CHAPTER IIX

THE USE OF PCR TECHNIQUE WITH OTHER ISOLATES OF MALARIAL PARASITE IN DRUG SUSCEPTIBILITY TEST

8.1 Introduction

According to the results of previous chapters, the most appropriate technique and primers for parasites detection in drug susceptibility test is PCR technique using *rap-1* primers However, the malaria parasites have been known to express genotypic diversity among their wild population and results in a unique character in each isolates. The newly developed technique which was tested against a parasitic clone, T9/94RC17 may not flexible enough to detect other isolates of malaria parasites. To ensure its applicability, the developed PCR technique must be tested against other malaria isolates.

In this chapter, the PCR technique was used to reveal the presence of three *Plasmodium falciparum* isolates collected from different geographical areas. Those isolates included; isolate MH20 collected from Mae Hong Son, isolate TD12 collected from Trad and isolate K160 collected from Kanchanaburi. Those isolates were subjected to antimalarial drug susceptibility tests (quinine, mefloquine, chloroquine and pyrimethamine) in four replicate. Two replicates were examined for MIC value using microscopy. Other two replicates were extracted for their DNA using phenol-chloroform extraction (Snounou, 1994). The obtained DNA was used for PCR amplification of *rap*-1 gene. Finally, the MIC values from two techniques were compared.

8.2 Results

The results, which performed in two replicates were similar, thus, only one replicate was reported.

8.2.1 Microscopic examination

From the quinine, chloroquine and pyrimethamine susceptibility test of *P. falciparum* MH20, TD12 and K160, high parasitized red blood cells with normal ring forms, early schizonts and trophozoites were observed in the negative control group and in drug-treated samples at lower concentration than the MIC level (data not shown). All parasite isolates were killed at the MIC level and higher. The MIC values of *P. falciparum* MH20 against quinine and chloroquine were lower than the MIC value of TD12 and K160 (Table 8.1a, 8.2a and 8.2b).

In the mefloquine susceptibility test of all isolates, normal ring forms, trophozoites and mature schizonts were observed in the negative control group and at the 10^{-8} M of mefloquine. The isolate MH20 was gradually decreased at higher mefloquine concentrations. The small amount of living parasite and parasite debris were observed at $5x10^{-8}$, $7x10^{-8}$ and 10^{-7} M of mefloquine (data not shown). The MIC values of isolates MH20, TD12 and K160 against mefloquine are $2x10^{-7}$, $3x10^{-8}$, and $2x10^{-7}$ M respectively (Table 8.1b).

a)

Quinine(M) / Isolates	0	10^{-8}	$5x\ 10^{-8}$	10^{-7}	$3x\ 10^{-7}$	$5x\ 10^{-7}$	10^{-6}
MH20	+	+	+	+	-	-	-
TD12	+	+	+	+	+	-	-
K160	+	+	+	+	+	-	-

b)

Mefloquine(M) / Isolates	0	10^{-8}	$3x\ 10^{-8}$	$5x\ 10^{-8}$	$7x\ 10^{-8}$	10^{-7}	$2x\ 10^{-7}$	$5x\ 10^{-7}$
MH20	+	+	+	(+)	(+)	(+)	-	-
TD12	+	+	-	-	-	-	-	-
K160	+	+	+	+	+	+	-	-

Table 8.1 The results of *P. falciparum* MH20, TD12 and K160 against a) quinine and b) mefloquine using microscopic detection. [+ = living parasites found, (+) = rarely found the living parasites, - = no living parasite is found, the gray-highlight cells in the table represent the MIC level of each isolate]

a)

Chloroquine(M) / Isolates	0	10^{-8}	$5x\ 10^{-8}$	10^{-7}	$2x\ 10^{-7}$	$5x\ 10^{-7}$	10^{-6}
MH20	+	+	+	+	-	-	-
TD12	+	+	+	+	+	-	-
K160	+	+	+	+	+	-	-

b)

Pyrimethamine(M) / Isolates	0	$5x\ 10^{-8}$	10^{-7}	10^{-6}	10^{-5}	$5x\ 10^{-5}$	10^{-4}	$2x\ 10^{-4}$
MH20	+	+	+	+	+	+	-	-
TD12	+	+	+	+	+	+	-	-
K160	+	+	+	+	+	+	-	-

Table 8.2 The results of *P. falciparum* MH20, TD12 and K160 against a) chloroquine and b) pyrimethamine using microscopic detection. [+ = living parasites found, (+) = rarely found the living parasites, - = no living parasite is found, the gray-highlight cells in the table represent the MIC level of each isolate]

8.2.2 PCR technique

In MH20, the MIC_p (The MIC level determined by PCR technique) against quinine and mefloquine were the same to MIC determined by microscopic examination (Figure 8.1a, 8.2a). The differences between MIC_p and MIC of this isolate were revealed by the detectable PCR products in the MH20 treated with chloroquine and pyrimethamine. The MIC_p was higher ($5x10^{-7}$ M) than the MIC against chloroquine ($2x10^{-7}$ M) while it was lower ($5x10^{-5}$ M) than the MIC against pyrimethamine (10^{-4} M) (Figure 8.3a, 8.4a).

The MIC_p (10^{-4} M) were equal to the MIC level in the mefloquine and chloroquine susceptibility test against TD12 (Figure 8.2b, 8.3b). On the contrary, the MIC_p level was higher than the MIC level in the test against quinine and pyrimethamine (Figure 8.1b and 8.4b). In K160 experiment, most of the MIC_p were the same as the MIC against all drug tested (Figure 8.2c, 8.3c, 8.4c), except against quinine. The MIC_p against quinine was 10^{-6} M while the MIC was equal to $5x10^{-7}$ M (Figure 8.1c).

8.3 Discussion and Conclusion

The amplification results showed that the *rap-1* primers can bind and specifically amplify the *rap-1* gene from wild isolates, even though; they were collected from different geographical areas of Thailand. The consistency of PCR product size also suggested that the *rap-1* gene is highly conserved in both sequence and size as previously reported (Howard and Peterson 1996). These results were confirmed by duplicate experiment and gave the same results which means that the experiment were repeatable.

Surprisingly, there was no tested isolate which gave the MIC_p at the same level as the MIC in all drug tests (Table 8.3). In any isolates, only two drug tests have MIC_p at the same level as MIC. For example, MIC_p obtained in quinine and mefloquine tests against MH20 were equal to MIC while K160 showed similar results in chloroquine and pyrimethamine.

Most of the MIC_p values were higher than the MIC which may cause by slightly higher sensitivity of PCR technique than that of microscopic technique. In microscopy technique, cell debris was considered as a dead cell, but its genome or the fragment contained *rap-1* gene may still intact. This genome of DNA fragment can be amplified by *rap-1* primers and leaded to higher sensitivity than microscopy

technique. Although, all of the MIC_p were not equal to the MIC of parasite isolates, they are very closed to the MIC enough to be used as the preliminary detection for MIC estimation with large sample size. More accurate MIC, then, can be measured later, if it is needed.

Isolate and MIC / Drugs	MH20		TD12		K160	
	MIC_p	MIC	MIC_p	MIC	MIC_p	MIC
quinine	$3x10^{-7}$	$3x10^{-7}$	10^{-6}	$5x10^{-7}$	10^{-6}	$5x10^{-7}$
mefloquine	$2x10^{-7}$	$2x10^{-7}$	$3x10^{-8}$	$3x10^{-8}$	$5x10^{-7}$	$2x10^{-7}$
chloroquine	$5x10^{-7}$	$2x10^{-7}$	$5x10^{-7}$	$5x10^{-7}$	$5x10^{-7}$	$5x10^{-7}$
pyrimethamine	$5x10^{-4}$	10^{-4}	$2x10^{-4}$	10^{-4}	10^{-4}	10^{-4}

Table 8.3 This table compares between MIC_p and MIC of each isolates against all tested drugs.

In conclusion, the PCR technique using the *rap-1* primers can be use to estimate the MIC of wild parasite isolates with large sample size. More efforts are needed to fine-tune the technique for practical used.

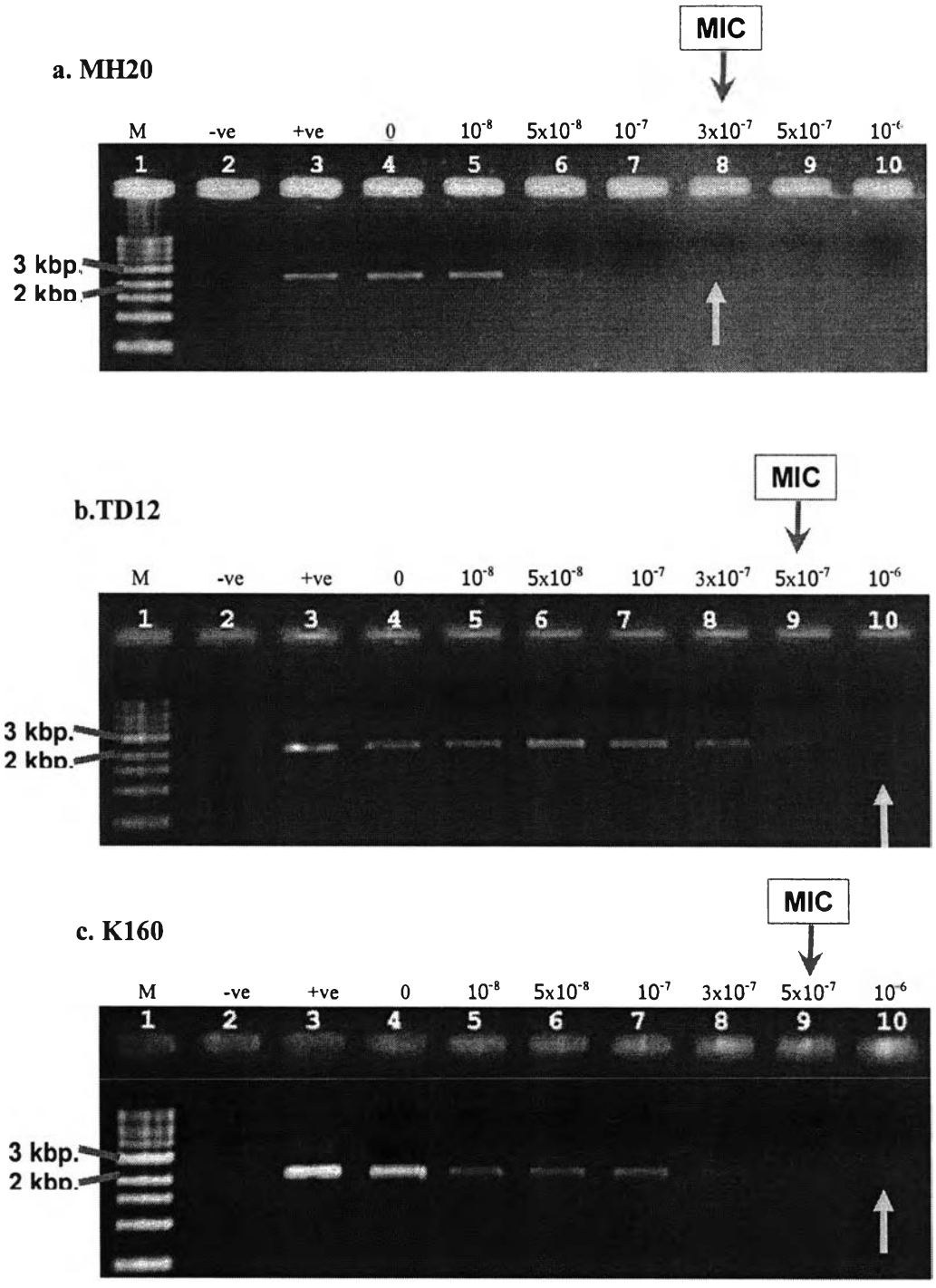

Figure 8.1 *rap-1* PCR products from *P. falciparum* isolate MH20 (a), TD12(b) and K160(c) against quinine (the numbers above each lane are the concentration of quinine) are subjected to agarose gel electrophoresis. The arrows show the MIC level (using microscopic technique) and the white arrows show the MIC_p. [M = 1 kb ladder, -ve = negative control and +ve = positive control (*P. falciparum* DNA)]

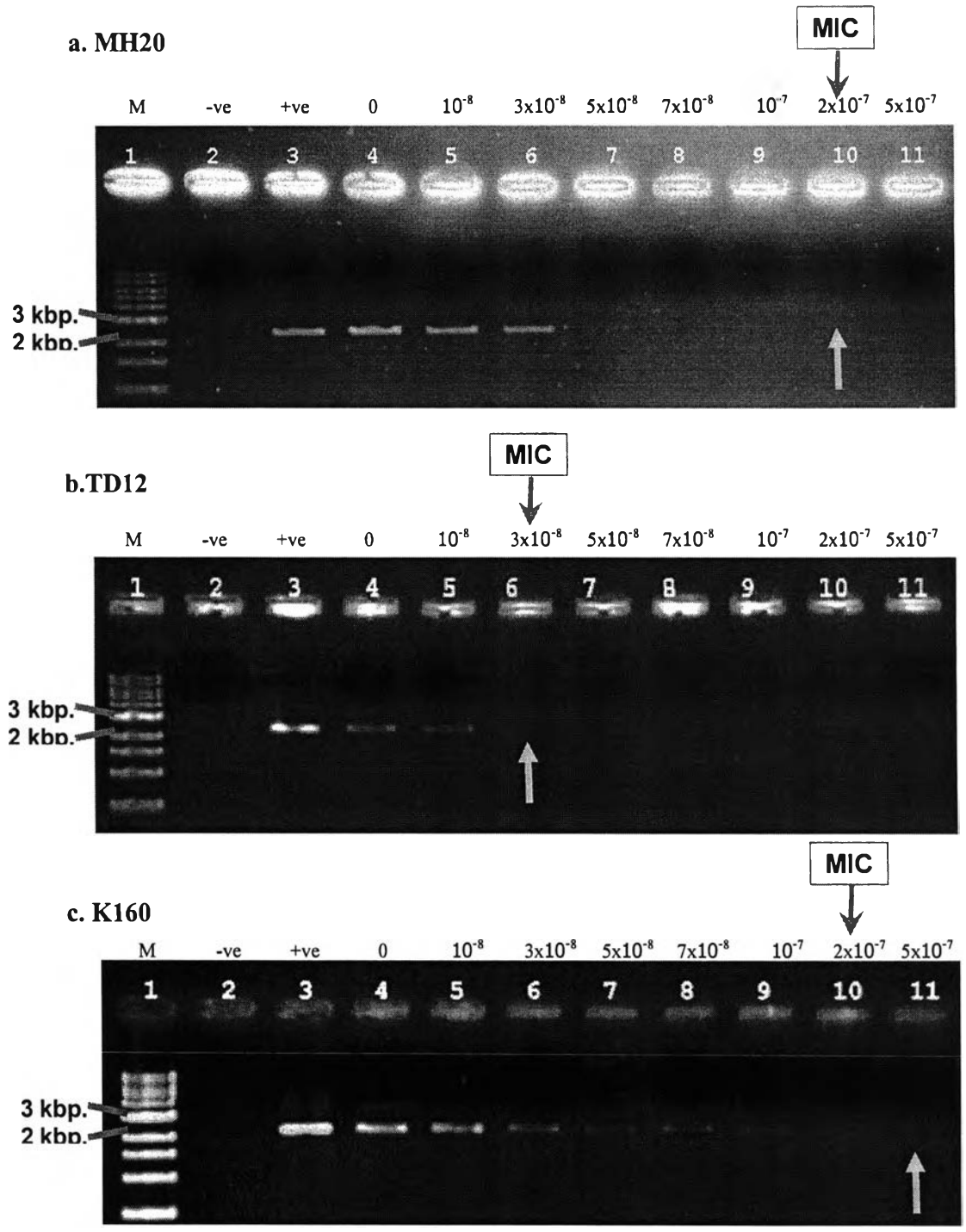

Figure 8.2 *rap-1* PCR products from *P. falciparum* isolate MH20 (a), TD12(b) and K160(c) against mefloquine (the numbers above each lane are the concentration of mefloquine) are subjected to agarose gel electrophoresis. The arrows show the MIC level (using microscopic technique) and the white arrows show the MIC_p. [M = 1 kb ladder, -ve = negative control and +ve = positive control (*P. falciparum* DNA)]

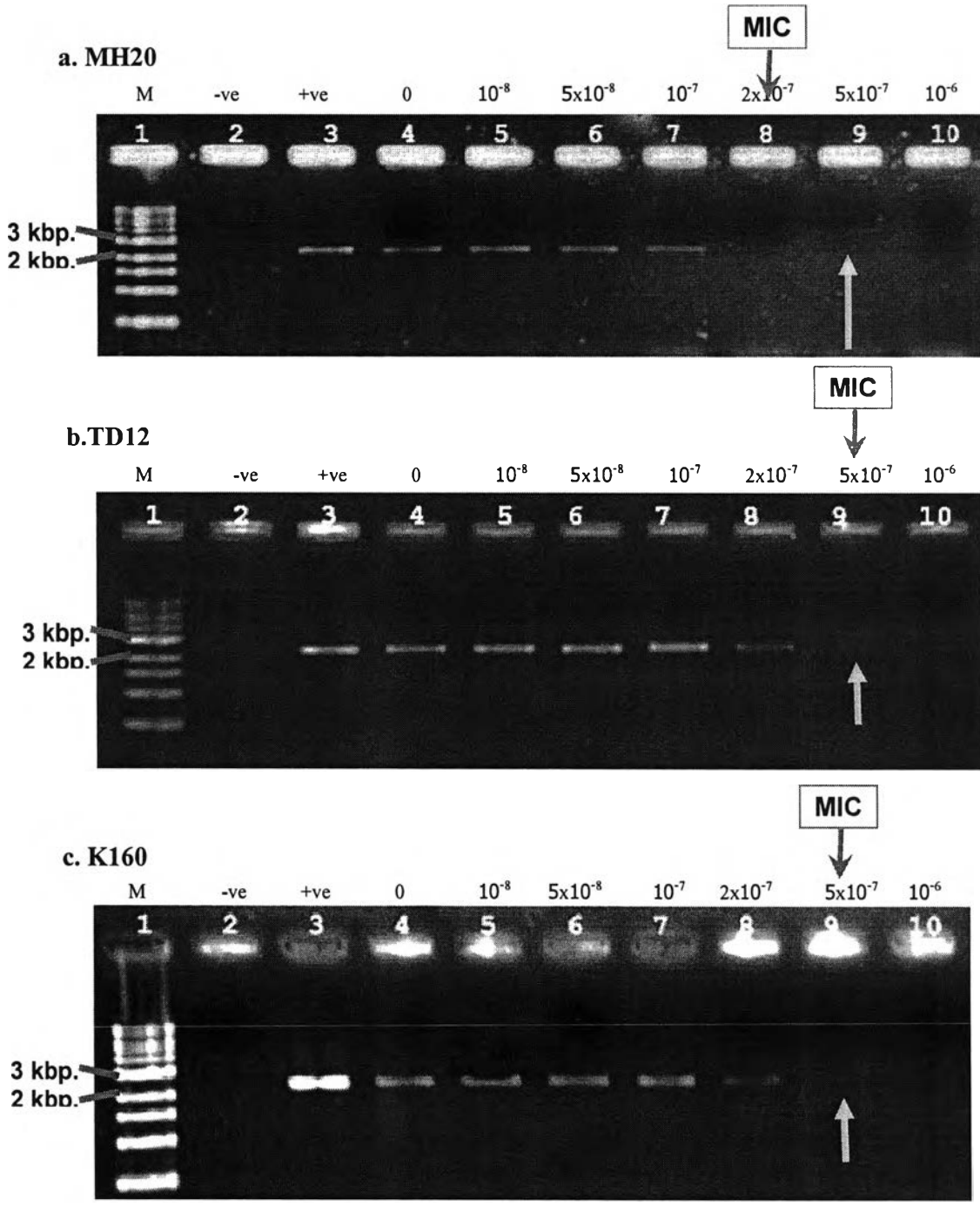

Figure 8.3 *rap-1* PCR products from *P. falciparum* isolate MH20 (a), TD12(b) and K160(c) against chloroquine (the numbers above each lane are the concentration of chloroquine) are subjected to agarose gel electrophoresis. The arrows show the MIC level (using microscopic technique) and the white arrows show the MIC_p. [M = 1 kb ladder, -ve = negative control and +ve = positive control (*P. falciparum* DNA)]

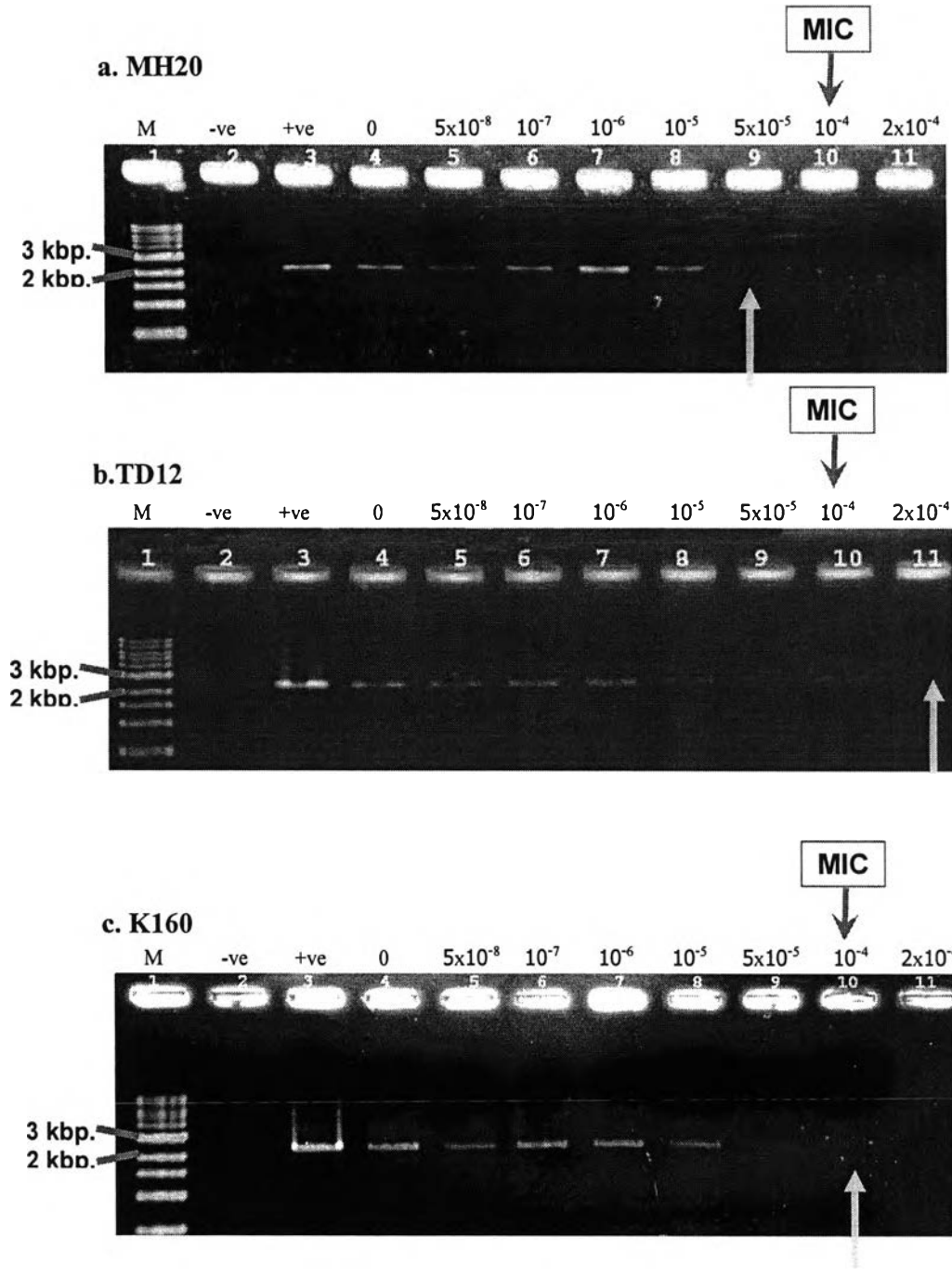

Figure 8.4 *rap-1* PCR products from *P. falciparum* isolate MH20 (a), TD12(b) and K160(c) against pyrimethamine (the numbers above each lane are the concentration of pyrimethamine) are subjected to agarose gel electrophoresis. The arrows show the MIC level (using microscopic technique) and the white arrows show the MIC_p. [M = 1 kb ladder, -ve = negative control and +ve = positive control (*P. falciparum* DNA)]