สำนักงานกองทุนสนับสนุนการวิจัย

รายงานวิจัยฉบับสมบูรณ์
โครงการ
การศึกษาระดับอณูของการเปลี่ยนแปลงที่เกิดขึ้นของเชื้อพลาสโมเดียม ฟัลซิปารั่มในการพัฒนาเข้าสู่ระยะแกมีโซท์

โดย
ผู้ช่วยศาสตราจารย์ ดร.พิสิฏฐ์ ประพันธ์วัฒนะ

30 พฤศจิกายน พ.ศ.2545

สัญญาเลขที่ RSA/01/2543

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จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

สำนักงานกองทุนสนับสนุนการวิจัย

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รหัสโครงการ RSA43-8-0001

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ชื่อโครงการ การศึกษาระดับอณูของการเปลี่ยนแปลงที่เกิดขึ้นของเชื้อพลาสโมเดียม ฟัลซิปารั่ม ในการพัฒนาเข้าสู่ระยะแกมีโซท์

Project Title Molecular studies on gametocytogenesis of *Plasmodium falciparum*

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คำหลัก (Keywords)

Plasmodium falciparum; malaria parasites; gametocytogenesis; sexual stage-specific gene expression; differential display; Real-time PCR

ABSTRACT

A switch to sexual development (gametocytogenesis) is essential for the malarial parasites to be transmitted to mosquito and therefore the spread of the disease. Eventhough, there are more and more knowledge on the gene expression in this stage coming out, the molecular mechanisms of the gametocyte development are still largely unknown. With the aim to explore the change in gene expression that may govern the gametocytogenesis process, differentially display (DD) technique was used to compare the gene expression in ring stages of gametocyte producing and non-gametocyte producing lines of *Plasmodium falciparum*. Both lines of falciparum parasite, KT-3 isolate, were cultured to obtain synchronized ring stages prior to subject to DD technique. There were about 15-20 bands that appeared only in ring stage of gametocyte producing line and about 80 bands that were more intense in sample from gametocyte producing line compared to the one from non-gametocyte producing line. The selectively intense DD bands in ring stage of gametocyte producing line were cloned and sequenced. The sequences obtained were blasted into the parasite genome data base to search for the possible homoloque sequences. Whilst some clones were identified as genes of known function such as Pfs16, PfEMP1, pfRingA, nucleoside/nucleobase transporter, P-type ATPase III, Ser/Thr protein kinase, MAPK, Rifin, some appeared to be homoloques of sequenced but unidentified genes. Further analysis on selected genes; Pfs16, nucleoside/nucleobase transporter, P0type ATPase III and PfRingA, using real-time PCR, showed that only the expression of Pfs16 gene could be demonstrated in the higher level in ring stage from gametocyte-producing line. The DHFR gene showed the similar level of expression in either parasite line. Other identifed cDNA tags are waiting for further analysis.

Keywords

Plasmodium falciparum; malaria parasites; gametocyte; gametocytogenesis; sexual stage; differential display; Real-time PCR

บทคัดย่อ

การพัฒนาของเชื้อเข้าสู่ระยะวงจรชีวิตแบบใช้เพศของเชื้อมาลาเรีย มีความสำคัญต่อการแพร่กระจายของโรค แต่การวิจัยศึกษาส่วนใหญ่มุ่งเป้าไปที่ระยะวงจรชีวิตแบบไม่ใช้เพศ เพราะเป็นระยะที่สัมพันธ์กับการแสดงอาการ และความรุนแรงของโรค เป็นเป้าหมายและมีผลต่อการรักษาในผู้ป่วย อย่างไรก็ตามหากมีความเข้าใจกระบวนการ หรือกลไกทางอณูชีววิทยาที่เกิดขึ้นในระยะแกมีโทไซท์ หรือการพัฒนาเข้าสู่ระยะแกมีโทไซท์ มากขึ้นเท่าใด ก็น่าจะยิ่งมีประโยชน์ในแง่การรักษาและควบคุมการแพร่กระจายของโรคได้มีประสิทธิภาพดียิ่งขึ้นเท่านั้น ด้วยจุดมุ่งหมายเพื่อศึกษาการแสดงออกของยีนที่จำเพาะ และอาจเป็นตัวกำหนดการเปลี่ยนแปลงเข้าสู่ระยะการใช้เพศ เทคนิคที่เรียกว่า ดิฟเฟอเร็นเชี่ยลดิสเพลย์ (ดีดี) ได้ถูกนำมาใช้เพื่อวิเคราะห์เปรียบเทียบระดับการแสดงออกเป็น เอ็มอาร์เอ็นเอ ของยีนต่าง ๆ ระหว่างเชื้อในระยะวงแหวนของเชื้อมาลาเรียพลาสโมเดี่ยม ฟัลซิปารั่มสายพันธ์ที่สร้างแกมีโทไซท์ได้ และสูญเสียความสามารถนั้นไป จากการทดลองพบว่ามีจำนวนแถบดีดี ที่เข้มขึ้นในระยะวงแหวนของสายพันธุ์สร้างแกมีโทไซท์ได้อยู่ประมาณ 100 แถบ โดยเป็นแถบที่พบเฉพาะระยะวงแหวนของสายพันธุ์สร้างแกมีโทไซท์ได้อยู่ประมาณ 20 แถบ หลังจากการโคลน หาลำดับเบสของแถบที่สนใจ พบว่า โคลนที่ได้จำนวนหนึ่งมีลำดับเบสเหมือนกับยีนที่ได้มีการรายงานไว้แล้ว เช่น PfS16, PfEMP, pfRingA, nucleoside/nucleobase transporter, Ser/Thr protein kinase, mitogen-activated protein kinase, และ P-type ATPase III ในขณะที่บางโคลนยังไม่ทราบว่าเป็นยีนที่เป็นรหัสของโปรตีนใด ยีน 4 ชนิดคือ PfS16, pfRingA, nucleoside/nucleobase transporter, และ P-type ATPase III ได้ถูกนำมาศึกษาต่อเพื่อดูระดับการแสดงออกโดยอาศัยเทคนิคเรียลไทม์พีซีอาร์ พบว่ามีเพียง PfS16 เท่านั้นที่สามารถวิเคราะห์ได้ และพบว่ามีการแสดงออกเฉพาะในเชื้อวงแหวนของสายพันธ์ที่ยังสามารถสร้างแกมีโทไซท์ได้ จากการใช้ยีนของไดไฮโดรโฟเลตรีดักเทสเป็นตัวศึกษาเปรียบเทียบพบว่าการแสดงออกของยีนไม่ต่างกันในทั้งสองสายพันธ์ สำหรับยีนอื่น ๆ ที่ได้วิเคราะห์หาลำดับเบสแล้วจะถูกวิเคราะห์ศึกษาต่อไป

คำหลัก

เชื้อมาลาเรีย; พลาสโมเดียมฟัลซิปารั่ม; ระยะการใช้เพศ; แกมีโทไซท์; แกมีไทไซโทเจเนซิส; เทคนิคดิเฟอเรนเชี่ยลดิสเพลย์; เทคนิคเรียลไทม์พีซีอาร์

Introduction

Malaria, resulting from infection by species of *Plasmodium*, is responsible for the highest mortality of parasitic disease. Currently, malaria causes more than 300 million clinical cases per annum, resulting in more than 1.5 million deaths worldwide in tropical and subtropical area (Trigg and Kondrachine 1998), with the species *Plasmodium falciparum* responsible for the majority of deaths. The emergence of multi-drug resistant strains of P. falciparum and the current lack of a vaccine have stimulated the search for novel control strategies. A better understanding of the various cellular and molecular processes at the different stages of the parasite will help to identify new targets for vaccine and drug development.

Malaria parasite exhibits a complex life cycles of asexual cycle or schizogony in vertebrate host and sexual cycle or sporogony in Anopheline mosquito vector. In host blood circulation, after the merozoite invades a red blood cell, it can either embark on a new cycle of asexual multiplication leading to the formation a schizont ultimately releasing 8-32 new merozoites to restart new asexual cycles or undergo sexual differentiation to produce gametocyte (gametocytogenesis), a process characterized by cell cycle arrest, a shift in the transcriptional repertoire and morphological changes (Reviewed in Alano and Carter 1990; Day *et al.*1996).

The asexual blood stage cycle is correlated to the severity of signs and symptoms of the disease and is the target for effective treatment on the patient, therefore, almost of the researches have been aimed to explore the knowledge on the parasite of this asexual cycle. The study and the understanding about the gametocytic stage and gametocytogenesis is still very little eventhough this stage is the principal stage responsible for the continued transmission of the disease. The more we understand the cellular and molecular processes of the gametocytogenesis, the better chance to achieve the goal in treatment and effective control or blocking of the transmission of the disease we will have.

Nearly all of developmental stages in the complex life cycle of the malarial parasite contains a single set of chromosomes (haploid) counting from the sporozoite in mosquito vector and all of the developmental stages in vertebrate host including gametocytes. Moreover, Van der Ploeg and his colleagues had been reported that using pulse field gel electrophoresis, they found no difference in both size and number of the chromosomes compring between the different stages of the parasite (Van der Ploeg *et al* 1985). Therefore, the haploid genome of the preceding asexual parasite should contain all of the

genetic materials to form either asexual or sexual stages. The switching of the parasite from asexual to sexual development should be the result of the switching of the specific gene and/or specific protein expression that commit the parasite to differentiate into sexual stage. Moreover the switching of the specific set of genes might govern the development into either male or female gametocytes.

Most of the research works in the past (Carter and Miller, 1979; Bruce et al, 1990; Dyer and Day, 2000) were studied about the influence on the sexual differentiation by the environmental factors including host immunity, host hormones and erythrocyte intracellular environment factors. Although there have been many reports of factors that influence the sexual commitment rate, there have been difficulty in reproducing some of these results, either with different lines of parasites, or even in other laboratories.

At present, only small numbers of genes and proteins from the sexual stages have been cloned and characterized. Since, the aim of the majority of the research works is to develop the transmission-blocking vaccines, most of the proteins studied so far are the sexual stage-specific surface antigen. (Sinden, 1983; Vermeulen *et al*, 1986; Carter *et al*, 1989; Alano & Carter, 1990; Alano, 1991; Feng *et al*, 1993; Day *et al*, 1998; Lobo & Kumar, 1998). The functions of these proteins are still poorly understood and not clear whether there are involved in the commitment of the parasite to differentiate into sexual stage. Therefore, the study on innate genetic factors that govern the sexual stage commitment is still a big interesting area waiting to explore.

Objective

To study gene expression that is specific for or predominant in gametocytic stages of *Plasmodiun falciparum* by

1.Identifying the gene(s) that is (are) specifically expressed in sexual blood stage of falciparum malaria parasite and might play roles in sexual differentiation commitment by differential display technique

2. Cloning and expression of the genes of interest

Experimental Methods

Malarial Parasites. *Plasmodium falciparum*, KT-3, which is gametocyte-producing line was isolated from a Thai patient in Kanchanaburi province, Western Thailand, in 1984. The non-gametocyte-producing line of *P. falciparum* was the adaptive culture of the

gametocyte-producing KT-3 line. Both isolates were cultivated in human erythrocytes group O, in RPMI1640 medium supplemented with human serum according to candle jar method of Trager and Jensen (1976). Gametocytogenesis was induced as described by Ifediba and Vanderberg (1981). Different stages of parasites were isolated and purified by the Percoll stepwise gradient centrifugation method of Knight and Sinden (1982). Infected erythrocytes at ring stage were collected from the lowest band of the gradient (bottom of the tube) and the parasites were released from their host erythrocytes by saponin lysis method.

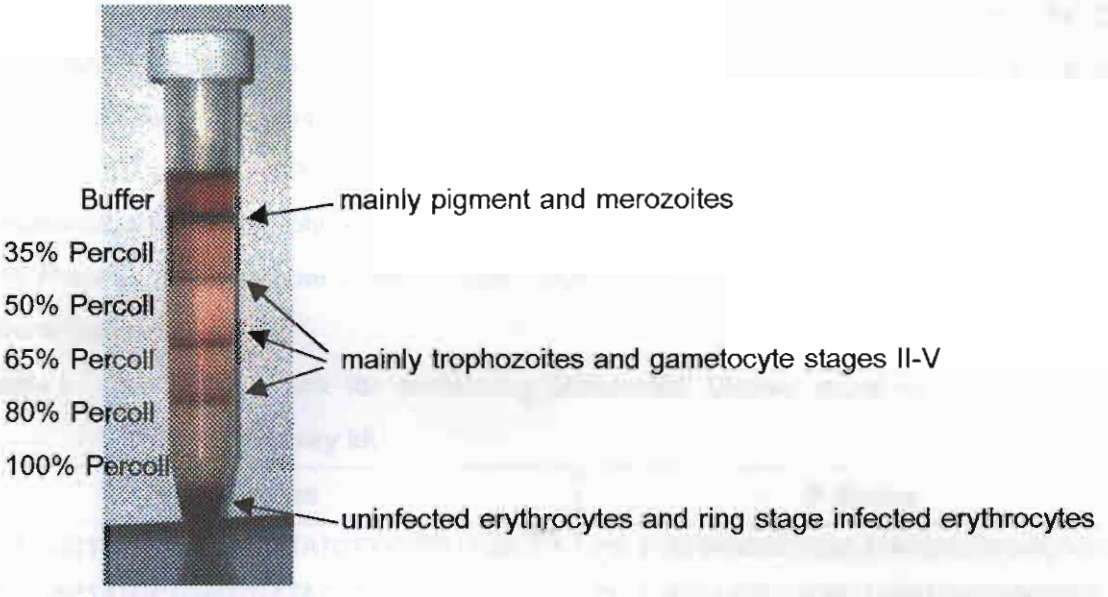

Figure 1: Percoll stepwise gradient centrifugation (method of Knight and Sinden,1982).

DNA and RNA extraction. Total DNA was extracted from the host cell-free parasites as previously described (Petmitr and Krungkrai 1995). Total RNA was extracted by the acid guanidinium isothiocyanate method of Chomezynski and Sacchi using Trizol reagent (Life Technologies). The RNA was precipitated by isopropanol using 5 μg of RNAse-free glycogen (Life Technologies) as precipitating carrier and redissolved in DEPC-treated water. The quality of the total RNA was checked on a 1.2% formaldehyde-agarose gel electrophoresis (Sambrook *et al* 1989). Plasmid DNA was extracted and purified from the interested clones using Wizard Miniprep preparation kit (Promega) and the methods were carried out as recommended by the manufacturer.

Differential Display. DD experiment was performed form total RNA extracted from ring stages of both gametocyte and non-gametocyte producing lines of KT-3 essentially as described by Liang and Pardee (1992) using Delta™ Differential Display kit (Clontech) in combination with Advantage cDNA PCR Kit (Clontech). The experimental steps were mainly as described in the User Manual supplied with the Kits.

Briefly, cDNAs were synthesized form 2 μg of each RNA sample using 1 μM of oligo-dT primer supplied by Advantage cDNA PCR kit. Each cDNA sample was further subdivided in to 2 diluted-portions: 10-fold dilution (named as dilution A) and 40-fold dilution (named as dilution B). All cDNA samples were stored at 60°C until using for DD experiment. To start the DD experiment, each PCR was performed using 1 μl of one cDNA-subpopulation sample and a pair of primers (20 μM each) in the presence 50 μM of dNTP, 50 nM of 1000-3000 Ci/mmol α-P^{33}-ATP (Amersham Pharmacia Biotech) and 0.4 μl of Advantage Klen Taq polymerase mix. Each pair of primer contained one arbitrary primer from P-series and one from T-series (See Table I, total of 18 pairs) supplied with Delta™ Differential Display kit.

Table I. Selected Primers for performing Differential Display supplied in the Delta™ Differential Display kit

T series	P Series
T7: 5'-CATTATGCTGAGTGATATCTTTTTTTTGA-3'	P5: 5'-ATTAACCCTCACTAAAGATCTGACTG-3'
T8: 5'-CATTATGCTGAGTGATATCTTTTTTTTGC-3'	P6: 5'-ATTAACCCTCACTAAATGCTGGGTG-3'
T9: 5'-CATTATGCTGAGTGATATCTTTTTTTTGG-3'	P7: 5'-ATTAACCCTCACTAAATGCTGTATG-3'
	P8: 5'-ATTAACCCTCACTAAATGGAGCTGG-3'
	P9: 5'-ATTAACCCTCACTAAATGTGGCAGG-3'
	P10:5'-ATTAACCCTCACTAAAGCACCGTCC-3'

Along with the experiment, positive and negative control reactions were performed. For a positive control, PCR was performed using positive cDNAs and the P10 and T8 primers supplied by the kit. Two negative controls were set up for each primer pair; one was H_2O control reaction using H_2O instead of cDNA sample and another was total RNA control using 1/100 dilution of the starting total RNA as a sample. The DD-PCR was performed using Hybaid Thermal Cycler and the conditions for the amplification reactions were as follows; 1 cycle of 94°C for 5 min; 40°C for 5 min, 68°C for 5 min; 2 cycles of 94°C for 3 min, 40 °C for 5 min, 68 °C for 5 min; 25 cycles of 94 °C for 1.30 min, 60 °C for 5 min, 68 °C for 5 min and 1 cycle of 68 °C for 7 min. The DD reactions were stored at -20 °C until ready to examine on the gel.

The DD-PCR products of mRNA from ring stages of gametocyte- and non-gametocyte-producing lines were compared site by site on a denaturating 5% polyacrylamide/8 M urea gel. After the finishing the run, the gel was taken out from the apparatus, dried and exposed to Kodak Biomax MR X-ray film (Kodak) at - 70 °C overnight with an intensifying screen. The exposed film was developed using Kodak developing and fixing reagents.

Cloning and Sequencing of DD products. The interesting DD bands that appeared only in the lanes representing the RNA from the gametocyte-producing isolate were excised from the dried gel. DD products were eluted from the gel sliced by heating at 100 °C for 5 min in 40 µl of sterile water. The eluted DD products were reamplified using the same pair of primers using in the original PCR. The reamplification was performed using Hybaid Thermal Cycler and the conditions for the amplification reactions were as follows; 24 cycles of 94 °C for 1.30 min, 60 °C for 1 min, 68 °C for 2 min and 1 cycle of 68 °C for 10 min. The PCR products were cloned into pT-Adv vector using Advantage™ PCR cloning kit (Clontech). Clones with insertion (white colonies) were picked up and plasmids were purified from these clones using Wizard Miniprep Kit (Promega). The obtained plasmids were analysed by restriction analysis with *Eco*R I. The clone with the insert size bigger than 100 bp were sent to Bioservice Unit for nucleic acid sequencing.

Sequenc analysis. cDNA tags identified by DD were compares with the available sequences in the GenBank by using BLASTN and BLASTX. Assignment of the sequences to specific chromosomes was done by customized BLASTN search of malaria sequence databases from Malaria Genome Project at the NCBI website (http://www.ncbi.nlm.nih.gov/Malaria/plasmodiumIcus.htm).

Real-time PCR. To study stage specific expression of identified genes, two-step real time PCR was performed on Light Cycler using LightCycler-Fast Start DNA Master SYBR Green I kit (Roche). Briefly, primers with the melting temperature of 54°C were designed for selective interesting cDNA tags known from the sequence analysis. Each pair of primers was designed to amplify a product of approximately 300 base pairs. Total RNA obtained from ring stages of both gametocyte- and non-gametocyte producing isolates were extracted as describe earlier. cDNA was synthesized from 100 ng of each RNA sample at 37°C in 20 µl of reaction with oligo-dT primer using Improm pt II reverse transcriptase (Promega) using conditions as described by the manufacturer. Real-time PCR was performed using 2-8 µl of cDNA sample in the final volume of 20-µl reaction with two

primers specific for each gene. PCR cycles were performed under the following conditions as suggested by the company with some modification: 1 cycle of 97°C for 5 min for pre-incubation period and followed by amplification for 50 cycles of 97 °C for 20 Sec; 56 °C for 5 Sec; 72 °C for 20 Sec and at 80 °C for 0 sec. The real time amplification was programmed to quantify the SYBR fluorescence at the step of the temperature of 80°C using single acquisition mode.

Results

RNA. All the extracted RNA used in this study gave the bright 28S and 18S ribosomal RNA bands at approximately 4.2 and 1.9 Kb respectively, when checking on a 1.2% formaldehyde-agarose gel electrophoresis as shown in Figure 2.

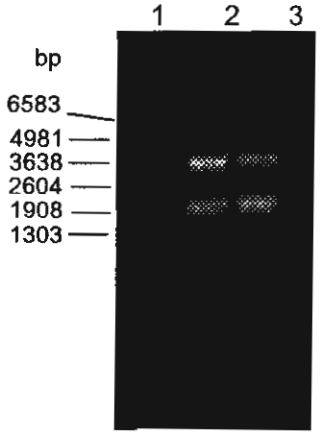

Figure 2. Gel electrophoresis of extracted total RNA. Lane 1: RNA markers; Lane 2: total RNA extracted from ring stage of non-gametocyte-producing line of *P. falciparum*, KT-3; Lane 3: total RNA extracted from ring stage of gametocyte-producing line of *P. falciparum*, KT-3.

Differential Display. To identify genes differentially expressed in early stage of gametocyte, different gene expression in ring stage parasite of gametocyte-producing line of was compared to the expression in ring stage of non-gametocyte producing line with a modified DD method. Using 6 arbitrary primers (P5-P10) in combination with 3 30-base-anchored oligo-dT primers, approximately 100 DD-bands were identified as differential expressed cDNA tags in ring stage of gametocyte producing line, of which about 15-20 bands were found to be expressed only in this line. There were also about 15 bands that appeared only in ring stage of non-gametocyte producing line and about 70 bands that

were more intense in sample from non-gametocyte-producing line compared to the one from gametocyte-producing line. For a positive control using primers T8 and P10 to amplify adult and fetal liver RNA, results showed the expected pattern as described in the kit manual (data not shown). The gel from the two controls gave two very similar displays, except that a single, strong band of ~380 base pairs was present only in the fetal liver display. For negative control that contained no template in the reaction, it showed few fainted bands in some areas that are acceptable as described in the kit manual.

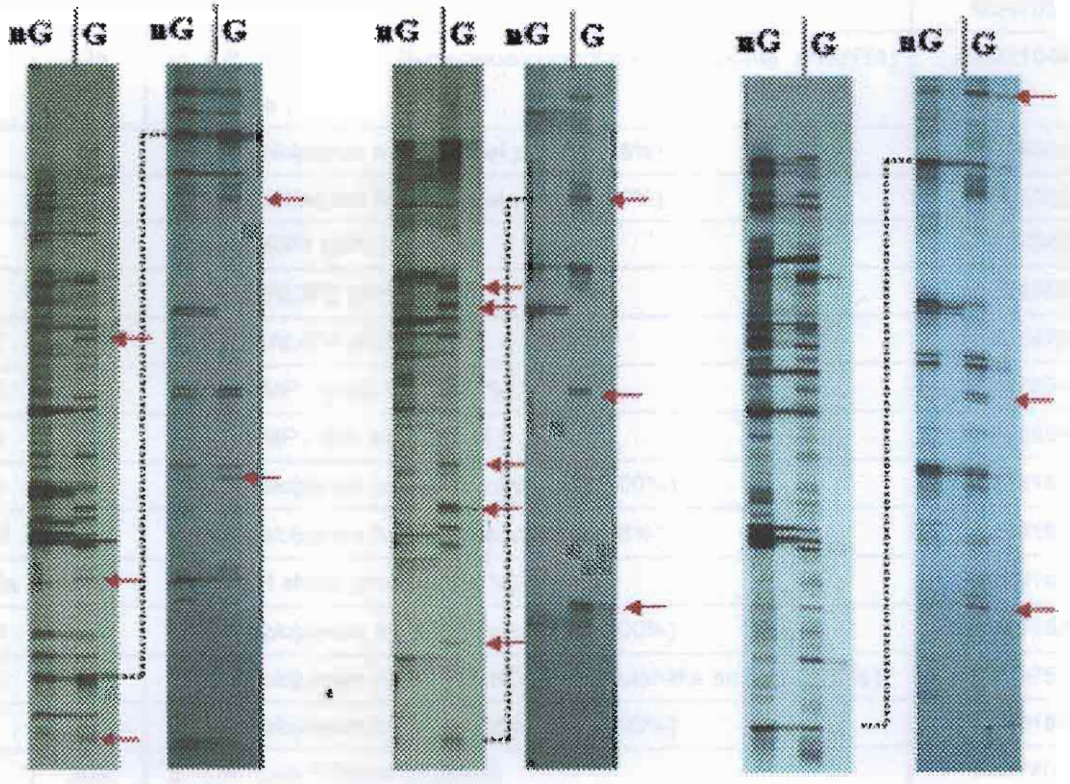

Figure 3. Examples of electrophoretic pattern of differential display. NG, DD reaction of RNA from ring stage of non-gametocyte-producing line; G, DD reaction of RNA from ring stage of gametocyte-producing line. Red arrows point to the bands that are differentially expressed in ring stage of gametocyte-producing line *P. falciparum*, KT-3.

Sequence analysis of the DD clones. Since the focus was on gametocyte-specific genes, twelve of the bands that appeared only in the lanes from gametocyte-producing line were excised from the gel and reamplified with the original primer pairs (Figure 4). After the PCR products were cloned and sequenced, the DD tags were compared to sequences from GenBank by BLASTN and BLASTX, and the results are presented in Table II.

Table II. Sequence Analysis of Differential Display Products with Significant Homology to Genbank Sequences

Sequence name	Length (bp)	CL	Homology to GenBank sequences	
			GenBank entries (% identity)	Accession No.
1.3A4	60	M	*P. falciparum* Cyt B, Cox I and Cox III (100%)	NC002375
1.3A5	68	2	*P. falciparum* merozoite surface protein-4 (PfMSP4) (94%)	U85260
1.3A12	50	11	*P. falciparum* Ag15 : 15 KDa vesicular-like antigen (100%)	Z28975
1.3A18	120	13	*P. falciparum* Nucleoside/nucleobase transporter (PfENT1) gene (100%)	AC221844
1.7A1	115	4	*P. falciparum* Pfs16 (96%)	M64705
1.7A2	425	13	*P. falciparum* Nucleoside/nucleobase transporter (PfENT1) gene (100%)	AC221844
1.7A4	256	12	*P. falciparum* hypothetical protein (96%)	AC005505
1.7A5	120	12	*P. falciparum* hypothetical protein (96%)	AC005505
1.7B7	125	9	PfEMP1 (90%)	AAC37240
1.7B8	32	3	PfMAL3P2 (90%)	AL034558
1.11AB2	292	4	PfMAL4P4 (86%)	AL035477
1.11AB3	53	*	PfEMP1 (HB3 Var I)(94%)	AF008994
1.11AB4	49	*	PfEMP1 (PA Var 8)(95%)	AF008989
1.11AB9	59	13	*P. falciparum* hypothetical protein (100%)	AL049180
1.11AB10	301	13	*P. falciparum* hypothetical protein (98%)	AL049180
1.11AB10a	94	13	Heat shock protein (100%)	AL049180
1.11AB11	34	13	*P. falciparum* hypothetical protein (100%)	AL049180
1.11AB12	43	11	*P. falciparum* Ag15 : 15 KDa vesicular-like antigen (100%)	Z28975
1.19A5	50	13	*P. falciparum* hypothetical protein (100%)	AL049180
1.19A9	303	5	P-Type ATPase III (94%)	X65740
1.19A10	50	11	*P. falciparum* hypothetical protein (96%)	AE014843
1.19A12	65	M	*P. falciparum* Cyt B, Cox I and Cox III (100%)	NC002375
2.7A1	50	M	*P. falciparum* Cyt B, Cox I and Cox III (100%)	NC002375
2.7A2	193	1	PfEMP1 (90%)	AF221828
2.7A4	30	14	Plasmodium falciparum protein Ser/Thr kinase-1 (psk1)	AF104915
2.7A6	162	14	Rifin	Af221828
2.7A7	110	13	PfRingA	AJ290924
2.7A9	60	14	Mitogen activated protein kinase	U36377

Notes. Bands identified by DD were excised from the gel, reamplified, cloned, sequenced, and compared to GenBank by BLASTN and BLASTX. Shown are the arbitrary names of the DD sequences (Sequence Names), their length in base pairs, and their chromosomal locations (CL). M is stand for mitochondrial location. Sequence with significant matches to P. falciparumsequences present on many chromosomal contigs is marked with an asterisk (*).

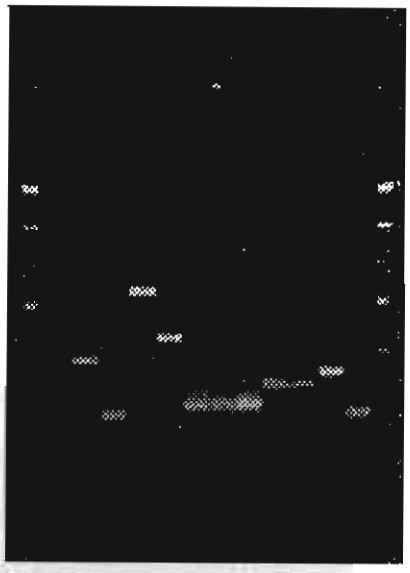

Figure 4. Reamplification of DD bands

All cDNA tags identified from DD analysis were perfect or near perfect matches (more than 85% homology) to *P. falciparum* sequences from GenBank and the Malarial Genome Project. These genes included putative structural or membrane proteins, enzymes, mitochondrial genes, and hypothetical proteins with unknown functions. Only one gene, Pfs16 was previously demonstrated as gametocyte specific (Baker *et al*, 1994; Bruce *et al*, 1990b; Bruce *et al*, 1994; Dechering *et al*, 1997; Moelans *et al*, 1991).

Confirmation of the Differential Display Results. Though DD technique is one of the commonly used methods for the identification of differentially expressed genes (Spielmann and Beck, 2000), one limitation of this technique is the tendency of generating false positives that need further verification. To verify DD results together with to study the expression of the DD products, 4 of the identified sequences (Pfs16, PfENT, P-type ATPase III, and PfRingA genes) has been selected for further quantitative RT-PCR analysis. cDNAs were synthesized from a new lot of total RNA samples extracted from ring stages of both non-gametocyte- and gametocyte-producing line of parasites. Since dihydrofolate reductase (DHFR) gene has been reported to be one of the malaria house-keeping gene and its expression level has been shown to be the same in all stages of development (Nirmalan *et al.*, 2002), it was used as control for the gene expression in this experiment. All of the primers were designed to have the same melting temperature of 54°C and to amplify the gene size of approximately 300 bp (Table IV). The condition of PCR to amplify all selcted genes such as $MgCl_2$ concentration, temperature for

denaturating, annealing, and polymerization steps, were optimized using DNA as the tested starting material (data not shown).

Table IV. Primer sequences for verification of the level of expression of the interest genes

Genes	Primer Sequences
DHFR	5' ATGGAACAAGTTCTGCGAC 3' 5' TCATTTACATTATCCACAGTT 3'
Pfs16	5' TGAATATTCGAAAGTTCATAC 3' 5' CTATAGCTAGCTGAGTTTC 3'
PfENT1	5' GTGTAGTGGCAGGATTAG 3' 5' ATATAAGATAATGTGGCATTG 3'
P-type ATPase III	5' ATGTGGAAAAATCCGTGTC 3' 5' TTTCATATCTTTAGATAGTTGT 3'
PfRingA	5' TTCTTTGCTATCATAGCAGT 3' 5' AGTAAGGCTAAAGCAG 3'

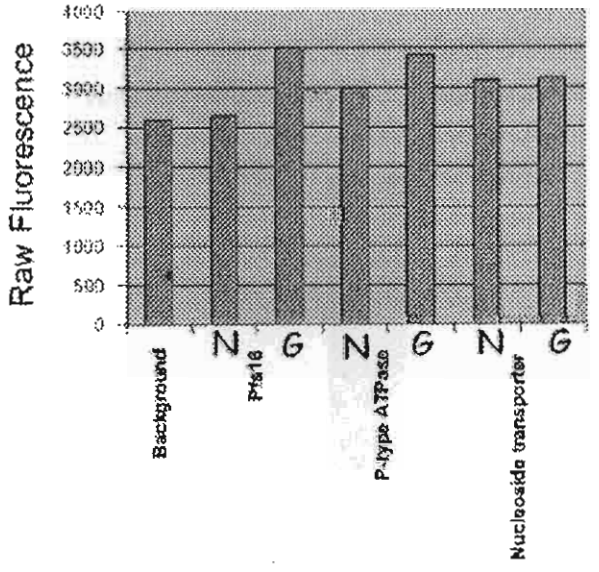

Figure 5. Fluorescence measurement of end-point PCR of selected genes comparing between ring stages of non-gametocyte- and gametocyte-producing lines of *P. falciparum* KT-3. Background: H_2O was added instead of cDNA sample; N, cDNA synthesized from total RNA extracted from ring stages of non-gametocyte-producing line; G, cDNA synthesized from total RNA extracted from ring stages of gametocyte-producing line.

At the beginning, the gene expression level was verified by end-point PCR measurement using Amplifluor™ Universal Amplification and Detection System (Intergen Co.) which is depending on the incorporation of fluorescein-labeled primer during amplification. The results obtained could not demonstrate the level of expression clearly because of the high fluorescence background (Figure 5). However, it showed the tendency that only Pfs16 gene was the only gene that showed the higher level of expression in the ring stages of gametocyte-producing line comparing to the non-gametocyte-producing one.

The next verification strategy has been changed to the real time quantitative PCR method. The first trial was based on reverse transcriptase-real time PCR using LightCycler-RNA Master SYBR Green I Kit (Roche) and performed on LightCycler Instrument. Total RNA samples were directly used as the starting material for the real-time reaction. Unfortunately, the reactions gave no amplification signal on any gene tested. This might be the result from the unsuccessful reaction either at the step of amplification or at the step of reverse transcription. To ovoid the possible effect from the failure of reverse transcription reaction, two-step quantitative real-time PCR strategy has been used. The cDNAs have been synthesized from the total RNA sample prior to commence the real-time PCR quantification. The integrity of cDNAs obtained were checked by performing normal PCR with DHFR primers. The cDNAs from both lines of *falciparum* malarial parasites gave the bands at expected size with the similar intensity (Figure V).

Figure V. Gel pattern of DHFR products from the amplification using cDNAs prepared from total RNA of ring stages of gametocyte- and non-gametocyte-producing lines of *P. falciparum* KT-3. Lane 1, 100-kb ladder marker; Lane 2, sample from non-gametocyte-producing line; Lane 3, sample from gametocyte-producing line.

The two-step real time PCR were performed using LightCycler-FastStart DNA Master STBR Green I kit (Roche) and the conditions were as suggested by the supplier with some modification. Normally, the company suggests to quantify the amount of amplification products by fluorescence detection at the end of amplification step of each cycle which is at the temperature of 72°C. It was found that at this temperature setting the true fluorescence signal of the products was interferred by the signal from the accumulated primer-dimers (PD). To ensure of the accurate quantification of the desired products, the problem was solved by setting the aquisition mode for detecting the signal at the temperature of 80°C. At this temperature, the primer-dimers will melt and is no further bound to SYBR Green I (http://www.wzw.tum.de/gene-quantification/optimization.html).

Figure VI showed the results of two-step real time PCR using 2 µl of cDNA samples (synthesized from 100 ng totalRNA in 20 µl reaction) as starting material for the analysis. The graph revealed that the fluorescence signal could be demonstrated only for the Pfs16 gene in the sample from ring stage of gametocyte producing line. The amount of the cDNA in the reaction was estimated to be 1.37 x 10^2 molecules.

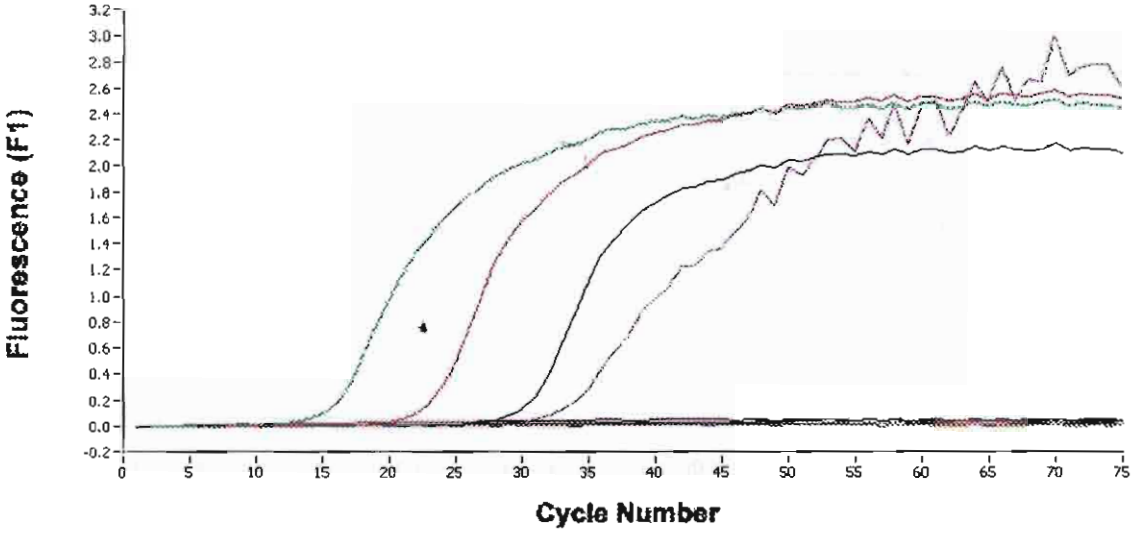

Figure VI. Fluorescence signal of amplified products during the real time PCR. Standard curve: green, 1 x 10^7 molecules/reaction; red, 1 x 10^5 molecules/reaction; black, 1 x 10^3 molecules/reaction. Margenta curve, sample from ring stage of gametocyte-producing line amplified with primers specific for Pfs16 gene. Other curves: negative control using H_2O to replace cDNA samples for each primer pair; sample from ring stage of gametocyte-producing line amplified with primers specific for DHFR gene, PfENT1 gene, P-type ATPase III gene and PfRingA gene; sample from ring stage of non-gametocyte-producing line amplified with primers specific for DHFR gene, Pfs16 gene, PfENT1 gene, P-type ATPase III gene and PfRingA gene.

In order to increase the amplification signal, the volume of cDNA samples as starting material for the analysis were increased to 8 µl/reaction. The graph (Figure VII) revealed that the fluorescence signal could be demonstrated in the amplification reactions for the Pfs16 gene only in the sample from ring stage of gametocyte producing line and DHFR gene in ring stage samples form both gametocyte- and non-gametocyte-producing lines. cDNA of the Pfs16 gene from ring stage of gametocyte producing line per reaction was estimated to be 9.40 x 10^2 molecules where as DHFR-cDNAs were estimated to be at the same level of expression which were 8 and 10 molecules per reaction for gametocyte- and non-gametocyte-producing lines, respectively.

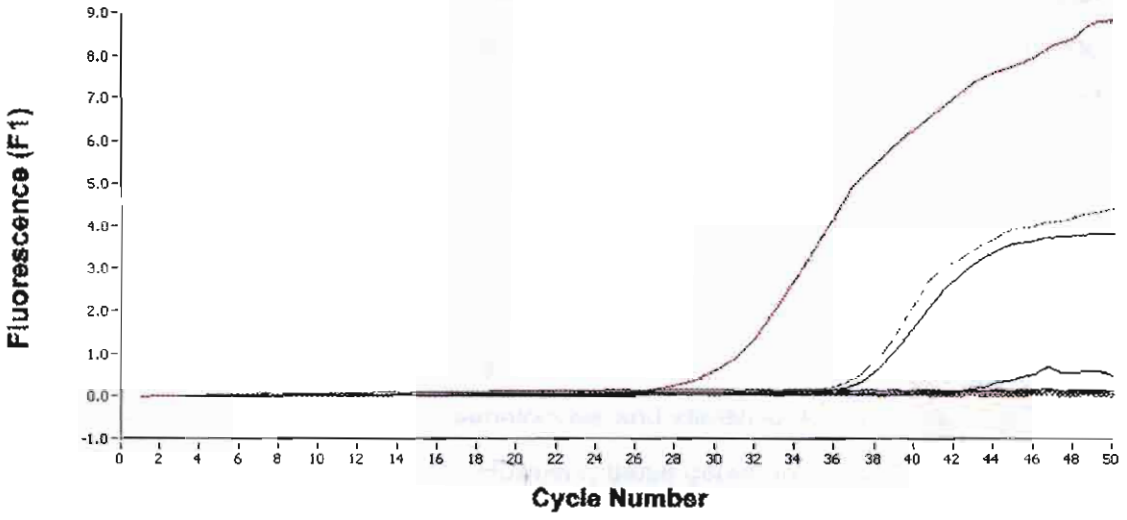

Figure VII. Fluorescence signal of amplified products during the real time PCR. Red curve, sample from ring stage of gametocyte-producing line amplified with primers specific for Pfs16 gene; Grey curve, sample from ring stage of non-gametocyte-producing line amplified with primers specific for DHFR gene; Margenta curve, sample from ring stage of gametocyte-producing line amplified with primers specific for DHFR gene; Other curves: negative control using H_2O to replace cDNA samples for each primer pair; sample from ring stage of gametocyte-producing line amplified with primers specific for PfENT1 gene, P-type ATPase III gene and PfRingA gene; sample from ring stage of non-gametocyte-producing line amplified with primers specific for Pfs16 gene, PfENT1 gene, P-type ATPase III gene and PfRingA gene.

Discussion

Based on the estimated density of one gene per 4.5-4.8 kb from sequence information of chromosomes 2 and 3 and a genome size of 25 mb, *P. falciparum* has approximately 600 genes (Gardner et al. 1998; Bowman et al. 1999). Although computer programmes have been developed to annotate the *P. falciparum* genome and putative gene fuunctions can sometimes be deduced by searching sequence databases for similarities to

known genes from other organisms, >60% of open rreading frames (ORFs) are without identifiable homologues in the GenBank. In other words, transcripts and protein products of most ORFs and their expression patterns, developmental regulatoin, and biological functions remain to be determined.

DD is a powerful tool for identifying and cloning differentially expressed genes and has been used successfully in many biological systems (Liang and Pardee 1992). This technique allows one to isolate genes based solely on differences in mRNA levels without prior knowledge of DNA and proteins sequences and an exact time of any gene expresion. It has been widely used in studying developmentally regulated gene expression in several parasites of human diseases. However, there have been only three articles so far that reported on the successful apllication of this technique in malaria research. One of the reason might be due to the high AT-rich in the genome. Two earlier attemps have a small success in identifying few genes that were differentially expressed in *Plasmodium* under different situation. In one study, Thelu et al. (1994) identified two genes with up-regulated expression in *P. falciparum* culture in response to chloroquine treatment. Another study by Lau *et al.* (2000) they adopted this technique to isolate liver-stage genes from *P. yoelii*-infected mouse erythrocyte. More recently, Cui *et al.* (2001) had used this technique to examine gene expression in stage IV-V gametocytes and identified 49 cDNA tags that were shown to be upregulated in these stages. However, these genes were not genes that might commit the sexual stage differentiation and there has been still no such report. Up to now, the gene demonstrated to be the earliest expressing gene in gametocytic stage was Pfs16 which is surface antigen of unknown function.

There are two models postulated by Cater and Miller (1979) regarding the stage at which sexual commitment occurs in the parasite. According to one model, the merozoite is uncommitted and early asexual blood-stage parasite after invasion has the full potential either to differentiate into a gametocyte or remain as an asexual parasite. The alternative model proposes that the merozoites of an individual schizoint might already be committed either to become asexual parasites or to develop into gametocyte after invasion. However ring form is the common stage shared by both models, thus, there is very high possibility that gene expression(s) that might govern the sexual commitment occur(s) at this stage.

In this project, in order to search for the gene that might play role in the sexual stage commitment, ring stages from both gametocyte- and non-gametocyte-producing line of *P. falciparum*, KT-3, were chosen as the materials for gene expression comparison. Using 6 upstream arbitrary primers combining with 3 oligo-dT primers, approximately 100

DD bands were found to be more intense in gametocyte-producing line when compared to non-gametocyte-producing line. Of these elevated bands, 20 bands were demonstrated only in gametocyte-producing line and twelve most intense bands were chosen to analyse further. With most eukaryotic systems, the majority of DD products represent the 3'regions of mRNA because of the annealing of oligo-dT primers to the poly A-tails. In contrast, all cDNA bands from DD analysis in *Plasmodium falciparum* in this work were internal regions of the gene. Apparently resulted from the annealing of oligo-dT primers to internal A-rich regions of the individual mRNA. The same result was also found by other groups as they applied DD technique with *Plasmodium* parasite (Thelu *et al.* 1994;Cui *et al.* 2001)

The sequences obtained were blasted into the parasite genome data base to search for the possible homoloque sequences. Whilst some clones were identified as genes of known function such as pfs16, pfMSP4, pfRingA, nucleoside/nucleobase transporter, and P-type ATPase III, some appeared to be homoloques of sequenced but unidentified hypothetical protein encoding genes (Table II). Among the gene identified, only Pfs16 was previously characterized as sexual stage specific (Lobo *et al.* 1994; Dechering *et al.* 1997). The failure to demonstrate other known gametocyte-specific genes such as Pfg27, Pfs230 and Pfs48/45 is probably due to two main reasons. First, the primers are not specifically optimized or designed for malarial parasite genes. The malarial genome contain very high AT content of ~70-75% whereas the arbitrary primers used in this study contain ~50% AT content. Second, in this study, only 6 arbitrary primers from the entire set of 10 were used in combination with 3 oligo-dT primers from the entire set of 9. A complete set would likely provide a better representation of the whole mRNA repertoire (~99%). Of other sequences that matched the previously known *P. falciparum* genes, some have been reported to be up-regulated in gametocyte (stages III-V) including Ser/Thr kinase, cytochrome b, Mitogen-activated protein kinase, P-Type ATPase III, products of var gene such as PfEMP and rifin, (Bracchi *et al.*, 1996; Dorin *et al.*, 1999; Learngaramkul *et al.*, 1999; Cui *et al.*, 2001;Rozmajzl *et al.* 2001; Supplement table 2 of Florens *et al*, 2002)

Among the sequences identified, four of the first lot; Pfs16, PfENT1, P-Type ATPase III, and PfRingA, Consistent with the previous results (Lobo *et al.* 1994; Dechering *et al.* 1997), Pfs16 showed gametocyte-stage-specific expression using quantitative real time PCR (Figure VI, VII). For DHFR which used for reaction control, it showed the same level of expresion comparing between ring stage from gamtocyte- and non-gametocyte-producing line. This results was supported by the previous results that the amount of this gene was

similar in either stages (Learngaramkul *et al.*, 1999). Moreover, the transcription of DHFR gene was recently reported to be the same across the cell cycle (Nirmalan et al., 2002).

For other genes tested, their expression could not be demonstrated in neither stages. The failure may pobably due to they presented in the sample in a small amount that could not be detected by the RT-PCR programme set in this project. Another possible reason may be because the clones picked up for analysis did not represent the real upregulated band. An individual DD band did not represent only one cDNA tags since the results of sequence analysis of the clones obtained from any single band yielded more than one nucleotide sequences. This is one of the limitation of DD technique. This phenomenon might obscure the real differential expressed products and led to the need of more clones to be analysed per one single DD band.

In conclusions, this project has used differential display technique to explore the genes that up-regulated in the ring stage of gametocyte-producing line compared to the same stage of non-gametocyte-producing line. Though the results obtained from this project sofar still can not identify any new gene that might govern the sexual stage commitment, there are still some gene left for further characterized.

Future perspectives

Our knowledge of the biology of the gametocyte is not as advanced as that of asexual erythrocytic stages. However, concentration of effort is still required to overcome obstacles to the study of some important questions concerning the basic biology of the gametocyte. The search for other genes encoding products that have specific roles to play in the sexual differentiation of *P. falciparum* must carry on if we are to unravel the molecular mechanisms of sexual commitment and development in *Plasmodium*. The deeper our understanding of the gametocyte biology, the better equiped we are to develop rationally designed drugs, assist our immune response and design effective intervention programmes.

The future works may be included:

1. Further chcracterized the level of the residual identified sequences whether they are really up-regulated in sexual stage.
2. Explore the proteomic pattern of rings stage from both lines.

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Outout

1. Poster Presentation on "Identification of genes differentially expressed in ring stages from gametocyte-producing line of *Plasmodium falciparum*." at Gordon Research Conference on Malaria, Queen's College, Oxford University, England. August 5th-10th, 2001.
2. Poster Presentation on "Identification of genes differentially expressed in ring stages from gametocyte-producing line of *Plasmodium falciparum*." at The International Conference on Bioinformatic 2002: North-South Networking. LeRoyal Meridien, Bangkok, Thailand. February 6th-8th, 2002.