CHAPTER VI

MEASUREMENT PARASITE FROM DRUG SUSCEPTIBILITY TEST BY USING PCR AND RT-PCR

6.1 Introduction

Antimalarial drugs are used by physicians and public health personal for the controlling and treatment of malaria infection. To decide which antimalarial drugs and doses should be applied in a specific area, the policy makers must take several factors into account. One of those factors is the drug susceptibility data of the parasites in the area. Since the drug susceptibility of an isolate or clone against each drugs is a unique property of tested isolate or clone, the changes of drug susceptibility status would allow the policy makers to modify the drug regimens in that area. Drug susceptibility test, *in vitro*, can be performed by collecting the parasite from a patient, culturing, treating with various concentrations of drug and measuring for the survived parasite in each drug concentration (Thaithong *et al.*, 1983). Results from drug susceptibility test could be illustrated in term of IC50 or MIC (minimum inhibitory concentration). The MIC, the lowest concentration of drug eliminating all or almost all of the parasites, of several isolates can be compared to evaluate the drug resistance status of the parasites in those isolates. However, to determine MIC value, researchers require high-resolution microscopes and specialized skill to check for the lowest drug concentration at which all parasites are killed. Besides, parasite detection in a large number of samples could not be accomplished because the process is time-consuming and labor-intensive. To solve this problem we attempt to explore the PCR and RT-PCR technique to determine the presence of parasitic DNA and RNA in drug-tested samples compared to conventional drug susceptibility test.

In this chapter, four-antimalarial drugs (quinine, mefloquine, chloroquine and pyrimethamine) were applied in drug susceptibility tests against *P. falciparum* clone T9/94RC17 in octaplications. Two sample sets were examined for MIC values using microscopy while other two sets were utilized for PCR and RT-PCR without DNA or RNA extraction (chapter 7). Other two duplicates, their DNA and RNA were extracted using phenol-chloroform extraction (Snounou, 1994) and RNA purification kit (Perfect RNA Eukaryotic Mini, Eppendorf Germany) respectively (Figure 6.1). Those

extracted samples were subjected to PCR and RT-PCR using primers for both *rap-1* and *ssrRNA* gene respectively. At the end of reactions, all samples were analyzed their PCR and RT-PCR products by agarose gel electrophoresis.

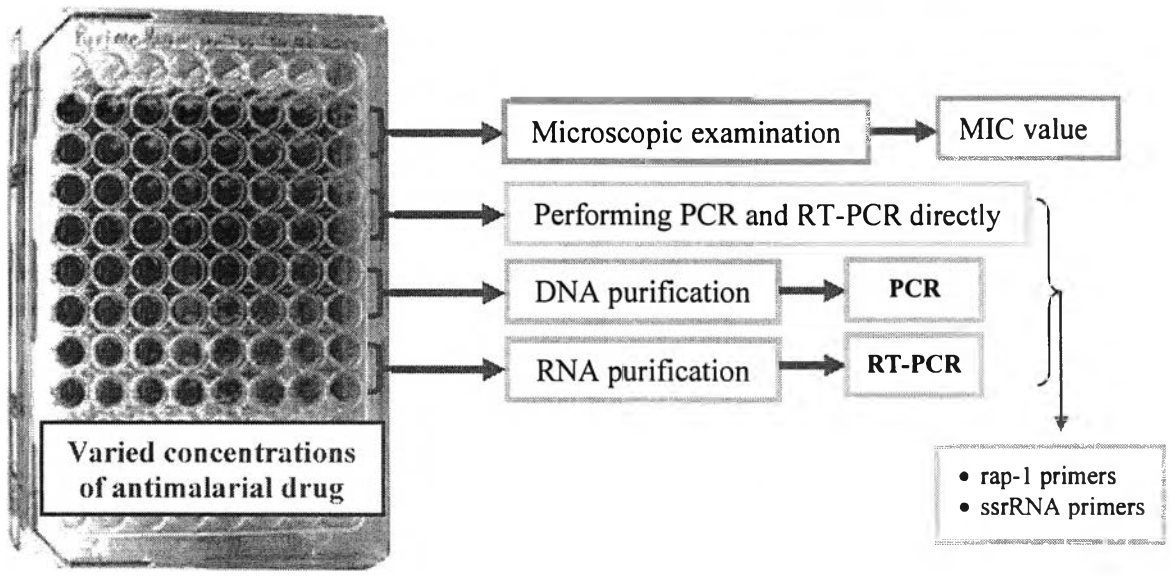

Figure 6.1 Octaplications of antimalarial drug susceptibility test were grouped into four sets. One was for MIC value determination by microscopy; other two were for PCR and RT-PCR without DNA and RNA extraction (chapter 7); the others were used for DNA and RNA extraction before PCR and RT-PCR analysis.

6.2 Results

The results of each experiment which perform in duplications were similar, thus, only one result was reported.

6.2.1 Microscopic examination

Malaria parasites as seen in blood samples taken from drug susceptibility tests can be measured by using thin blood films stained with Giemsa and examined under the microscope. In quinine susceptibility test (Figure 6.2A), high parasitized red blood cell, healthy ring forms and early schizonts were observed in the control group (no quinine). At 10^{-8} and $5x10^{-8}$ M of quinine, trophozoites and ring forms were found to be normal with high parasitemia. However, abnormal ring forms, with initially degraded cytoplasm, (green arrow), and the abnormal trophozoites, with shrinked

cytoplasm (blue) and their purple-stained chromatin (light green arrow), were observed at 10^{-7} M of quinine. The degradation of trophozoite cytoplasm (green arrow) and the parasitemia were marked decreased at $3x10^{-7}$ M. At the MIC level, $5x10^{-7}$M of quinine and higher, although, some of the parasite debris was found (data not shown), no living parasite could be found.

In mefloquine susceptibility test (Figure 6.2B), healthy ring forms, trophozoites and mature schizonts were found to be normal in the control group (no mefloquine). At 10^{-8} M of mefloquine, several ring forms and mature schizonts could be seen. The figure showed the infected red blood cell became ruptured releasing merozoites which were immediately invaded new red blood cells. The grains of dark brownish pigment which were by-products of hemoglobin digestion by the parasite (orange arrow). At $3x10^{-8}$, $5x10^{-8}$ and $7x10^{-8}$ M of mefloquine, the cytoplasm of ring forms degraded (green arrows) and the shrinkage of early schizonts were noticed (light green arrow). At 10^{-7} M, the parasite debris was observed (green arrow). In those four concentrations of mefloquine, the numbers of parasitized erythrocytes were gradually decreased correlated with the drug concentrations. At the MIC level ($2x10^{-7}$ M) of mefloquine and above, no parasite could not be visualized in the stained blood film.

In chloroquine susceptibility test (Figure 6.3A), normal trophozoites and early schizonts were observed in the control group (no chloroquine). Ring forms and mature schizonts were noticeable with high parasitemia and the dark brownish pigments were also clearly visible at 10^{-8}, $5x10^{-8}$ M of chloroquine (orange arrows). At 10^{-7} M, some abnormal parasite were observed. The parasitemia become markedly decreased at the MIC concentration of chloroquine. At the MIC level ($5x10^{-7}$ M) and above (data not shown), only few parasite debris was found.

In pyrimethamine susceptibility test (Figure 6.3B), several numbers of healthy ring forms, trophozoites and mature schizonts were observed in the control group (no pyrimethamine) as well as at $5x10^{-9}$ M of pyrimethamine. At 10^{-8} M, mature schizonts and ring forms were visualized similar to the control group, with lower parasitemia. At the MIC level ($5x10^{-8}$M) and above (data not shown) only few parasite debris were noticeable (green arrow). In brief, the MIC values of tested antimalarial drugs against T9/94RC17 were listed in the table below.

antimalarial drugs	MIC value (M)
quinine	$5x10^{-7}$
mefloquine	$2x10^{-7}$
chloroquine	$5x10^{-7}$
pyrimethamine	$5x10^{-8}$

Table 6.1 The MIC values of each antimalarial drugs against *P. falciparum* T9/94RC17 using microscopic detection

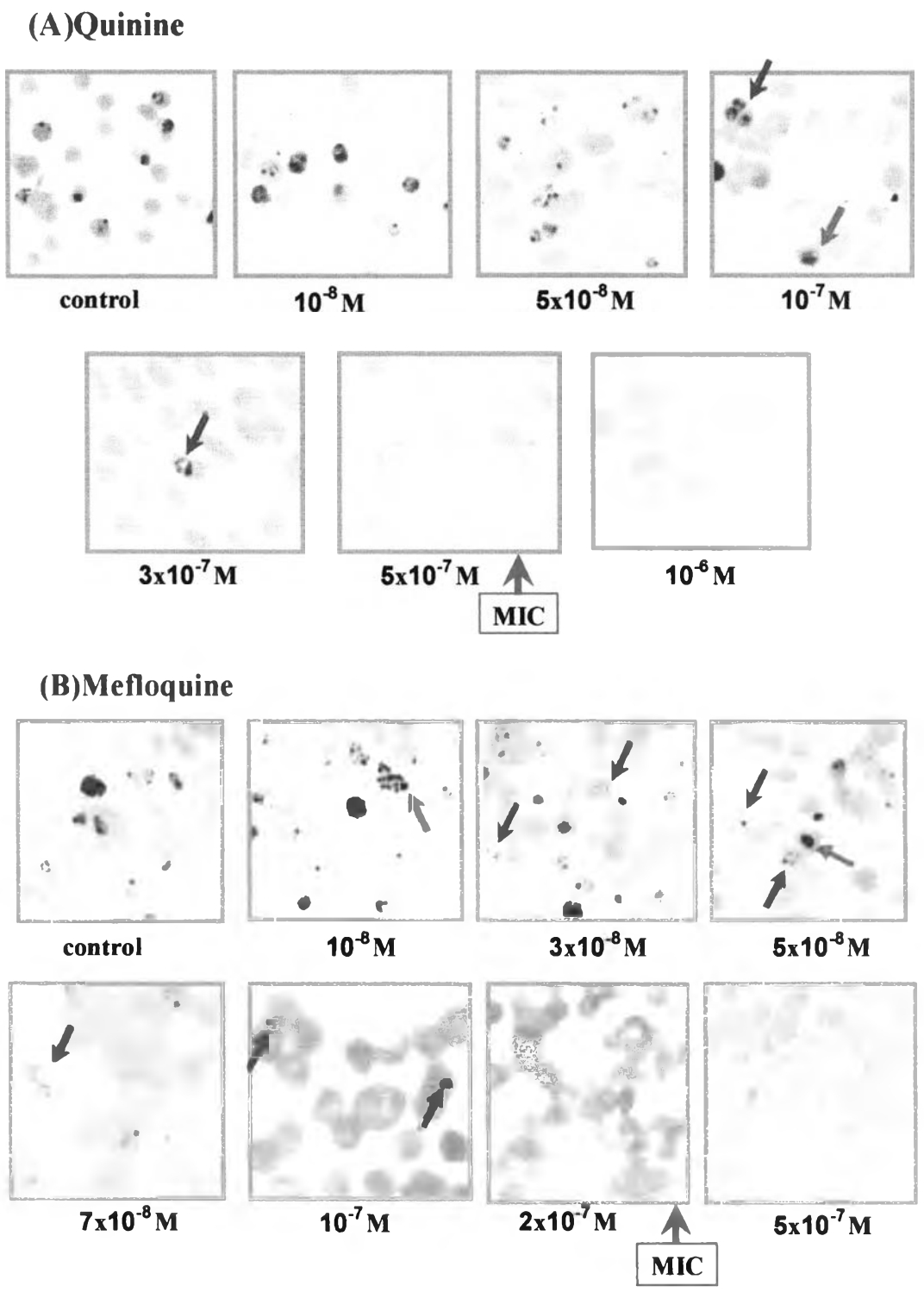

Figure 6.2 These photographs illustrate the result from quinine (A) and mefloquine (B) susceptibility tests which were observed by microscopic examination. Digits below pictures represent the concentrations of both drugs. Red arrows point at the photographs at the MIC values. An orange arrow points at a ruptured red blood cell. Green arrows point at the abnormal parasites.

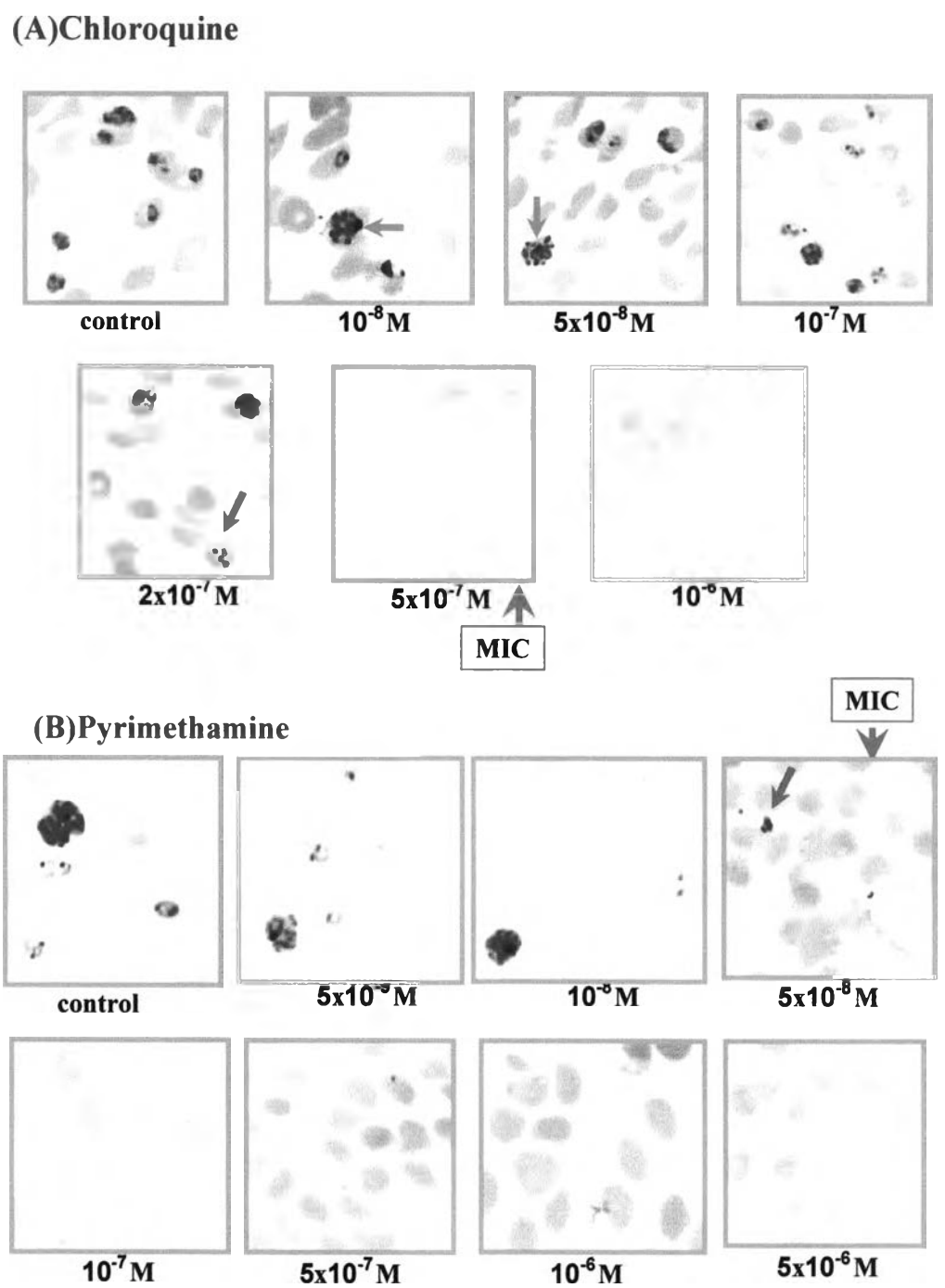

Figure 6.3 These photographs illustrate the result from chloroquine(A) and pyrimethamine(B) susceptibility tests which were observed by microscopic examination. Digits below pictures represent the concentrations of both drugs. Red arrows point at the photographs at the MIC values. Green arrows point at parasite debris.

6.2.2 PCR Technique

To detect the presence of parasites in drug-added test, all samples were extracted for their existing DNA and subjected to PCR. After the PCR reactions, the existing parasitic DNA in a sample would be revealed by agarose gel electrophoresis in the form of PCR product as a result of amplification activity of *Taq* DNA polymerase. From quinine, chloroquine and pyrimethamine susceptibility test, the *rap*-1 primers were able to amplify DNA template extracted from all samples except those extracted from the samples treated with drugs at MIC concentrations and above (Figure 6.4a, 6.6a and 6.7a)

However, the *rap*-1 primers failed to amplify PCR product from the parasites treated with mefloquine at lower than MIC concentration ($2x10^{-7}$ M). Although, some parasites survived in the presence of $7x10^{-8}$ and 10^{-7} M of mefloquine, detected by microscopy examination, the *rap*-1 primers was incapable of producing any PCR product using samples treated with both concentration of mefloquine, together with samples treated with MIC concentration and above (Figure 6.5a).

On the other hand, the *ssrRNA* primes can produce PCR products from all samples treated with drugs at all concentration (Figure 6.4b, 6.5b, 6.6b, 6.7b). To clarify the PCR experiment, the negative and positive control had been included into all experiment. All negative control samples, gave no PCR product while all positive control samples gave the single band of PCR products clearly seen in agarose gel analysis.

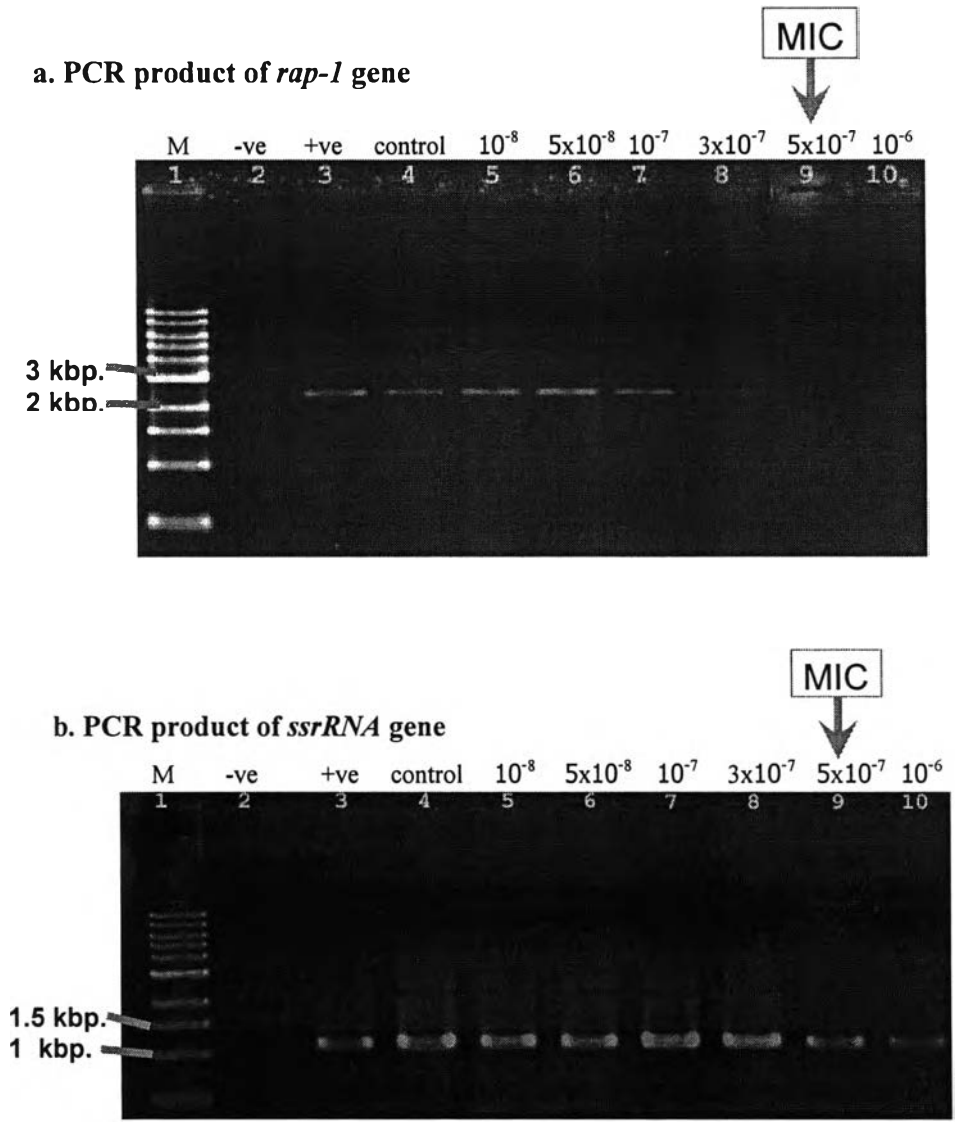

Figure 6.4 *rap-1* PCR products (a) and *ssrRNA* PCR products (b), which are amplified using DNA sample from *P. falciparum* T9/94RC17 treated with various concentrations of quinine, are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (using microscopic technique). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

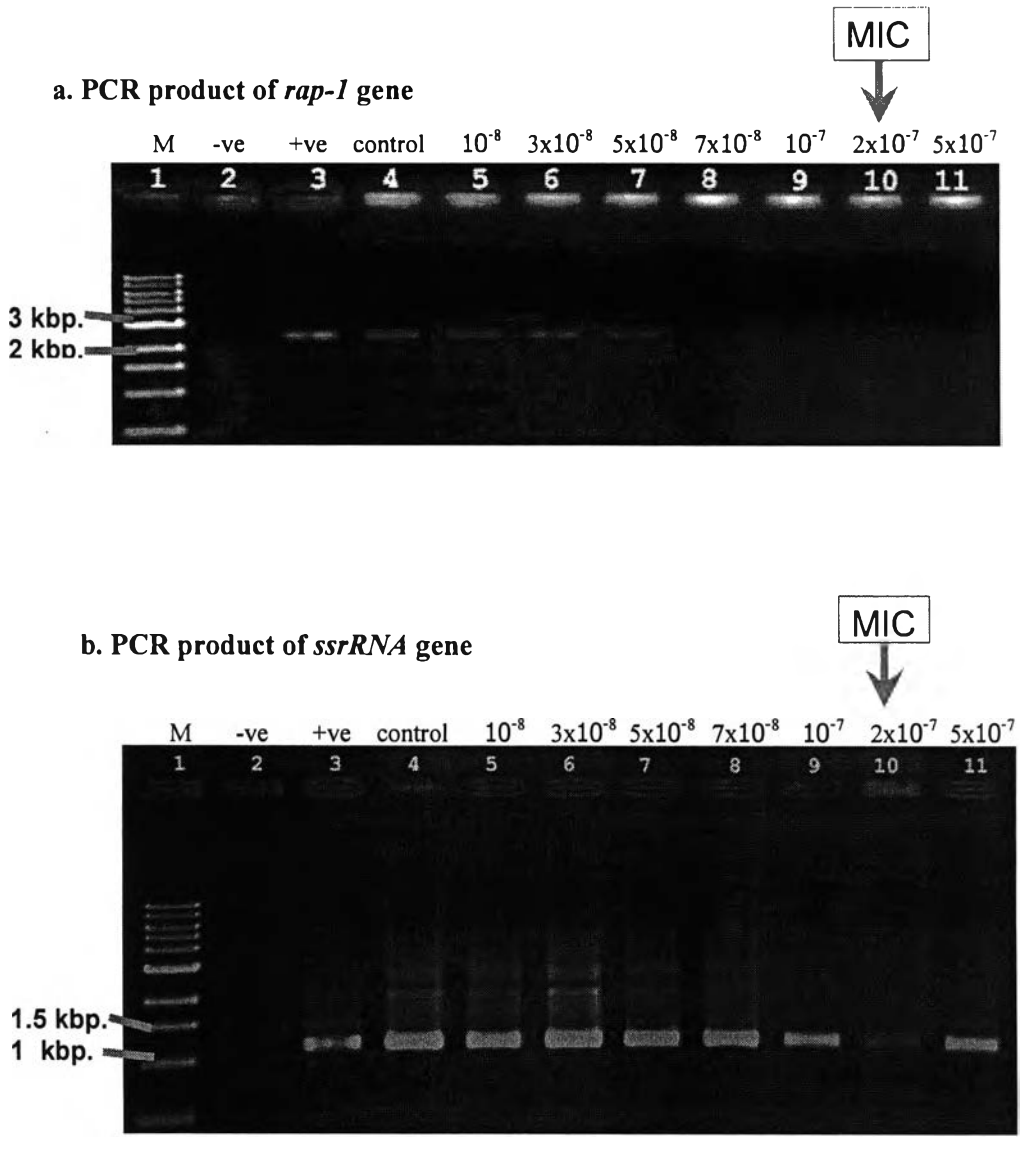

Figure 6.5 *rap-1* PCR products (a) and *ssrRNA* PCR products (b), which are amplified using DNA sample from *P. falciparum* T9/94RC17 treated with various concentrations of mefloquine, are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (using microscopic technique). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

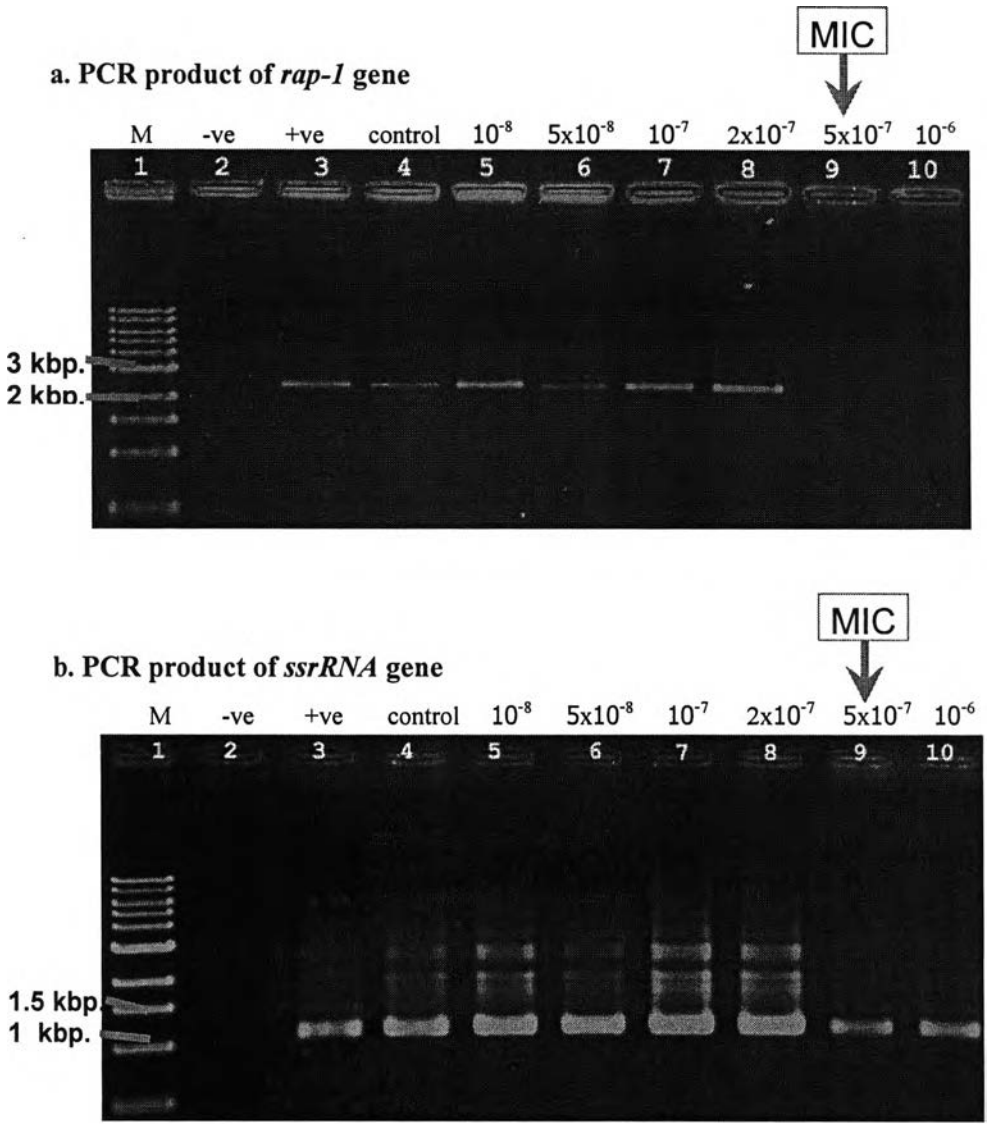

Figure 6.6 *rap-1* PCR products (a) and *ssrRNA* PCR products (b), which are amplified using DNA sample from *P. falciparum* T9/94RC17 treated with various concentrations of chloroquine, are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (using microscopic technique). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

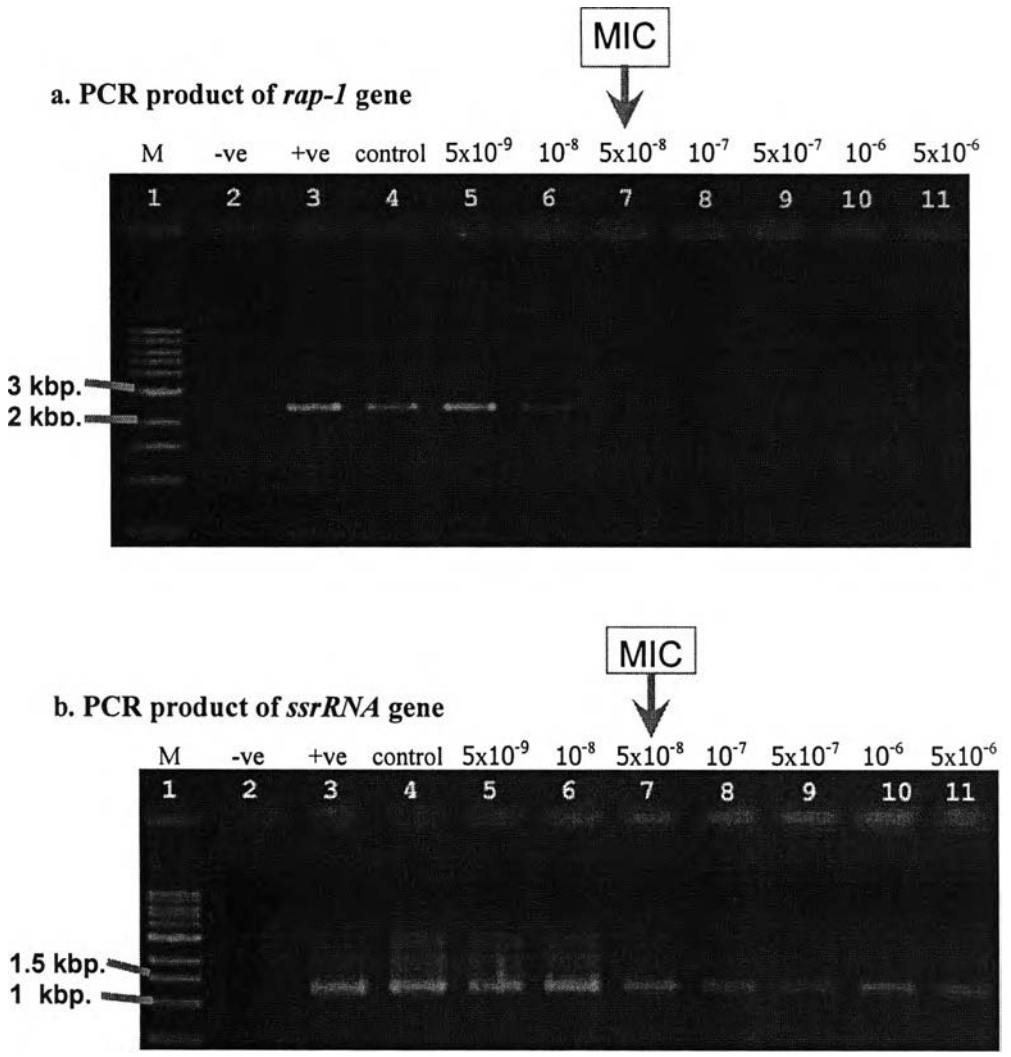

Figure 6.7 *rap-1* PCR products (a) and *ssrRNA* PCR products (b), which are amplified using DNA sample from *P. falciparum* T9/94RC17 treated with various concentrations of pyrimethamine, are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (using microscopic technique). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

6.2.3 RT-PCR Technique

The RT-PCR technique was also explored to determine the MIC value of malaria parasite compared to microscopy technique. Other two sets of drug susceptibility samples were subjected to RNA extraction and converted to cDNA by reverse transcriptase. The cDNA were used as DNA templates in PCR reaction with *rap*-1 and *ssrRNA* primers. From quinine, chloroquine and pyrimethamine susceptibility test, PCR products were detected, with *rap*-1 primers, in samples from parasites treated with all drug concentrations. However, the intensity of PCR product bands markedly decreased when the parasites were treated with drugs at MIC concentration compared to those treated with lower concentration (Figure 6.8a, 6.10a, 6.11a).

The result from mefloquine-treated parasites was different from other drugs. Although, parasitic RNA from the samples treated with the MIC concentration of mefloquine ($2x10^{-7}$ M) can not be detected with RT-PCR technique using *rap*-1 primers, samples treated with mefloquine at higher concentration ($5x10^{-7}$ M) gave very low amount of PCR products, seen as a very faint band (Figure 6.9a).

Again, the *ssrRNA* primers were able to amplified PCR products in samples treated with all drugs at all concentrations as detected by agarose gel electrophoresis (Figure 6.8b, 6.9b, 6.10b, 6.11b).

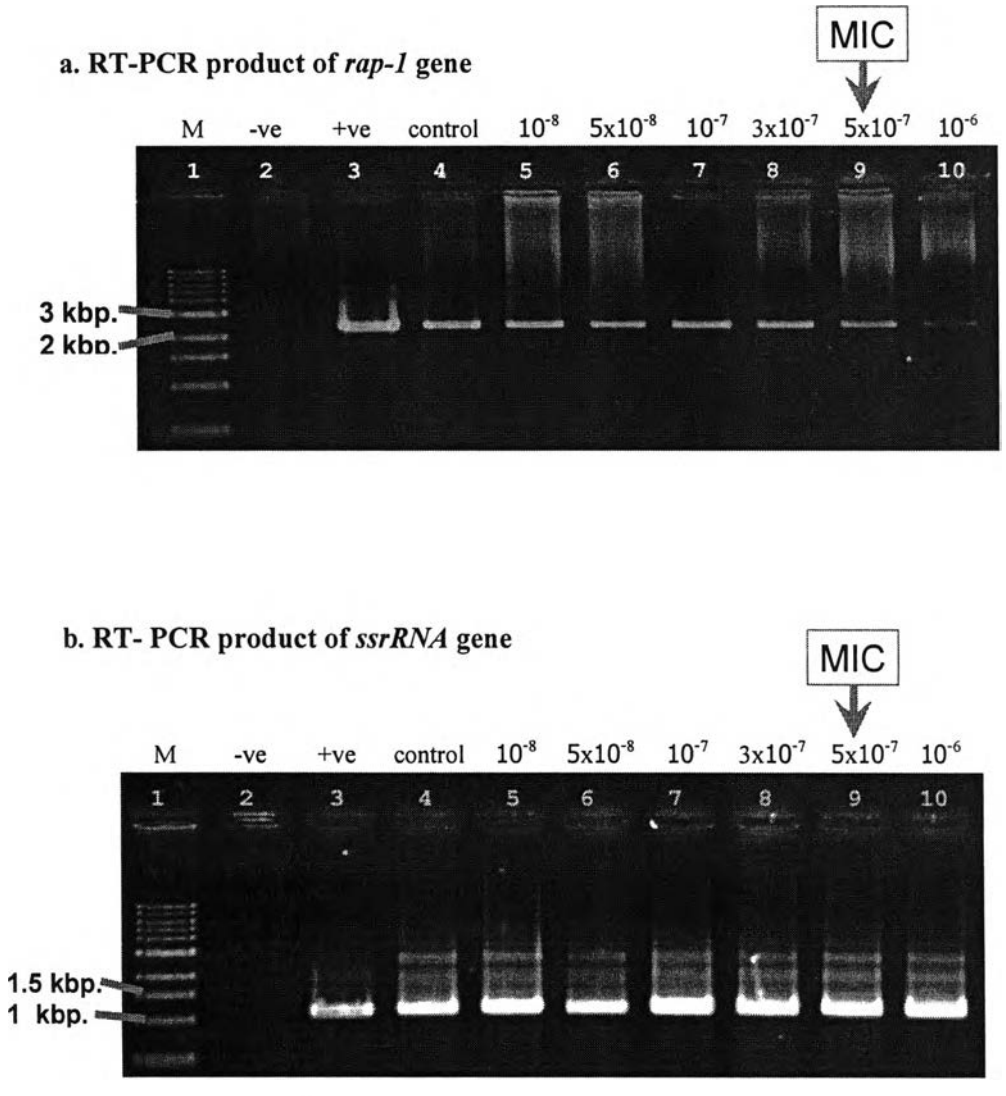

Figure 6.8 *rap-1* RT-PCR products (a) and *ssrRNA* RT-PCR products (b) which are amplified using DNA sample from *P. falciparum* T9/94RC17 treated with various concentrations of quinine, are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (using microscopic technique). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

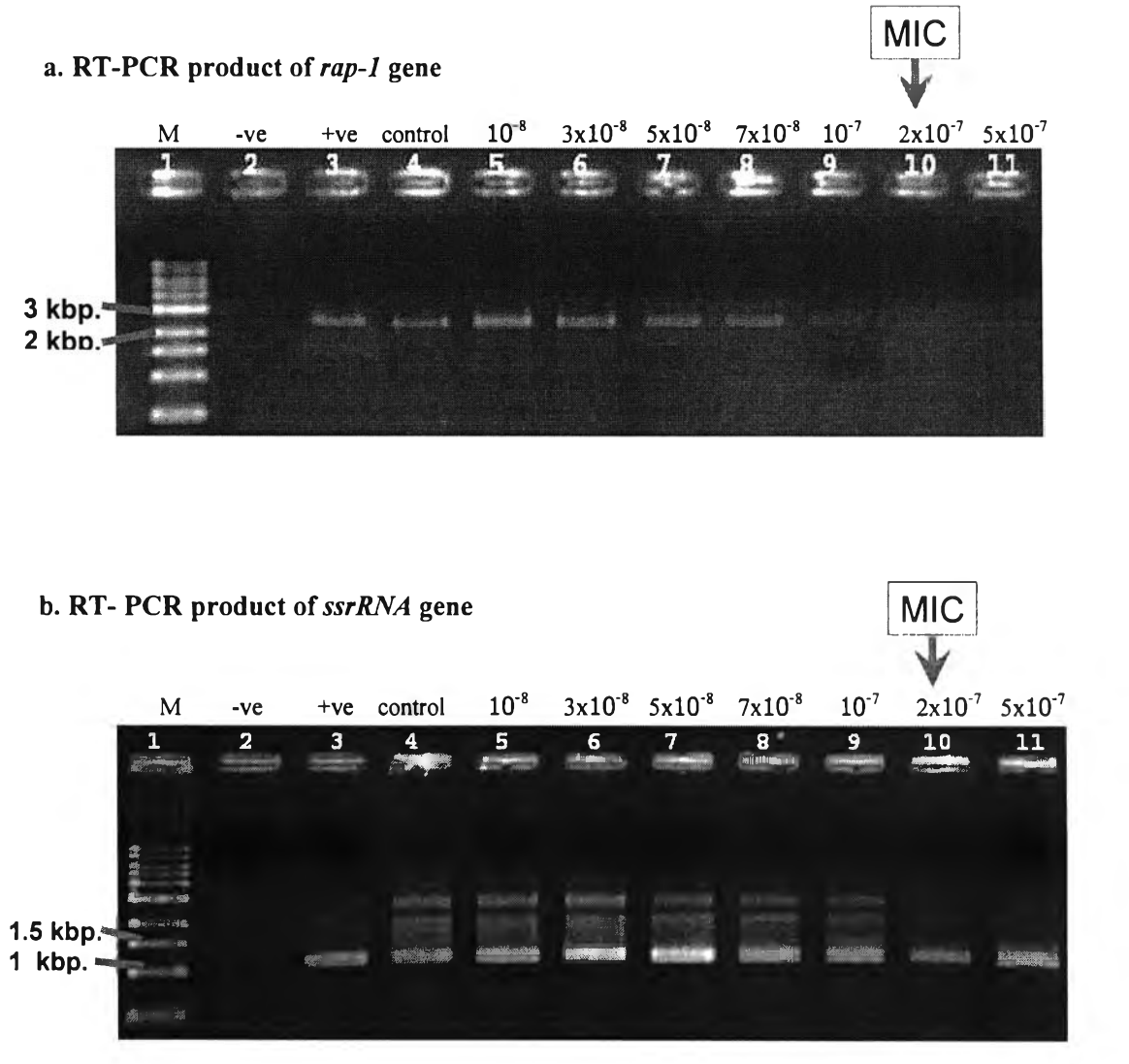

Figure 6.9 *rap-1* RT-PCR products (a) and *ssrRNA* RT-PCR products (b), which are amplified using DNA sample from *P. falciparum* T9/94RC17 treated with various concentrations of mefloquine, are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (using microscopic technique). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

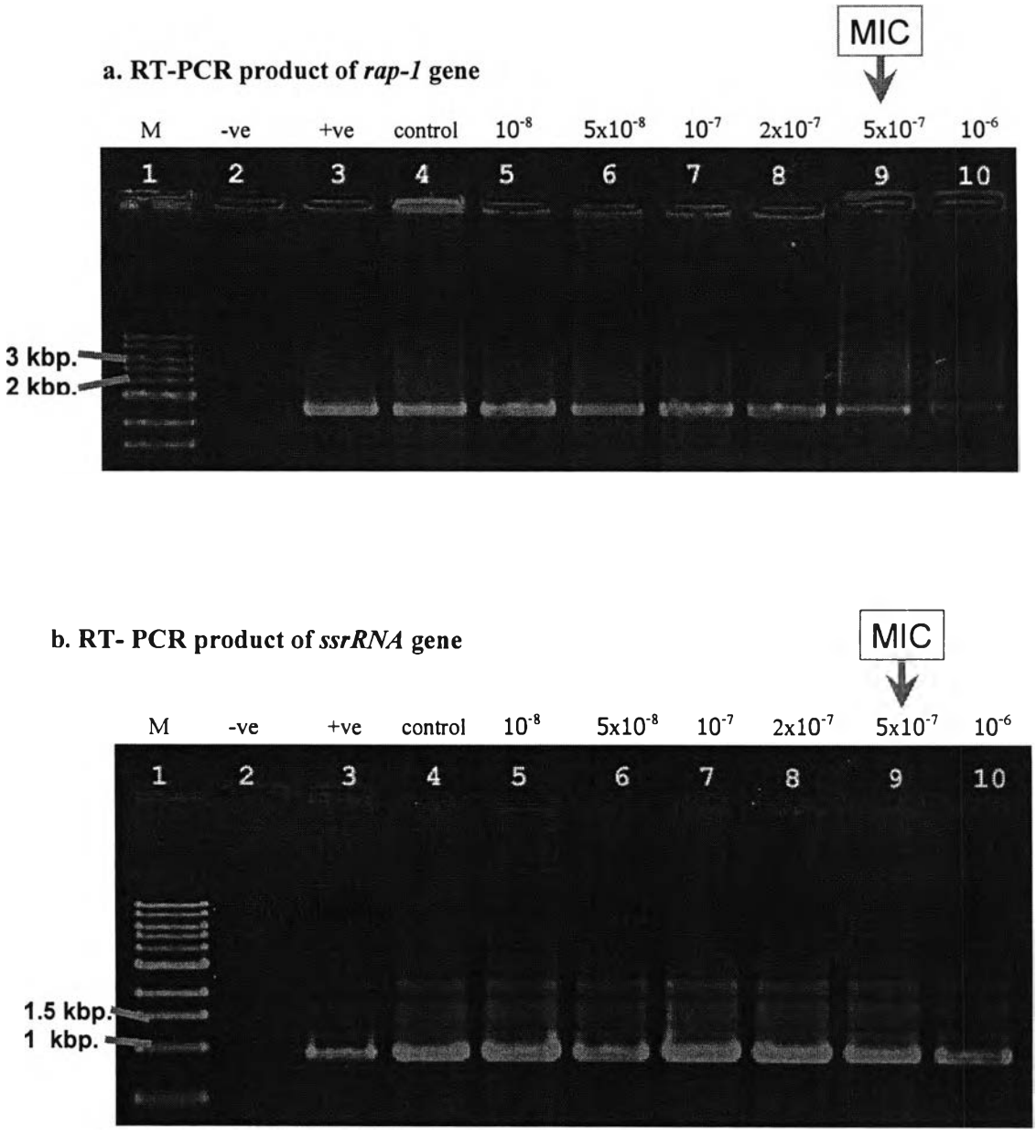

Figure 6.10 *rap-1* RT-PCR products (a) and *ssrRNA* RT-PCR products (b), which are amplified using DNA sample from *P. falciparum* T9/94RC17 treated with various concentrations of chloroquine, are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (using microscopic technique). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

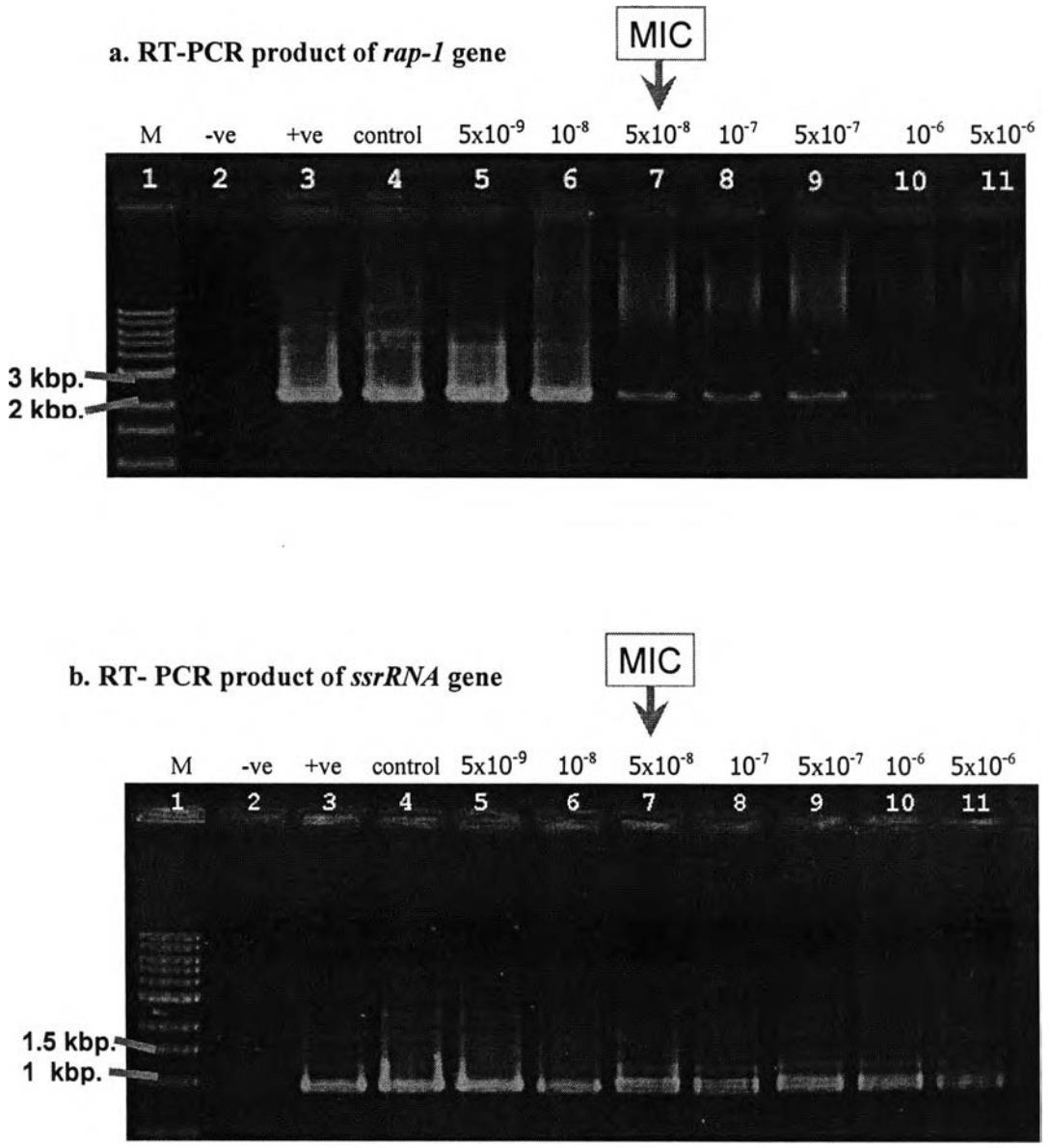

Figure 6.11 *rap-1* RT-PCR products (a) and *ssrRNA* RT-PCR products (b), which are amplified using DNA sample from *P. falciparum* T9/94RC17 treated with various concentrations of pyrimethamine, are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (using microscopic technique). Figure [M = 1 kb ladder, -ve = negative control, +ve = positive control (*P. falciparum* DNA), number on each lane represent the drug concentration in Molar]

6.3 Discussion and Conclusion

According to the microscopic results, from quinine and chloroquine susceptibility tests (Figure 6.2A and 6.3A), both drugs showed similar effect against *P. falciparum* T9/94RC17. The microscopy determination revealed that the parasite not only had the same MIC value ($5x10^{-7}$ M), but also showed noticeable effect when the culture media contained at least 10^{-7} M of drug, about 5 times less than the MIC value. The similarity outcome of quinine and chloroquine susceptibility tests suggested that both drugs may have related mode of action. For example, as a quinine derivative, chloroquine may interfere with haemoglobin degradation by complexing with hematin and kill the parasite (Carlton *et al.*, 2001). However, from the fact that a quinine sensitive parasite may be chloroquine resistance, more studies are needed before précised conclusion can be made.

However, from mefloquine susceptibility test (Figure 6.2B), the parasitized erythrocytes were gradually decreased, from $3x10^{-8}$ M to MIC concentration ($2x10^{-7}$ M), correlated with the drug concentration. Abnormal parasites became noticeable at $3x10^{-8}$ M (nearly 7 times less than the MIC value). This result suggested that, although, mefloquine is also the derivative of quinine, the mode of action may be different. Previous study also showed that mefloquine is active against chloroquine-resistant strains of parasites, but resistance can develop rapidly to each of this drugs (Dorsey *et al.*, 2001). The experiment supports the idea that mode of action of mefloquine may differ from that of chloroquine and quinine.

From pyrimethamine susceptibility test (Figure 6.3B), pyrimethamine at 10^{-8} M had no significant effect on the parasites, except slightly decreased of the parasitemia. At the MIC level ($5x10^{-8}$ M), however, the parasitemia dropped markedly (5 times more drug). Pyrimethamine which inhibits the folate biosynthetic pathway essential for the production of nucleotide for DNA synthesis (antifolate drug) may be the reason for the different of MIC value and the visual effect of parasites (Hyde, 2002). Accordingly, activity of antifolate drug is exerted at all growing stages of the asexual erythrocytic cycle (Olliaro, 2001) thus pyrimethamine may inhibit parasite growing and reproducing, caused the parasite death..

Using *rap*-1 primers to detect the presence of DNA from quinine, chloroquine and pyrimethamine susceptibility tests , no PCR product was amplified from samples treated with each drugs at MIC levels ($5x10^{-7}$, $5x10^{-7}$ and $5x10^{-8}$ M, respectively) and

above (Figure 6.4a, 6.6a and 6.7a). In other word, the MIC values obtained from microscopy and PCR are comparable.

The parasites treated with $7x10^{-8}$ M mefloquine or above failed to amplify the PCR product (Figure6.5a). That is the MIC value obtained from PCR technique differs from the MIC determined from microscopy. The results suggested that the drug may leads to the reduction of parasite or the degradation of parasitic genome below the sensitivity limitation of the *rap-1* primers, before the drug concentration reached microscopic MIC value.

The trial of RT-PCR technique showed that the sensitivity of RT-PCR was higher than that of PCR. This may be because, when the parasites died, both parasitic DNA and RNA were degraded. More *rap-1* mRNA molecules had more chance to survive degradation than those single copy *rap*-1 genes on the genome. Those survived mRNA can act as RNA template for cDNA conversion. Therefore, to measure the living parasite in drug susceptibility test, the PCR of a single copy gene, for example *rap*-1 gene, is more appropriate to get comparable result to those microscopy examinations.

In all drugs susceptibility test, the considerably bands of *ssrRNA* RT-PCR and PCR product could be observed at every concentration of all drugs (Figure 6.8b, 6.9b, 6.10b and 6.11b). This gene is not appropriate for measuring the living parasites because it is too sensitive to detect the survived parasite, but it should be used in diagnostic purposed.

In conclusion, according to the drug susceptibility results by microscopic examination, the MIC values of *P. falciparum* T9/94RC17 against quinine, mefloquine, chloroquine and pyrimethamine are $5x\ 10^{-7}$, $2x10^{-7}$, $5x\ 10^{-7}$, and $5x\ 10^{-8}$ M respectively. From the PCR and RT-PCR outcomes, the *ssrRNA* primers could not be used for evaluating the parasite during drug susceptibility test because these primers are too sensitive to measure definite living parasite as well as the RT-PCR technique of *rap-1* primers. Consequently, this study reveals that the PCR of *rap-1* primers could be used for detecting the presence of survived parasite during the drug susceptibility test.

Techniques	Quinine (M)									
	M	-ve	+ve	control	10^{-8}	$5x10^{-8}$	10^{-7}	$3x10^{-7}$	$5x10^{-7}$	10^{-6}
PCR										
• *rap-1* gene	+	+	+	+	+	+	+	+	-	-
• *ssrRNA* gene	+	+	+	+	+	+	+	+	+	+
RT- PCR										
• *rap-1* gene		+	+	+	+	+	+	+	+	+
• *ssrRNA* gene		+	+	+	+	+	+	+	+	+

Table 6.2 The amplification of *rap-1* and *ssrRNA* gene from quinine susceptibility test (+ and - show the presence and absence of the PCR products analyzed with agarose gel electrophoresis)

Techniques	Mefloquine (M)										
	M	-ve	+ve	control	10^{-8}	$3x10^{-8}$	$5x10^{-8}$	$7x10^{-8}$	10^{-7}	$2x10^{-7}$	$5x10^{-7}$
PCR											
• *rap-1* gene	+	+	+	+	+	+	+	-	-	-	-
• *ssrRNA* gene	+	+	+	+	+	+	+	+	+	+	+
RT- PCR											
• *rap-1* gene	+	+	+	+	+	+	+	+	+	-	+
• *ssrRNA* gene	+	+	+	+	+	+	+	+	+	+	+

Table 6.3 The amplification of *rap-1* and *ssrRNA* gene from mefloquine susceptibility test (+ and - show the presence and absence of the PCR products analyzed with agarose gel electrophoresis)

Techniques	Chloroquine (M)									
	M	-ve	+ve	control	10^{-8}	$5x10^{-8}$	10^{-7}	$2x10^{-7}$	$5x10^{-7}$	10^{-6}
PCR										
• *rap-1* gene	+	+	+	+	+	+	+	+	-	-
• *ssrRNA* gene	+	+	+	+	+	+	+	+	+	+
RT- PCR										
• *rap-1* gene		+	+	+	+	+	+	+	+	+
• *ssrRNA* gene		+	+	+	+	+	+	+	+	+

Table 6.4 The amplification of *rap-1* and *ssrRNA* gene from chloroquine susceptibility test (+ and - show the presence and absence of the PCR products analyzed with agarose gel electrophoresis)

Techniques	Pyrimethamine (M)										
	M	-ve	+ve	control	$5x10^{-9}$	10^{-8}	$5x10^{-8}$	10^{-7}	$5x10^{-7}$	10^{-6}	$5x10^{-6}$
PCR											
• *rap-1* gene	+	+	+	+	+	+	-	-	-	-	-
• *ssrRNA* gene	+	+	+	+	+	+	+	+	+	+	+
RT- PCR											
• *rap-1* gene	+	+	+	+	+	+	+	+	+	+	+
• *ssrRNA* gene	+	+	+	+	+	+	+	+	+	+	+

Table 6.5 The amplification of *rap-1* and *ssrRNA* gene from pyrimethamine susceptibility test (+ and - show the presence and absence of the PCR products analyzed with agarose gel electrophoresis)