CHAPTER V

MONITERING OF PRIMERS SENSITIVITY

5.1 Introduction

Assessment of MIC value in drug susceptibility test requires the detection of parasite at low levels of parasitemia. Although, the sensitivity of microscopic examination allows the detection of 20 to 50 parasite/μl (0.0004 to 0.001 % parasitemia) (Guerin *et al.*, 2002), the interpretation of blood smear, particularly at low levels of parasitemia, requires considerable skill (Warhurst and Wiliams, 1996). Hence, it is an infeasible task to perform within limited time with large numbers of samples. Moreover, at low parasitemia, the information obtained by microscopy might be biased and may lead to false negative results. On the other hand, PCR technique had been developed to detect parasites at very low numbers of the human malaria parasites (Snounou *et al.*, 1993). This technique can be performed with the large numbers of samples in shorter time.

In this chapter, we monitor the sensitivity of two primer sets, *rap-1* and *ssrRNA*, using PCR and RT-PCR technique. Initially, we diluted parasite, from 10% parasitemia, into 9 different parasitemias using uninfected red blood cell. The actual parasitemias were evaluated by microscopic examination (Table 5.1).

As indicated by previous experiment (chapter 4), parasitic DNA was prepared by phenol chloroform extraction method (Snounou, 1994) to maximize the amplification result. To assess the primer sensitivity in PCR reaction, extracted DNA was used as DNA templates for the primers in PCR reaction.

For the RNA preparation in RT-PCR reaction, the RNA purification kit (Perfect RNA Eukaryotic Mini, Eppendorf Germany) were used to increase the speed and yield of purified RNA. RT-PCR amplification of *rap-1* and *ssrRNA* gene was performed using cMaster RT_{plus}PCR System, Eppendorf Germany in the presence of tested primers. Consequently, we could monitor the sensitivity of each primers through results obtained from PCR and RT-PCR amplification technique.

5.2 Results

Nine serial dilutions of the parasite were prepared and their blood films were counted for parasitemia evaluation. Parasites as low as 0.01% parasitemia can be detected by using microscopy (Table 5.1). Nine concentrations of % parasitemia were divided in two identical sets; one was used for DNA preparation while another was used for RNA purification.

Expected % parasitemia	Observed % parasitemia
10%	9.27%
1%	0.86%
0.5%	0.45%
0.1%	0.09%
0.05%	0.04%
0.01%	0.02%
0.005%	< 0.005%
0.001%	< 0.001%
0.0005%	< 0.0005%

Table 5.1. Table shows the percentages of parasitized red blood cell measured from thin blood films stained with Giemsa.

5.2.1 PCR technique

Samples with different parasite concentrations were subjected to DNA purification and gene amplification, the *rap-1* gene and the *ssrRNA* gene, by PCR technique. DNA from 10%, 1%, 0.5%, 0.1% and 0.05% parasitemia samples can be amplified in PCR reaction while DNA from 0.01%, 0.005%, 0.001%, and 0.0005% parasitemia samples could not produce any PCR product of *rap-1* gene (Figure 5.1a).

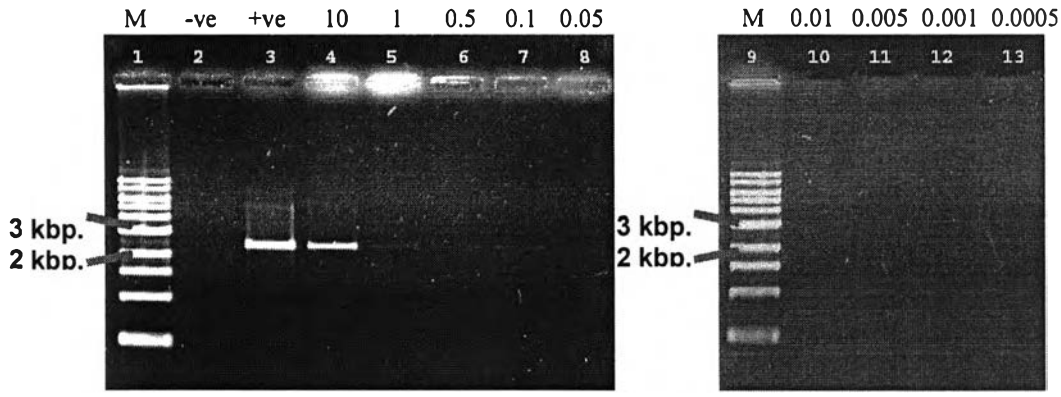
For the amplification of *ssrRNA* gene, PCR products can be obtained using DNA extracted from 10%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% parasitemia samples while DNA from 0.0005% parasitemia sample, failed to give any *ssrRNA* PCR product(Figure 5.1b).

5.2.2 RT-PCR technique

The other set of serial dilution was purified for parasitic RNA. Subsequently, those RNA samples were converted into cDNA and amplified by reverse transcriptase PCR technique. From this study, the results revealed that the RT-PCR products of the *rap-1* gene can be obtained using RNA extracted from 10%, 1%, 0.5%, 0.1%, 0.05%, and 0.01% parasitemia samples while no RT-PCR product can be seen using RNA extracted from 0.005%, 0.001%, and 0.0005% parasitemia samples(Figure 5.2a).

In performing RT-PCR of the *ssrRNA* gene, RT-PCR products can be obtained from 10%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% parasitemia DNA whereas from 0.0005% parasitemia, cDNA template cannot produce any *ssrRNA* PCR product (Figure 5.2b).

(a) *rap-1* gene



(b) *ssrRNA* gene

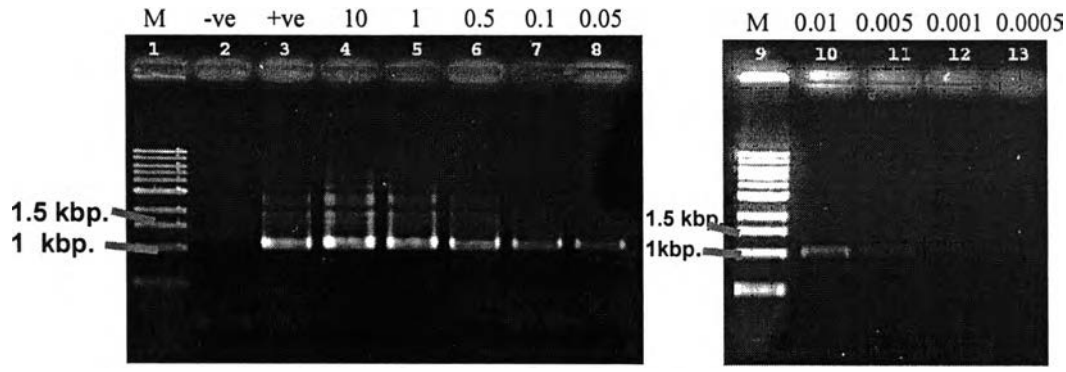
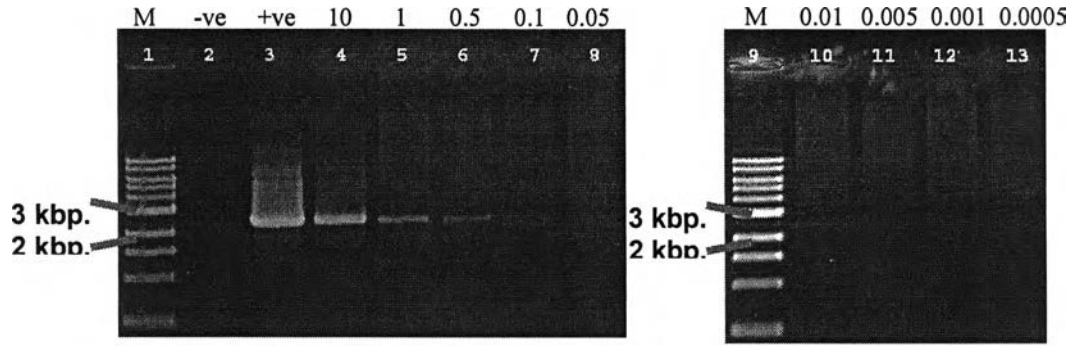


Figure 5.1 These figures show the agarose gel electrophoresis analysis of the PCR products obtained from the amplification reaction of DNA template extracted from the samples contained different parasitemias using a) *rap*-1 gene primers; b) *ssrRNA* gene [M: 1 Kb ladder marker, -ve: negative control (no parasitic DNA), +ve: positive control (*P. falciparum* DNA), the numbers on the top of each lane represent the parasitemias.]

(a) *rap-1* gene



(b) *ssrRNA* gene

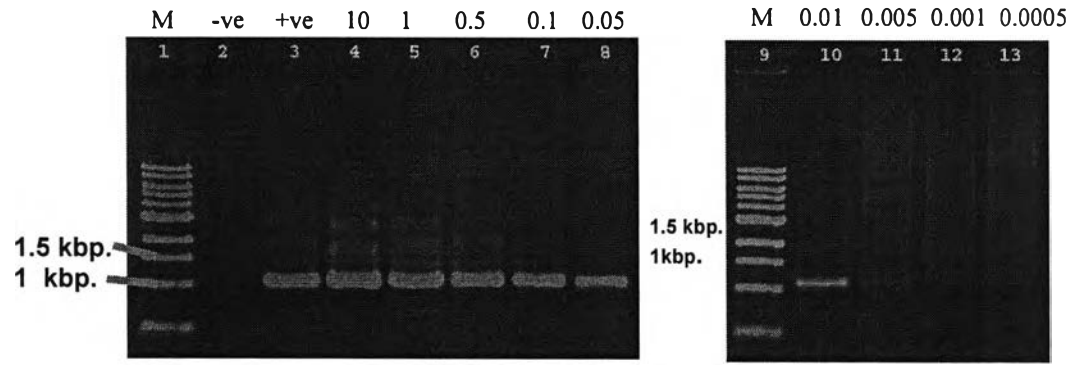


Figure 5.2 These figures show the agarose gel electrophoresis analysis of the RT-PCR products obtained from the amplification reaction of cDNA template converted from RNA extracted from the samples contained different parasitemias using a) *rap*-1 gene primers; b) *ssrRNA* gene [M: 1 Kb ladder marker, -ve: negative control (no parasitic DNA), +ve: positive control (*P. falciparum* DNA), the numbers on the top of each lane represent the parasitemias.]

5.3 Discussion and Conclusion

According to the results, microscopic assay can detect malaria infection as low as 0.01% parasitemia (Table 5.1). Under the optimum condition, however, it had been shown that this technique could detect parasitemia as low as 0.0004 to 0.001 % parasitemia (Guerin *et al.*, 2002). Although, microscopic examination is a standard technique at the most reasonable cost, to achieve high sensitivity, training and quality control of expertises, adequate equipment and maintenance are required. Furthermore, when the number of samples increase, it become more difficult to determine the parasite at very low level of parasitemia. As shown by Milne and his group that more than 10% of the blood films submitted to the London-UK Malaria Reference Laboratory had been read as false negative (Milne *et al.*, 1994) or those 10 to 15% misdiagnosis because of low parasitemia by laboratories in Quebec, Canada (Ndao *et al.*, 2005). During drug susceptibility test, researchers have to routinely examine the low level of parasite to determine the MIC value – the lowest concentration of drug that can eradicate the entire parasite. Therefore, using only microscopy to evaluate the MIC value is very time-consuming and can also lead to inaccurate outcome when there are many samples. To confirm the result from microscopic examination, PCR technique is introduced for determining parasite in drug susceptibility test. The use of PCR technique to detect the low levels of parasite requires the sensitivity data of primers used; *rap-1* and *ssrRNA* gene.

According to the results from PCR technique, the *rap-1* and *ssrRNA* primers can detect malaria infection as low as 0.05% and 0.001% parasitemia respectively (Table 5.2). The sensitivity of *rap-1* primers is near to the sensitivity of microscopy (0.01% parasitemia). The results reflect the single copy status of the *rap-1* gene while *ssrRNA* gene presents in multiple copies inside the malarial genome. This suggested that PCR assay using *ssrRNA* primers is more sensitive than microscopic assay which is similar to the recent study by Scopel and his groups (Scopel *et al.*, 2004). Moreover, it has been estimated that the nested PCR method based on amplification of *p126 P. falciparum* gene can detect malaria infections with parasitemia as low as 0.0001% parasitemia which is more sensitive than that of microscopy (Zalis *et al.*, 1996).

Techniques	% parasitemia								
	10	1	0.5	0.1	0.05	0.01	0.005	0.001	0.0005
PCR									
• *rap-1* gene	+	+	+	+	+	-	-	-	-
• *ssrRNA* gene	+	+	+	+	+	+	+	+	-
RT- PCR									
• *rap-1* gene	+	+	+	+	+	+	-	-	-
• *ssrRNA* gene	+	+	+	+	+	+	+	+	-

Table 5.2 The amplification of *rap-1* and *ssrRNA* gene from serial dilution samples at different %parasitemia (+ and - show the presence and absence of the PCR products analyzed with agarose gel electrophoresis)

The results from RT- PCR technique reveal that *rap-1* and *ssrRNA* primers can detect the infection of *P. falciparum* as low as 0.01% and 0.001% parasitemia respectively (Table 5.2). They also show that the *rap-1* gene primers are more sensitive in the RT-PCR than the PCR. This may be because, in the RT-PCR, more than one mRNA molecules were changed into cDNA and acted as DNA template compared to the single copy of *rap*-1 gene in the genome. As a result, at vary level of parasites, more cDNA template, in the RT-PCR, can be amplified and gave out PCR products which can be detected by agarose gel electrophoresis. On the other hand, there was no different of sensitivity in the RT-PCR and the PCR technique using *ssrRNA* gene primers. It is possible that the number of DNA template reached the lowest detectable limit in both reactions.

In conclusion, The results suggested that the sensitivity of *rap-1* primers is similar to microscopic assay (lower in PCR and as sensitive as microscopy assay in RT-PCR) whereas the sensitivity of *ssrRNA* primers is much higher than microscopy assay (both PCR and RT-PCR). Therefore, it may be possible to use PCR or RT-PCR technique of the *rap*-1 gene to assess drug susceptibility status of the malaria parasites *in vitro*.