ความแตกต่างของบริเวณจดจำของเอนไซม์ตัดจำเพาะ *Eco* **RI** ในจีโนมของเชื้อมาลาเรียชนิดฟัลซิปารัม สายพันธุ์บริสุทธิ์ที่เลี้ยงในอาหารเลี้ยงเชื้อที่มียาไพริเมทามีน

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บทคัดย่อ

การศึกษารูปแบบการเพิ่มจำนวนชุดของยีนไดไฮโดรโฟเลท รีดักเทส-ไทมิดิเลท ซินเทส ด้วยเทคนิค restriction fragment length polymorphisms (RFLPs) ของเชื้อ *Plasmodium falciparum* สายพันธุ์บริสุทธิ์ T9/94RC17 ที่ชักนำให้เกิดการดื้อต่อยาไพริเมทามีนในห้องปฏิบัติการ พบว่าในการเพิ่มจำนวนชุดของยีนดังกล่าวนั้นไม่ได้เพิ่มเฉพาะในส่วนที่เป็นบริเวณของยีนเท่านั้นยังรวมถึงบริเวณปลาย 5′ และปลาย 3′ ซึ่งอยู่นอกยีนด้วย นอกจากนี้ยังพบแถบดีเอ็นเอซึ่งเป็นส่วนของยีนที่มีขนาดเล็กลงเพิ่มขึ้นถึงสองแถบในรูปแบบของดีเอ็นเอที่ได้จากเชื้อดื้อต่อยาไพริเมทามีนสายพันธุ์บริสุทธิ์ T9/94RC17 Py 10^{-7} ซึ่งแตกต่างจากเชื้อดื้อต่อยาไพริเมทามีนสายพันธุ์บริสุทธิ์อื่นๆ อาจเกิดเนื่องจากความผิดพลาดในการจำลองสารพันธุกรรมของเชื้อเมื่ออยู่ในภาวะที่ต้องสัมผัสกับยา หรือเกิดการกลายพันธุ์เฉพาะจุด (point mutation) ซึ่งทำให้เกิดบริเวณจดจำของเอนไซม์ตัดจำเพาะแห่งใหม่ขึ้นและใกล้กับยีนดังกล่าวมากขึ้น หรือเกิดการตัดบางส่วนของยีนออก (deletion) จากผลการทดลองนี้ชี้ให้เห็นว่าอาหารเลี้ยงเชื้อที่มียาไพริเมทามีนเข้มข้น 10^{-7} โมลาร์เป็นภาวะที่เชื้อมีการปรับตัวสูงสุดเพื่อสามารถอยู่รอดและมีการดื้อต่อยามากขึ้น

คำสำคัญ: ความแตกต่างทางพันธุกรรม, การเพิ่มขยายยีน, ยีนไดไฮโดรโฟเลท รีดักเทส-ไทมิดิเลท ซินเทส, การชักนำให้ดื้อต่อยาไพริเมทามีน, เชื้อมาลาเรียชนิดฟัลซิปารัม

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Plasmodium falciparum: *Eco* RI site Polymorphism in the Genome of a Parasite Clone Grown *In Vitro* in Presence of Pyrimethamine

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Abstract

The restriction fragment length polymorphisms (RFLPs) patterns of the increasing number of dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) gene copy in the parasite genome were analyzed. The RFLPs profile of pyrimethamine resistant clone line of *Plasmodium falciparum*, T9/94RC17, grown *in vitro* in the presence of pyrimethamine showed the amplification of this gene including the 5′ and 3′ flanking region outside the gene. Moreover, one of the resistant progenies, T9/94RC17 Py 10^{-7}, had two extra small gene fragments in the DNA profile. An error during DNA replication under drug pressure condition or point mutation that caused new restriction site nearer to the gene or DNA deletion may be a cause of this finding. At 10^{-7}M of drug may be a crucial condition for parasite adaptation to survive and has higher resistant to this drug.

Key words: genetic polymorphism, gene amplification, dihydrofolate reductase-thymidylate synthase, pyrimethamine induction, *Plasmodium falciparum*

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Original Article

Introduction

Around 40 percent of the world's population lives in malaria-transmitted areas and it is estimated that up to 2.7 million deaths from malaria per year, especially children under 5 years, live in sub-Saharan Africa, account for 90 percent of all deaths due to malaria[1]. Although the total number of malaria cases has decreased in some endemic areas such as Thailand[2], resistance of falciparum malaria against many antimalarial drugs has become serious and growing problem in most malaria endemic areas. It is a major obstacle to provision of the effective treatment and to control the disease. The mechanisms for the mode of action and resistance development of most antimalarial drugs are unknown.

Although, pyrimethamine is little or no value for the malarial treatment in many endemic areas, its biochemical action is best known. This drug acts as a dihydrofolate reductase (DHFR) inhibitor whose affinity is greater to the parasite enzyme than the host enzyme. Inhibition of DHFR leads to the reduction of tetrahydrofolate co-factors level and, finally, inhibits the DNA synthesis[3,4]. Two genetic changes had been proposed as drug resistance mechanisms. These mechanisms are (i) the decreased affinity to the drug target as a result of structurally altered enzyme by point mutations in the sequence of *dhfr* gene[5,6,7] and (ii) the increasing of enzyme production due to the *dhfr* gene amplification[8]. It was found that mutation in codon 108, from serine or threonine to asparagine, was the important change for pyrimethamine resistance[9], with additional point mutation at position 16, 51, 59 and 164 are associated with the higher pyrimethamine and cycloguanil resistance[10,11]. Thaithong and colleagues had also confirmed that amplification of *dhfr* gene and some mutations occur among the population of *Plasmodium falciparum* grown *in vitro* in the presence of pyrimethamine[12]. From this study, some of pyrimethamine resistant lines of *P. falciparum* had different number of the *dhfr* gene copies. In the first set, T9/94RC17, after the long period of pyrimethamine selection the parasites were analyzed and compared with their original sensitive clone. It was found that there is no change in amino acid sequence in the *dhfr* gene but the gene had been increased in copy number up to 44-fold in the resistant populations. The TM4CB8-2.2.3 clone, after the drug selection, the *dhfr* gene has been amplified and has a mutation from isoleucine to methionine at position 164. The last set of resistant lines, G112CB1.1, has developed a single mutation from alanine to serine at position 16 without the amplification of the *dhfr* gene[12].

The aim of this work was to analyze the restriction fragment length polymorphisms (RFLPs) in the genome of a pyrimethamine resistance *P.falciparum* clone after prolong drug induction[12].

Materials and Methods

Parasites

The parasite clones used in this experiment were T9/94RC17, pyrimethamine sensitive clones, and their pyrimethamine progenies. The pyrimethamine susceptibity of these parasites were shown in the table 1.

DNA preparation, electrophoresis and Southern blotting

The parasite genomic DNA were extracted by standard phenol-chloroform extraction method and cut with restriction endonuclease, *Eco* RI, then size fractionated on an 0.7% agarose gel. The gel was blotted onto nylon membrane filter (Hybond N^+, Amersham). A Southern blot of parasite fractionated genomic DNA was hybridized with labeled probes and hybridization conditions were previously described[12].

Hybridization probes

The probes for hybridization were labeled with [α-^{32}P dATP] using a High Prime DNA labeling kit (Roche Diagnostics). These probes namely DHFR, TSl and TSs were the fragments of the dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) gene as shown in Figure 1. The DHFR probe was amplified by polymerase chain reaction (PCR) with the 5′ primer 5′- AAT ATT CCA ACA TTT TCA AGA TTG ATA CA –3′ and the 3′ primer 5′ – GAA TTC TTC ATC ATC ATC ATC ATT TTC A –3′. For the other two probes, the TS probes were amplified with the same 3′ primer (5′- AAG CTT AAC CGT TCA GGT AAT TTT GCT A –3′) using different 5′ primer (5′- GCA TGC CTT TAA AGA ATG ATG ACA AAG A –3′ and 5′- ACC AAA TGG CAT TAC CTC CT –3′ for TSl and TSs probe, respectively). The PCR conditions were described elsewhere[12]. The DHFR probe was used for the *dhfr*-end detection while the TSs probe was used for the *ts*-end detection. The TSl probe, intended to use as a specific probe for both junction and *ts*-end of the gene (Figure 1).

Table 1 Drug susceptibility test.

Clone	MIC† of pyrimethamine (M)	Clone	MIC† of pyrimethamine (M)
T9/94RC17	$5x10^{-8}$	T9/94RC17 Py $5x10^{-7}$	10^{-5}
T9/94RC17 Py‡ 10^{-8}	$5x10^{-7}$	T9/94RC17 Py 10^{-6}	10^{-5}
T9/94RC17 Py $5x10^{-8}$	$5x10^{-7}$	T9/94RC17 Py $5x10^{-6}$	$5x10^{-5}$
T9/94RC17 Py 10^{-7}	10^{-6}	T9/94RC17 Py 10^{-5}	$5x10^{-5}$

† MIC is stand for a minimum inhibitory concentration.

‡ The number behind Py letter showed the concentration of pyrimethamine in culture medium.

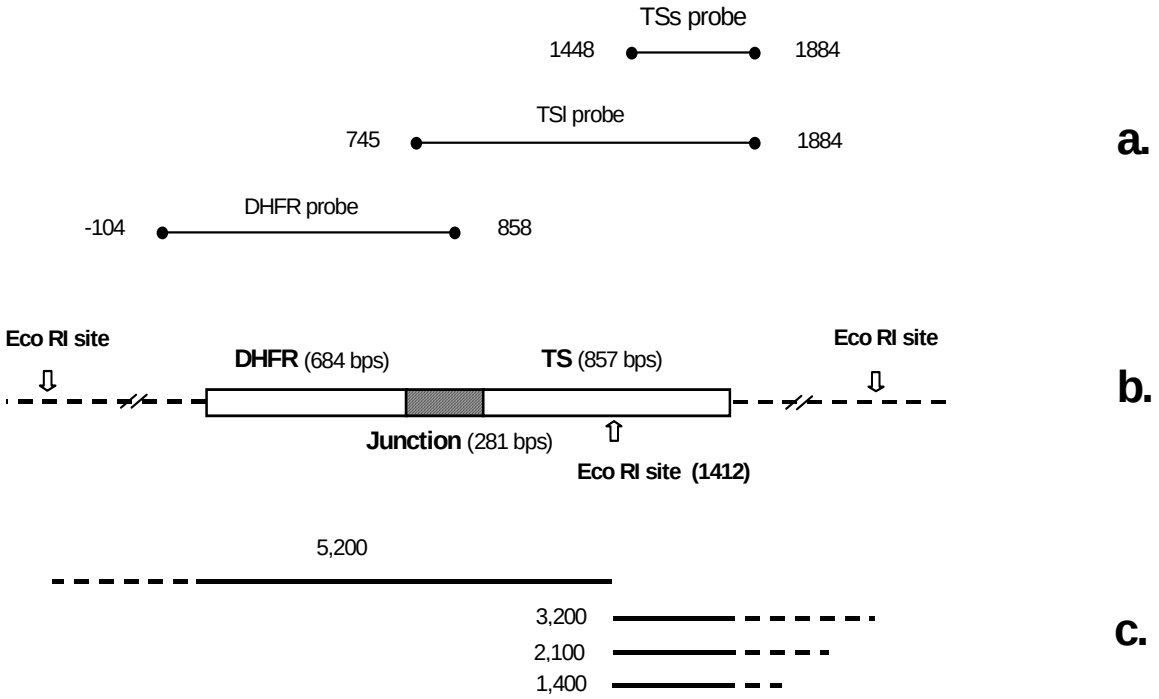


Figure 1. Schematic diagram of the *Plasmodium falciparum dhfr-ts* gene. This showed a) the position of probes on the *dhfr-ts* gene, b) the *Eco*RI recognition sites and c) the RFLP fragments.

Results

The hybridization, with the DHFR probe, result suggested that the 5′ fragments of the *dhfr* gene were amplified, as a 5,200 bp fragment, in all parasite clones without size polymorphism detected by agarose gel electrophoresis separation (Figure 2a). In contrast, the DNA band profiles of a drug resistant clone, hybridized with the TSl and TSs probes were different from its parent clone. Two DNA bands, 5,200 and 3,200 bp fragments, hybridized with the TSl probe, were found in the parent clone, T9/94RC17, and all its pyrimethamine resistance progeny. Only T9/94RC17 $Py10^{-7}$ clone which was selected and grown in 10^{-7} M pyrimethamine has two extra DNA bands, 2,100 and 1,400 bp in size (Figure 2b). Similar DNA band profiles were also confirmed using the TSs probe (Figure 2c).

The DNA profile of T9/94RC17 suggested that there are two *Eco*RI recognition site flanking *dhfr-ts* gene on the chromosome 4. The 5′ and 3′ recognition site located approximately 5,200 bp upstream and 3,200 bp downstream of the internal *Eco*RI recognition site respectively (1412^{th}–1417^{th}). In comparison to DNA profiles of other progenies, it could be implied that not only the *dhfr-ts* gene but also

the flanking region, at least 8,400 bp (5,200 bp+3,200 bp), were coamplified. Using this estimated size, the 44 copies amplification in T9/94RC17 $Py10^{-5}$ [12] would result to chromosome 4 size increasing by at least 0.37 Mb in this particular clone. Although there is no direct evidence how these amplified fragments were arranged in our drug-selected clones, previous experiment suggested that these fragments may arrange as the tandem head-to-tail repeats[13,14].

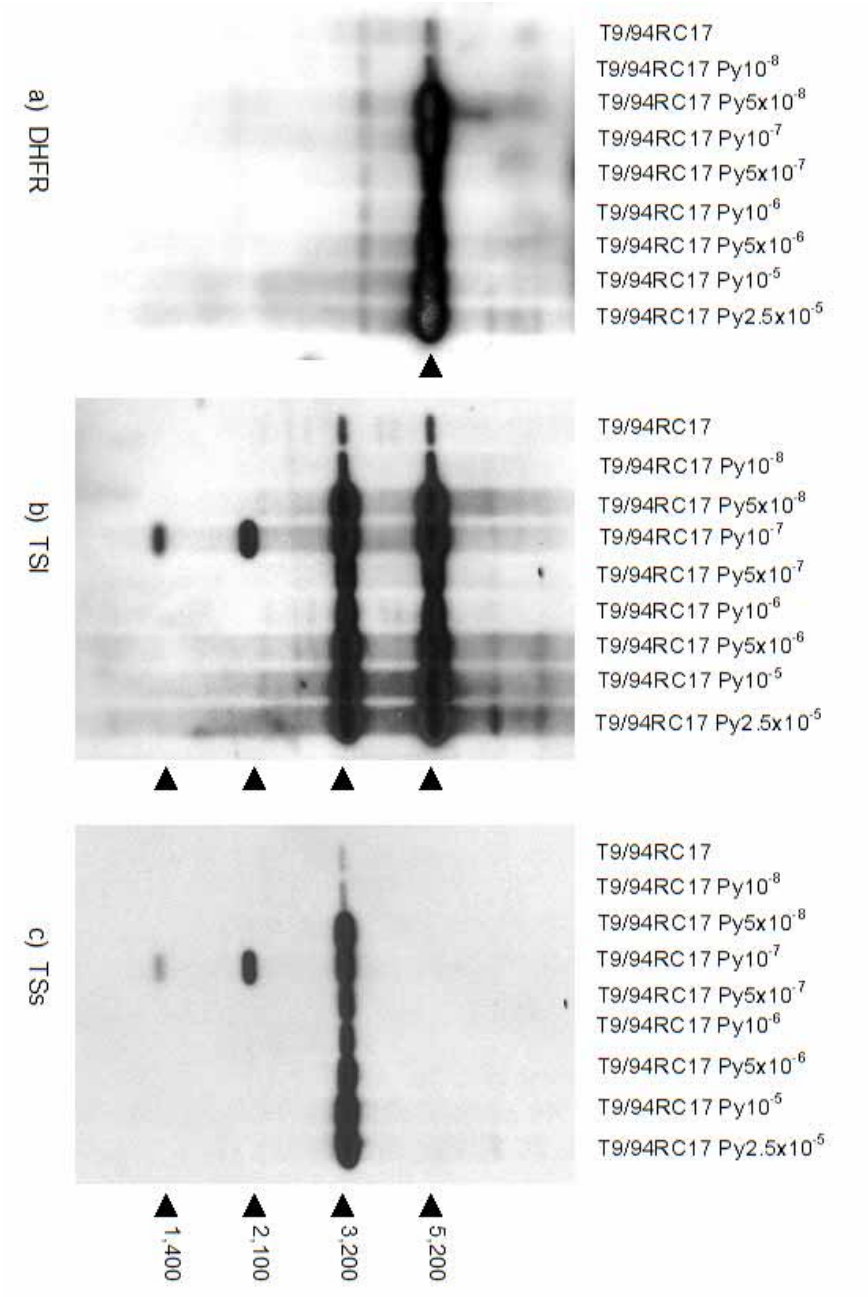


Figure 2. Southern analysis of T9/94RC17 clone lines. One microgram genomic DNA was digested with 10 U of *Eco*RI and fractionated by 0.7% agarose gel electrophoresis. A Southern blot was hybridized with a) DHFR probe, b) TSl probe and c) TSs probe.

Discussion

The RFLPs found in these clones may be caused by (i) error during DNA replication (ii) DNA deletion (iii) point mutation. It is possible that the parasite may make a mistake during DNA replication in threatening environment of drug pressure. As a result, some parts of the *dhfr-ts* gene flanking region, at the 3/ of the gene, was missing from the parasite genome.

Since a total copy number of the shorten *dhfr-ts* gene per parasite genome was still unknown. The RFLPs may cause by DNA deletion at both the early or later time of DNA replication. Most or all of the progenies, then, contained shorten copies of flanking region.

Point mutation of genomic DNA nearer to the *dhfr-ts* gene may create new *Eco*RI recognition site nearer to the gene. It is also possible that the RFLPs may be generated by combination of these mechanisms during T9/94RC17 Py5x10^{-8} to T9/94RC17 Py10^{-7} or from T9/94RC17 Py10^{-7} to T9/94RC17 Py5x10^{-7} transition.

The *dhfr-ts* gene amplification has been considered as one of the drug resistance mechanisms adapted by malaria parasite and also methotrexate-resistant mouse tumor cells and several cell lines[15].

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