CHAPTER IV

EVALUATION METHOD FOR DNA PREPARATION

4.1 Introduction

Apart from microscopic examination, PCR technique has been known as a technique could be used for parasite detection in diagnosis with high sensitivity. This knowledge would be applicable to drug susceptibility test which needs a method to reveal parasites at very low parasitemia. However, DNA template quality plays an important role in the success of PCR reaction, loss of DNA during the extraction process or the contamination of hemoglobin may inhibit, partly or the whole of amplification reaction. To extract high quality parasitic DNA at low parasitemia, DNA extraction methods have to meet the following criteria: (1) rapid preparation and large scale throughput, (2) high reliability, (3) good quality of DNA for long-termed storage, (4) avoidance of contaminants, and (5) reasonable cost (Henning *et al.*, 1999).

In this chapter, we compare three different methods of DNA preparation from pyrimethamine susceptibility test of *P. falciparum* in order to find out the most compatible approach for performing PCR. The following methods were compared: rapid boiling method, Phenol chloroform extraction (Snounou, 1994), and a commercially available DNA purification kit (Perfect gDNA Blood Mini, Eppendorf Germany).

Parasitic DNA prepared from three different techniques was compared in a PCR amplification of rhoptry associated protein-1 gene (*rap-1*) and small subunit ribosomal RNA gene (*ssrRNA*) of *P. falciparum*. The *rap-1* is, a large size (2493 base pairs), single copy gene and highly conserved (Ridley *et al,* 1990) while the *ssrRNA* is rather small size, multiple copies and has been frequently used in detection of *P. falciparum* (Rougemont *et al.*, 2004). Accordingly, we can use *rap-1* and *ssrRNA* for parasite detection in drug susceptibility test. We performed four replicates of pyrimethamine susceptibility test. One replicate was for MIC value determination using microscopic examination. The others were extracted their DNA using three different techniques for PCR amplification of *rap-1* and *ssrRNA*.

4.2 Results

4.2.1 MIC value from thin blood film

From the data obtained from microscopy method of pyrimethamine susceptibility test to T9/94RC17, the result showed that the lowest concentration which killed nearly all of the parasites after exposure to drug-containing media for 72 hours (MIC, minimum inhibitory concentration) was 5 x 10^{-8} M (Table 4.1).

Pyrimethamine (M) / T9/94RC17	control	$5x10^{-9}$	10^{-8}	$5x10^{-8}$	10^{-7}	$5x10^{-7}$	10^{-6}	$5x10^{-6}$
Replication 1	+	+	+	-	-	-	-	-

Table 4.1 Table shows the result of pyrimethamine susceptibility test to T9/94 RC17 from microscopic examination. (+) normal parasites found, (-) could not find normal parasite

4.2.2 Different DNA extraction methods

Parasites in each well from the drug susceptibility test were subject to DNA extraction. Three DNA extraction methods were used to compare their capabilities. Agarose gel electrophoresis analysis of DNA samples from these extraction methods revealed DNA amplification reaction. DNA acquired from three DNA preparation methods was amplified using two pairs of primers, *rap-1* (rhoptry associated protein-1) and *ssrRNA* (small subunit ribosomal RNA). The parasitic DNA (from drug susceptibility test) from boiling method was amplified with the *rap-1* and *ssrRNA* primers. From this study, PCR products of *rap-1* were amplified from the parasites treated with pyrimethamine at 10^{-8} M, $5x10^{-8}$ M and 10^{-7} M (Figure 4.1a) while the *ssrRNA* PCR products were found from the samples treated with the drug at all concentrations (Figure 4.1b)

The samples extracted by phenol chloroform extraction were also amplified by the *rap-1* and the *ssrRNA* primers. The samples treated with pyrimethamine at all concentrations can generate *rap-1* and *ssrRNA* PCR products (Figure 4.2a, 4.2b).

Parasitic DNA obtained from DNA purification kit was determined by the *rap-1* gene and the *ssrRNA* gene amplification. The PCR product of *rap-1* was found only from the positive control group (Figure 4.3a). The *ssrRNA* PCR product was amplified

from the samples treated with $5x10^{-9}$ M, 10^{-8} M and $5x10^{-8}$ M pyrimethamine and the positive control group of parasite (Figure 4.3b).

4.3 Discussion and Conclusion

The microscopic examination provides a standard for MIC against pyrimethamine as $5x\ 10^{-8}$ M. At this pyrimethamine concentration, all parasites were killed. If the parasitic DNA and/or RNA degraded, no DNA template would be available for PCR reaction; as a result, no PCR product can be visualized by agarose gel electrophoresis. On the contrary, if there was a DNA and/or RNA molecule left in the sample, PCR product will be detected after the amplification reaction. However, from microscopic result, the dead parasite at MIC level and higher pyrimethamine concentrations had large vacuole, the nucleus was dense and some of the dead parasites had degraded cytoplasm (Figure 4.4). It was suggested that the DNA of dead parasites may not rapidly degraded. Hence, it would be possible that the *rap-1* PCR product could be detected after the parasites had been exposed to drug at higher concentration than the MIC.

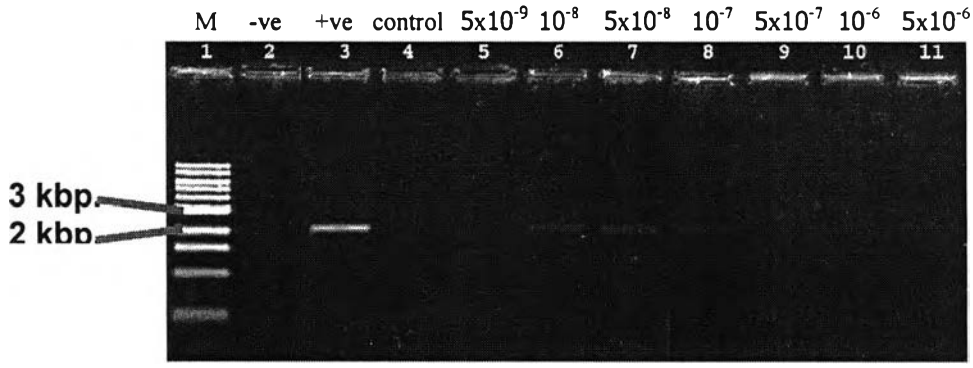
From all three methods of DNA extraction, the phenol-chloroform method gave clear signals of *rap-1* PCR product from the samples treated with pyrimethamine up to $5x10^{-6}$ M. The intensity of PCR products dramatically decreased at the MIC value (Table 4.2).

The boiling method gave very faint bands of PCR product in a narrow range of drug tested. Because the over simplified procedure in the method, no protein or other contaminated inhibitors had been removed from the samples. These contaminants may play an important role in holding back the amplification reaction of PCR.

Surprisingly, the samples purified by DNA extraction kit failed to give satisfactory results. From this result together with the cost of extraction, this extraction method was not suitable in favor of economy and efficiency.

The parasite detection using *ssrRNA* primers gave out very strong band of the PCR product, but its sensitivity was beyond the use in this application. Large amount of PCR products may cause by the numerous copies of the gene in a genome. As a result, DNA templates were abundant, even though some DNA molecules had already been degraded.

(a) *rap-1* gene



(b) *ssrRNA* gene

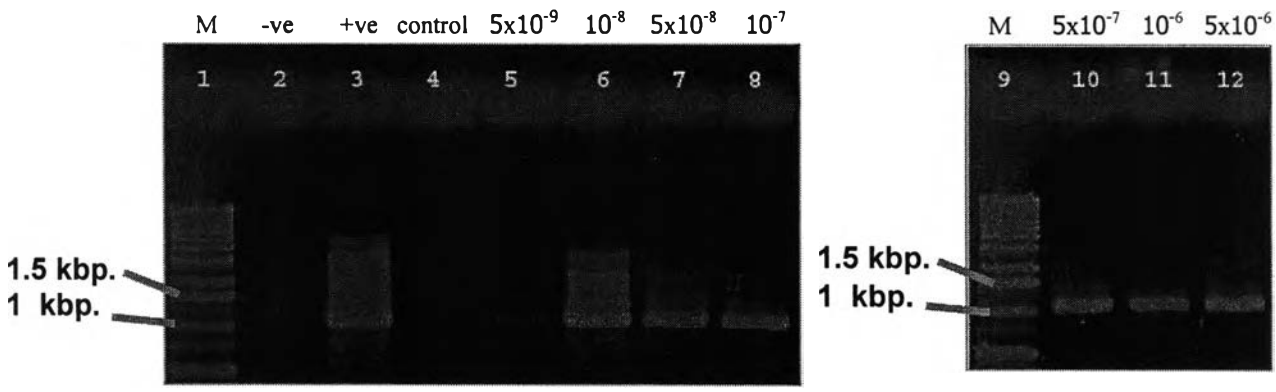
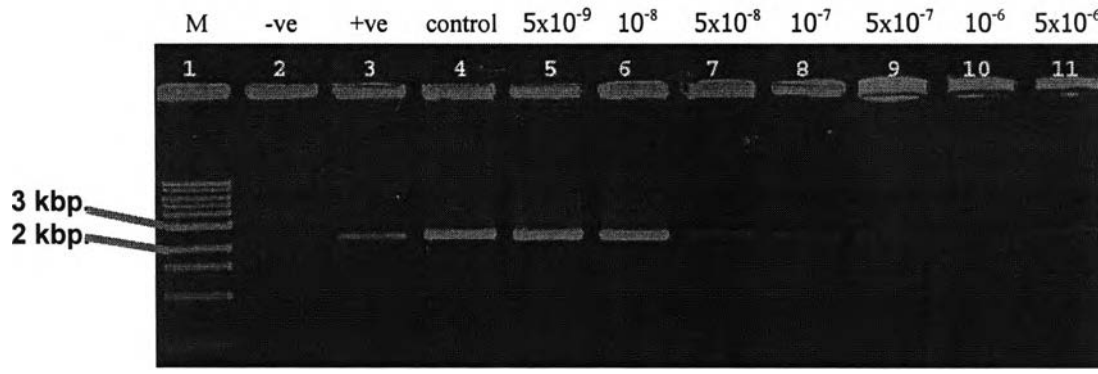


Figure 4.1 These figures show the PCR product from the parasitic DNA extracted by rapid boiling method. Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

(a) *rap-1* gene



(b) *ssrRNA* gene

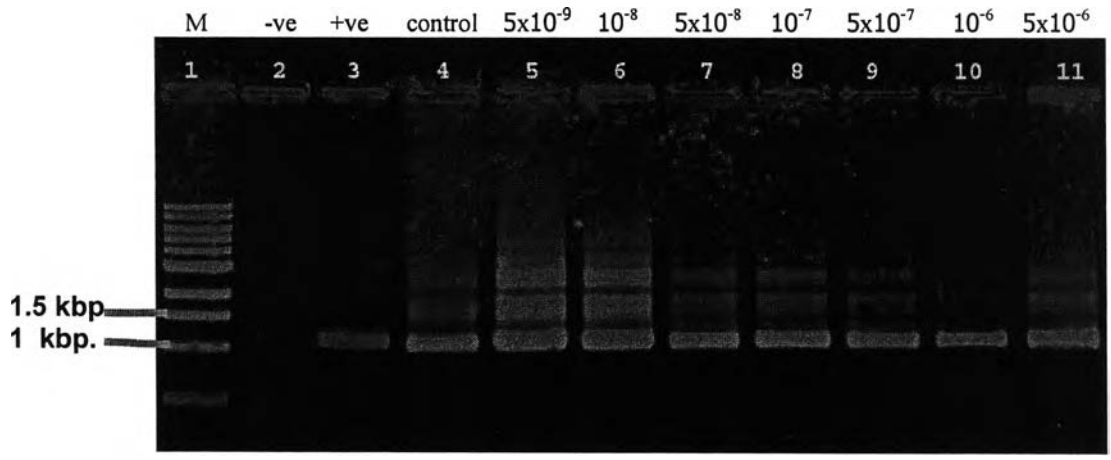
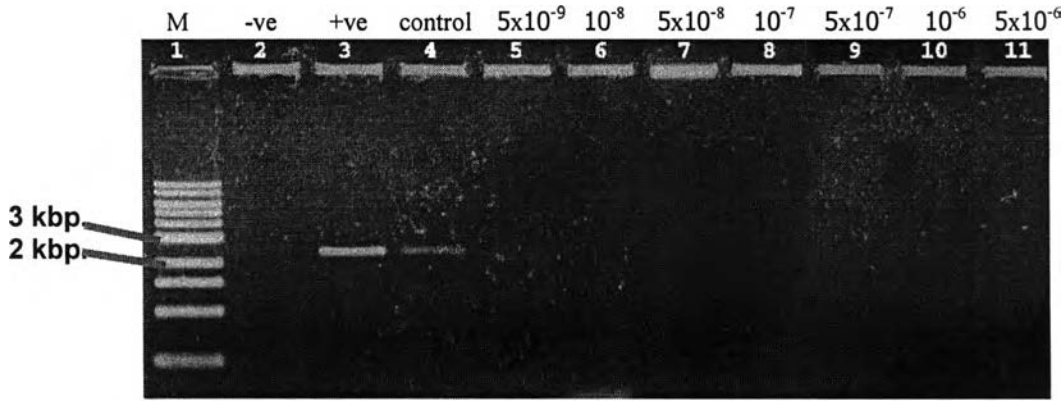


Figure 4.2 These figures show the PCR product from the parasitic DNA extracted by phenol chloroform extraction method. Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

(a) *rap-1* gene



(b) *ssrRNA* gene

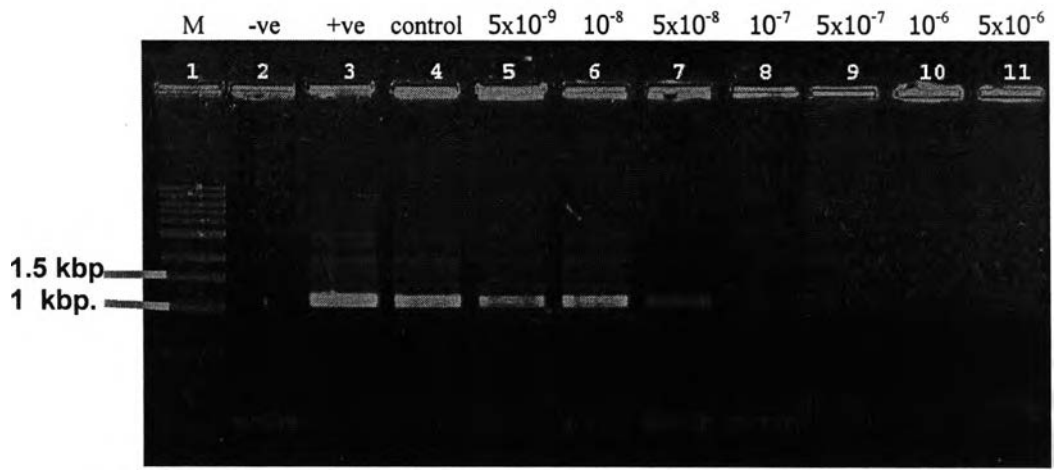


Figure 4.3 These figures show the PCR product from the parasitic DNA extracted by Perfect gDNA Blood Mini (Eppendorf). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

Pyrimethamine (M) / T9/94RC17		control	$5x10^{-9}$	10^{-8}	$5x10^{-8}$	10^{-7}	$5x10^{-7}$	10^{-6}	$5x10^{-6}$
Replicate 2 is extracted with rapid boiling method	*rap-1*	-	-	+	+	+	-	-	-
	ssrRNA	-	+	++++	++++	++++	++++	++++	++++
Replicate 3 is extracted with phenol chloroform extraction	*rap-1*	++	++	++	+	+	+	+	+
	ssrRNA	++++	++++	++++	++++	++++	++++	++++	++++
Replicate 4 is extracted with Perfect gDNA Blood Mini	*rap-1*	+	-	-	-	-	-	-	-
	ssrRNA	+++	+++	+++	+	-	-	-	-

Table 4.2 Table shows the result of pyrimethamine susceptibility test to T9/94 RC17 from PCR detection. Column in grey shows the MIC value from microscopic examination. (++) found PCR product, (+) slightly found PCR product, and (-) could not find PCR product.

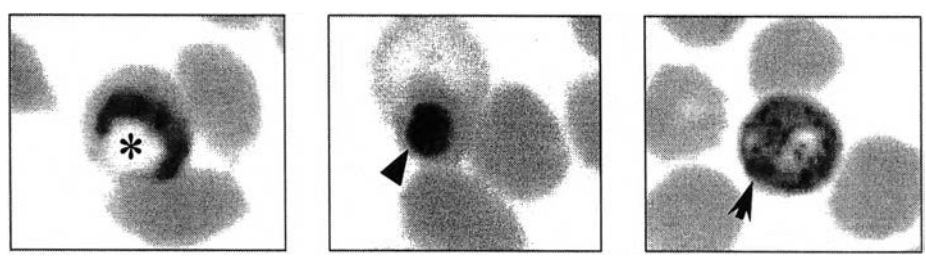


Figure 4.4 illustrates the conformation of dead parasite at MIC level of pyrimethamine. (*) vacuole, (▲) dense nucleus, and (▲) degraded cytoplasm

In conclusion, the most appropriate technique for DNA preparation is phenol chloroform extraction method because this technique supplies the high quality of DNA and consistency of amplification products as well as has reasonable cost. Particularly, this method can perform in a large quantity of samples in the same time.