CHAPTER VII

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

7.1 Introduction

Using PCR and RT-PCR technique to determine the presence of living parasite in the drug susceptibility test, several steps were included: 1) drug susceptibility test, 2) DNA and RNA extraction, 3) PCR or RT-PCR and 4) analysis the PCR and RT-PCR product with agarose gel electrophoresis. For DNA and RNA purification step, several solutions have to be prepared and lengthy procedures must strictly follow. These steps are not only time consuming, but also increases the risk of external-DNA contamination as well as the losing of parasitic DNA. Although, commercial kit had been used to purify RNA molecules which were more complicate to perform than DNA molecule purification, the procedures were difficult to achieve when dealing with a large number of samples. The commercial kit (Perfect RNA Eukaryotic Mini kit, Eppendorf) was also considered as an expensive way to purify RNA in laboratory (about 200 baths per preparation).

To solve these problems, an alternative method for DNA amplification, called direct PCR has been introduced (Mercier *et al,* 1990). A target DNA can be directly amplify from a blood sample without DNA extraction. The direct PCR has several advantages 1) save time, 2) decrease external-DNA contamination, 3) preserve DNA template number and 4) diminish purification cost. In this study, the RT-PCR was also adapted to direct RT-PCR in order to obtain advantages mentioned above.

In this chapter, two duplications of drug susceptibility test, from previous chapter, were subjected to direct PCR and RT-PCR. According to the data obtained from the previous chapter, the *ssrRNA* primers were not suitable for detecting the presence of parasites during drug susceptibility test because these primers are too sensitive. For this reason, only the *rap-1* primers were used for direct PCR and RT-PCR in this chapter.

7.2 Results

Although the PCR reactions can amplify DNA template in positive control samples in all experiments, they failed to amplify DNA template in drug-treated samples from all tested drugs at all concentrations (Figure 7.1a, 7.2a, 7.3a and 7.4a).

In direct RT-PCR experiment, DNA and RNA templates in all drug treated samples were amplified and gave out PCR products. Quinine and chloroquine treated samples produced PCR products at all drug concentrations (Figure 7.1b and 7.3b). In comparison, the intensity of PCR product bands showed no significant variation among different concentration in each drug. On the contrary, mefloquine and pyrimethamine treated samples gave PCR products with different intensity bands at different drug concentrations. The intensity of PCR product bands from mefloquine treated samples markedly decreased at $7x10^{-8}$ and 10^{-7} M and very faint bands were observed at $2x10^{-7}$ (MIC level) and $5x10^{-7}$ M (Figure 7.2b). Similarly, the intensity of PCR product bands from pyrimethamine treated samples dropped significantly at 10^{-7} and $5x10^{-7}$ M and very faint bands were noticeable at 10^{-6} and $5x10^{-6}$ M (Figure 7.4b).

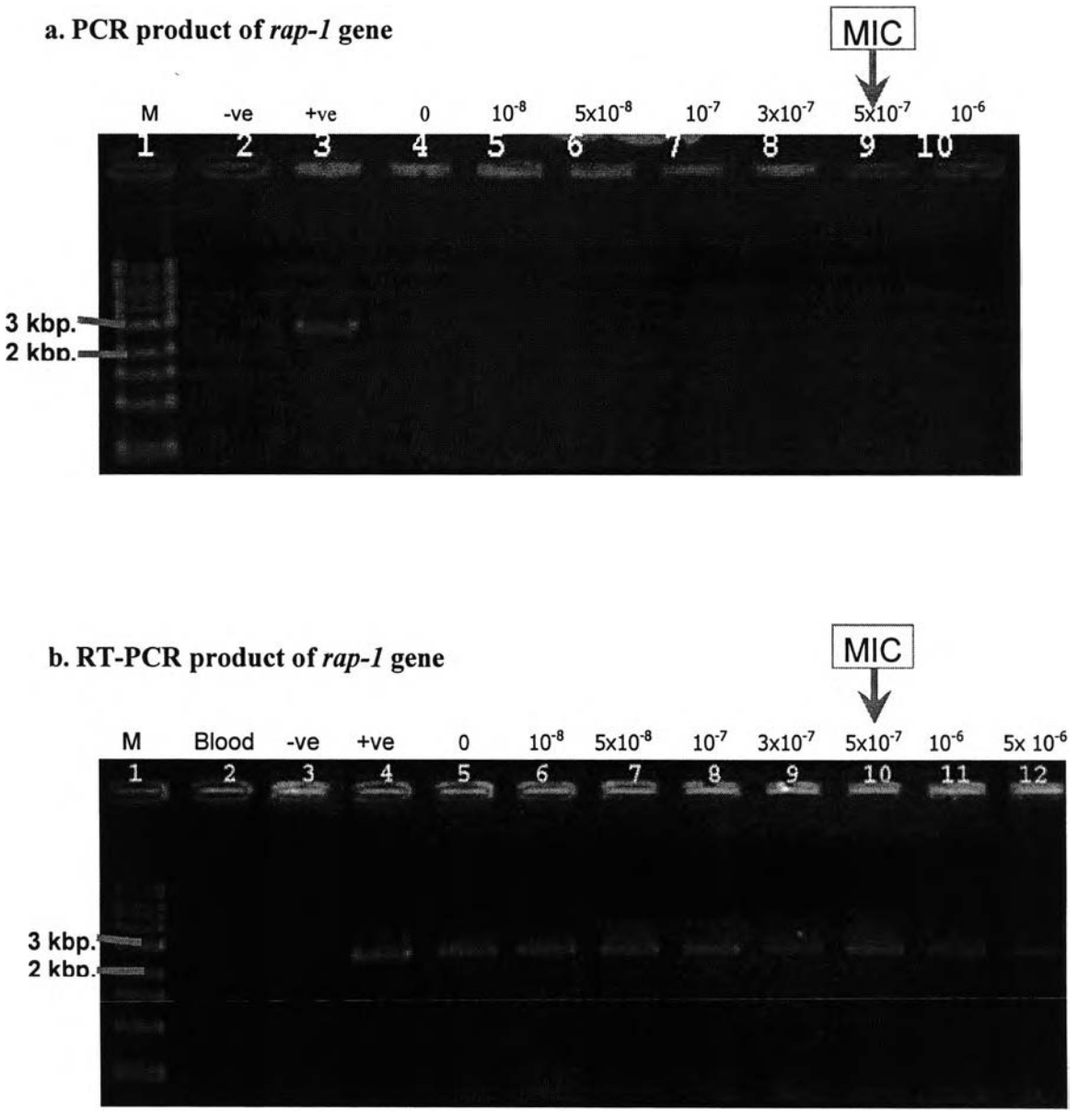

Figure 7.1 *rap-1*PCR products (a) and RT-PCR products (b), which are amplified using red blood cell culture of *P. falciparum* T9/94RC17 treated with various concentrations of quinine (the number above each lane), are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (M = 1 kb ladder, Blood = human red blood cell, -ve = negative control and +ve = positive control).

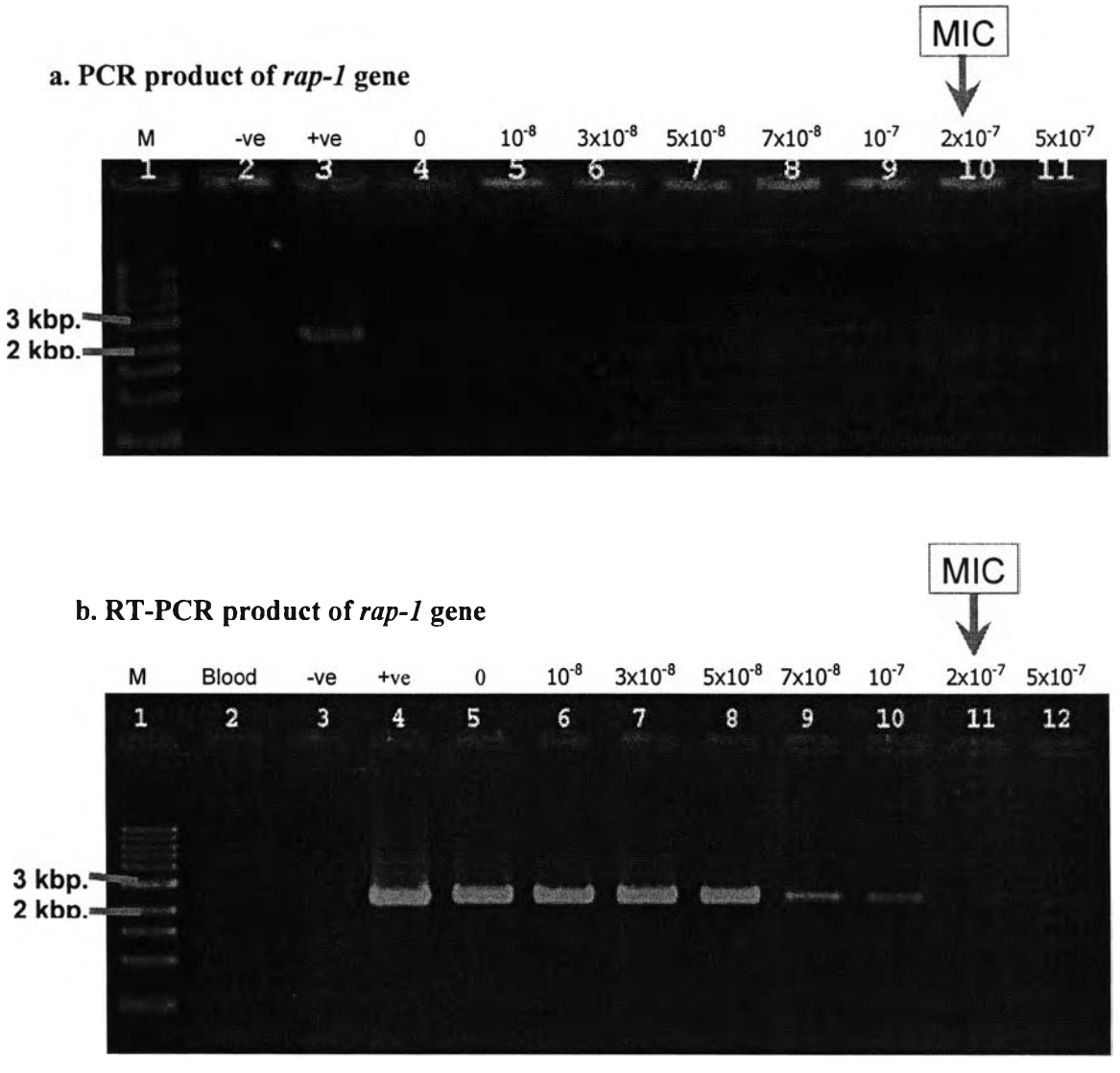

Figure 7.2 *rap-1*PCR products (a) and RT-PCR products (b), which are amplified using red blood cell culture of *P. falciparum* T9/94RC17 treated with various concentrations of mefloquine (the number above each lane), are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (M = 1 kb ladder, Blood = human red blood cell, -ve = negative control and +ve = positive control).

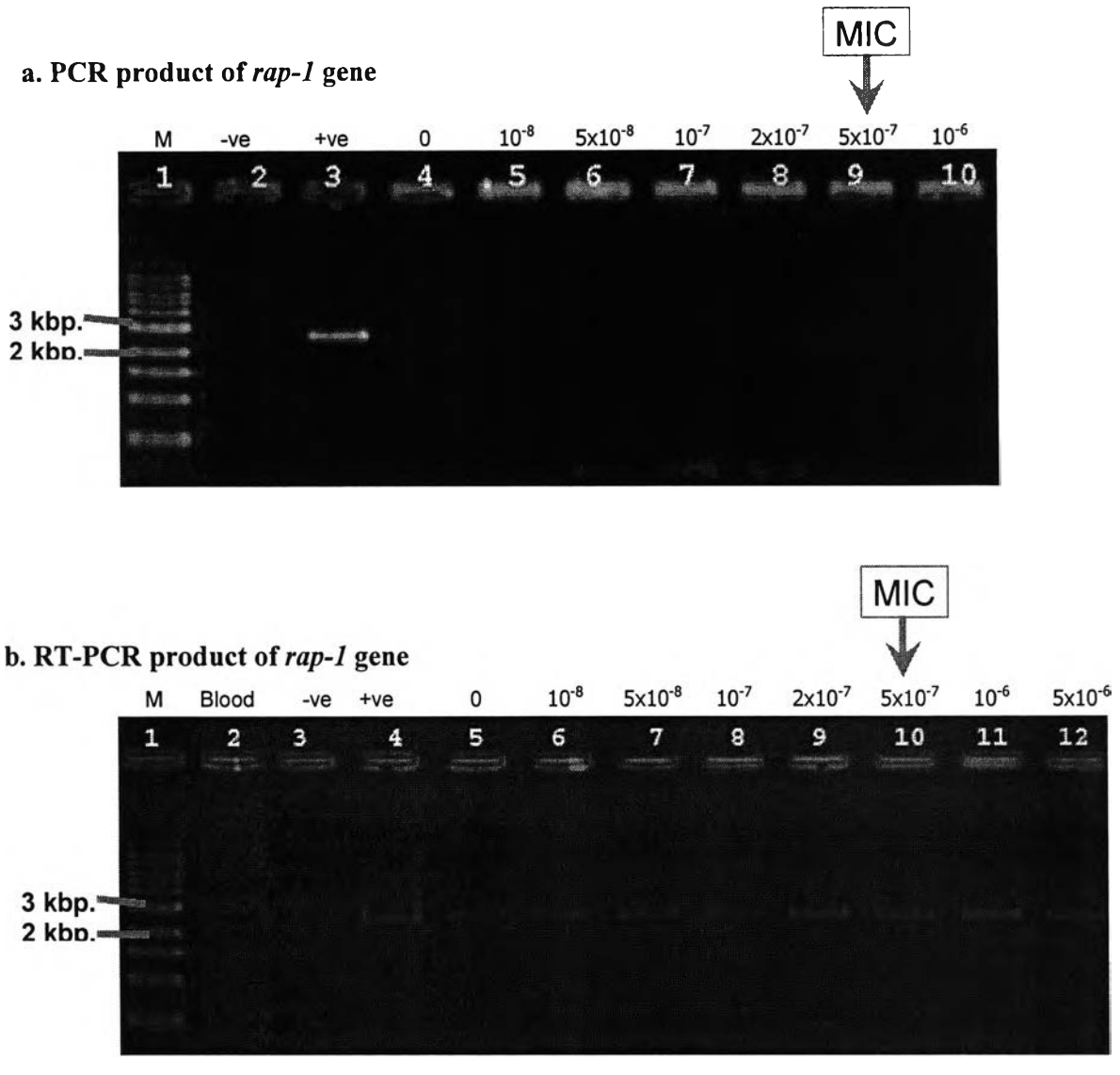

Figure 7.3 *rap-1* PCR products (a) and RT-PCR products (b), which are amplified using red blood cell culture of *P. falciparum* T9/94RC17 treated with various concentrations of chloroquine (the number above each lane), are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (M = 1 kb ladder, Blood = human red blood cell, -ve = negative control and +ve = positive control).

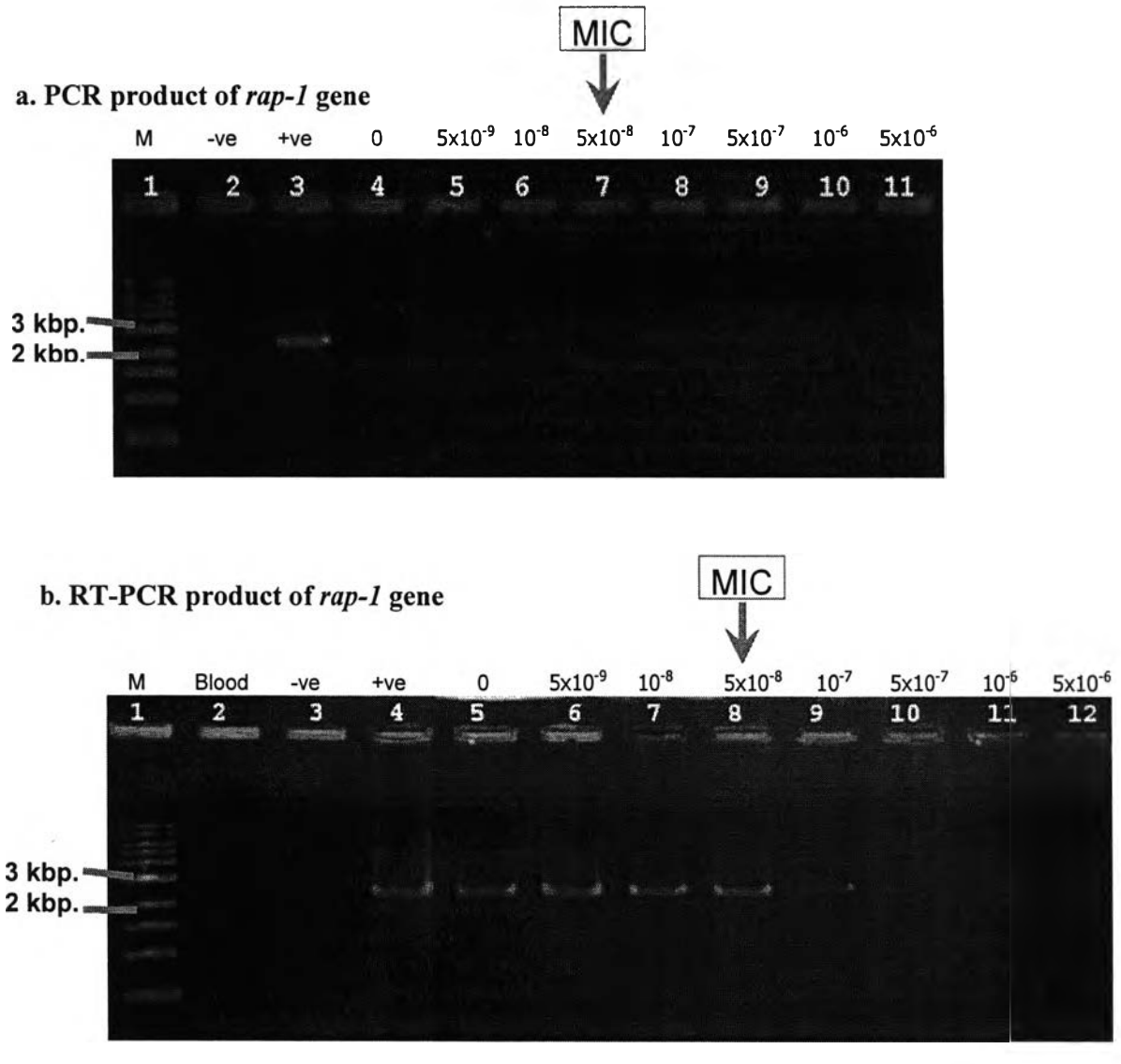

Figure 7.4 *rap-1*PCR products (a) and RT-PCR products (b), which are amplified using red blood cell culture of *P. falciparum* T9/94RC17 treated with various concentrations of pyrimethamine (the number above each lane), are subjected to agarose gel electrophoresis. The arrows show the concentration of drugs at the MIC level (M = 1 kb ladder, Blood = human red blood cell, -ve = negative control and +ve = positive control).

7.3 Discussion and Conclusion

To measure the living parasites from drug susceptibility test by detecting their DNA and RNA without purification, four antimalarial drug susceptibility tests were subjected to direct PCR and RT-PCR technique.

In all drug susceptibility tests, the direct PCR product of *rap-1* gene was not observed in all drug concentrations, even in control group (no drug) (Figure 7.1a, 7.2a, 7.3a and 7.4a). Although, many researchers were successful in applying this technique toward gene amplification, several failures have also been reported by several groups of researchers. Successful attempts included the amplification of a short fragment, 224 base pairs (bp) of the Insulin Growth Factor-1 gene (McCusker *et al,* 1992), to a long fragment, 900 bp from frozen whole blood samples (Mercier *et al,* 1990).

From some published works, few factors were proposed as the causes of direct PCR failure in malaria experiment and some approaches were developed to overcome these barriers. As proposed by Machado and his group, the causes of direct PCR failure included 1) high amount of PCR inhibitor in blood, 2) long target gene and 3) low amount of DNA template (Machado, 1998). These factors may be considered as the causes of failure in this experiment. The attempt to inactivate the PCR inhibitors in the blood with a preheating step (McCusker *et al,* 1992) may not be able to solve such problem. The size of *rap*-1, 2439 base pairs (Ridley *et al,* 1990), may also lead to the failure of PCR reaction. Using smaller target fragment in *rap*-1 gene may help improving the amplification reaction. To increase the efficiency of amplification at low DNA template condition, nested PCR or double PCR technique should be applied. The direct double PCR technique had been successfully applied to detect *Plasmodium falciparum* in the Solomon (Wataya *et al,* 1993). A glass fiber membrane GFM has also been reported to improve the direct PCR outcome.

In direct RT-PCR from all drugs susceptibility tests, RT-PCR product of *rap-1* was observed at all concentrations of four drugs. These results suggested that the sensitivity of direct RT-PCR was higher than that of direct PCR. This may be because the number of *rap-1* mRNA which are converted into DNA templates, together with contaminated genomic DNA molecule, for the direct RT-PCR reaction were greater than that of direct PCR which used genomic DNA containing single copy *rap-1* gene as the DNA template. The result of direct RT-PCR is resemble to the result from RT-PCR (chapter 6) which was proposed that the RT-PCR technique is not appropriate for

measuring the living parasites because it is too sensitive to detect the actual living parasites. Accordingly, the direct RT-PCR technique may not suitable for assessment the survived parasites during drug susceptibility test.

In conclusion, from the direct RT-PCR results, the RT-PCR technique should not be used for evaluating the parasite during drug susceptibility test because it is too sensitive than microscopic examination.