	Pf3D7_14:66269-67081(-)	270
30.	MAL13P1.415	Pf3D7_13:2788492-2789604(-)	371
31.	MAL7P1.59	Pf3D7_07:662489-663346(-)	223
32.	PFA0360c	Pf3D7_01:299776-301082(-)	399
33.	MAL8P1.90	Pf3D7_08:701035-701160(-)	41
34.	PF14_0754	Pf3D7_14:3219854-3220204(-)	116
35.	PF14_0741	Pf3D7_14:3170158-3171627(+)	489
36.	PFL0637c	Pf3D7_12:576390-576641(-)	83
37.	PFA0720w	Pf3D7_01:573708-574404(+)	118

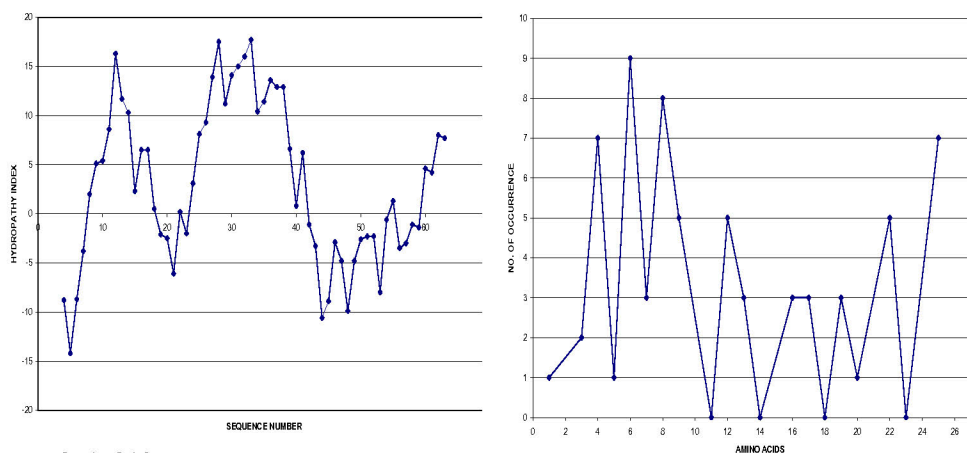
Hydropathy plots and helical structures

The hydropathy plots of selected proteins were obtained using a Microsoft Excel® program developed in our laboratory (Bewaji, 2000). This

program also provides information on the molar mass of each protein and the number of occurrence of each amino acid in the protein. The alpha helical structures in the proteins were identified using the helical wheel tools developed by Grisham (2000). Alpha helices are vital elements of protein structure. An appreciation of the nuances of helix design can yield important insights into the structure and function of proteins. The helical wheel is a useful device for studying alpha helices. The 'wheel' is a view along the long axis of the helix, with numbered positions and residues indicated. A Java applet which accepts user-entered peptide sequences and displays the peptide in a helical wheel presentation was developed by Grisham (2000). It can be used to study the sequence of a peptide or protein of any length up to 256 residues.

Results and Discussion

Proteins with short sequences are very likely to be useful for diagnostic purposes because of the ease of their detection in biological fluids with appropriate biochemical techniques. Some other proteins with peculiar amino acid contents, such as the histidine rich proteins (HRP) have also been used for this purpose (Biswas et al., 2005; Desakorn et al., 1997). In this study, we focused on proteins consisting of less than 100 amino acid residues (Table 3). Proteins with a large number of residues are more suitable as vaccine candidates as a result of their high molar mass and ability to stimulate immune response in animals injected with the proteins.



MAL8P1_210

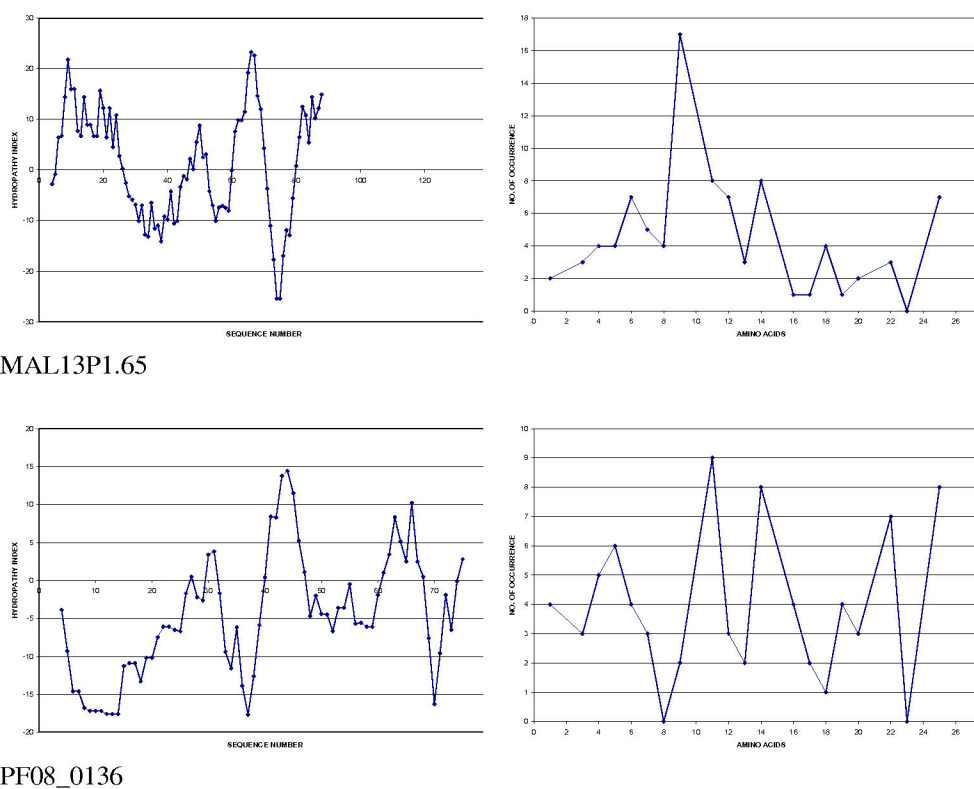
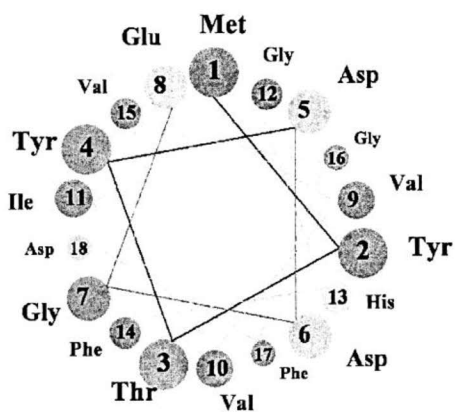
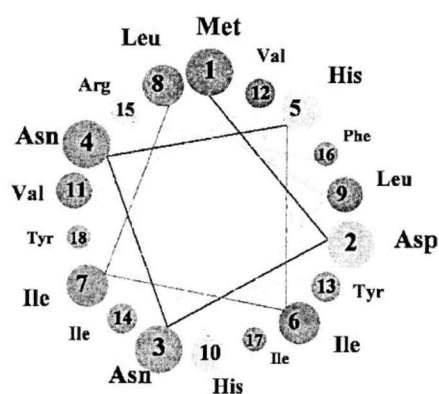


Fig. 1: Hydropathy plots (left panels) and amino acid counts (right panels) in three proteins MAL8P1_210, MAL13P1.65 and PF08_0136 among the sixteen studied in this experiment. These could be used as signatures to study the characteristics of each protein.

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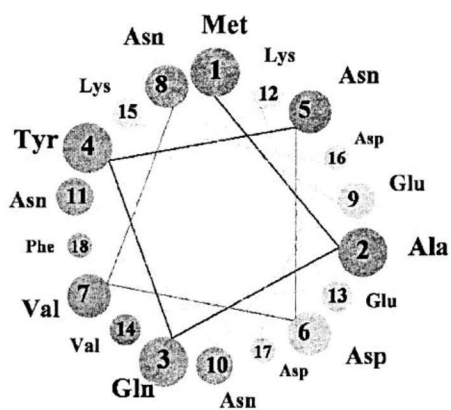


MAL8P1_210



MAL13P1.65

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PF08_0136

Fig. 2: Helical wheel representation of the first eighteen amino acids in each of three representative proteins MAL8P1_210, MAL13P1.65 and PF08_0136.

Table 3: Proteins that could be useful for the rapid diagnosis of malaria infection.

S/No	Gene number	No. of amino acid residues
1.	PFB0056c	70
2.	PFA0645c	81
3.	MAL7P1.350	70
4.	MAL8P1.210	66
5.	PFB0973c	64
6.	PFI0060c	70
7.	PF08_0136	78
8.	PFA0035c	67
9.	MAL13P1.65	91
10.	PFB0976w	66
11.	PFC0191c	87
12.	MAL8P1.211	72
13.	PFB0972w	62
14.	MAL8P1.90	41
15.	PF14_0754	116
16.	PFL0637c	83

The focus of the present paper is on the rapid diagnosis of malaria. The tools used for the characterization of the proteins listed in Table 3, as well as the molecular properties of these proteins consist of a Microsoft Excel® program developed in our laboratory for the quantification of the number of amino acids in a protein sequence and computation of the molar mass of the entire protein. This program is also designed to produce the hydropathy plot of the protein being studied. Fig. 1 shows the computations produced for three representative proteins among the sixteen studied in this experiment. Fig. 2 also shows the helical wheel representation of the first eighteen amino acids in each of these three proteins. These simple tools provide a framework for the design of appropriate biochemical tests for the detection of these proteins in biological fluids such as blood, urine or saliva. Work is in progress in our laboratory on the *in silico* characterization of proteins of high molar mass that could be useful as drug targets or vaccine candidates.

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