

CHAPTER V

RESULTS

1. *In vitro* drug susceptibility of *P. falciparum* isolates

A total of 50 isolates were tested for their susceptibilities to chloroquine (CQ), quinine (QN), mefloquine (MQ), and artesunate (ARS). Of these isolates, 19, 16 and 15 isolates were adapted during the periods 1988-1989, 1991-1993 and 2003, respectively. The levels of CQ, QN, and MQ susceptibility were determined using the criteria of Pickard and colleague, (Pickard *et al.*, 2003) to discriminate resistance and sensitive parasite isolates. For ARS however, there are no criteria to define ARS resistance.

1.1 CQ susceptibility: The CQ IC_{50} values of these isolates ranged from 13.8 to 174.2 nM. CQ susceptibility was categorised into three levels, *i.e.*, sensitive (S: $IC_{50} < 25$ nM), moderately resistant (MR; $25 < IC_{50} < 100$ nM), and highly resistant (HR: $IC_{50} \geq 100$ nM). Based on these criteria, 1 (2%), 29 (58%), and 20 (40%) isolates were categorised as S, MR, and HR, respectively. Of those isolates collected during 1988-1989, 12 (63%) and 7 (37%) isolates were categorised as MR and HR, respectively; the corresponding IC_{50} values for parasite isolates collected during 1991-1993 were 6 (37.5%) and 10 (62.5%), respectively. The parasite isolates collected during 2003; 1 (7%), 11 (73%), and 3 (20%) for S, MR, and HR, respectively (**Table 11**). The frequency distribution of CQ IC_{50} values for all isolates is shown in **Figure 15**. The distribution appears to be abnormally distributed, with the majority of isolates lying within the MR range and minority lying within the HR range. The mean (95%CI) of IC_{50} values for parasite isolates collected during 1988-1989, 1991-1993 and 2003 were, 91.1 (51.9-119.4), 110.6 (67.4-119.8) and 78.1 (46.3-96.1) nM, respectively (**Figure 16**). Mean CQ IC_{50} values of parasite isolates collected during 1991-1993 (the period of clinical use of MQ monotherapy) were significantly higher than those collected during 2003 (the period of clinical use combination of MQ plus ARS) ($p = 0.027$).

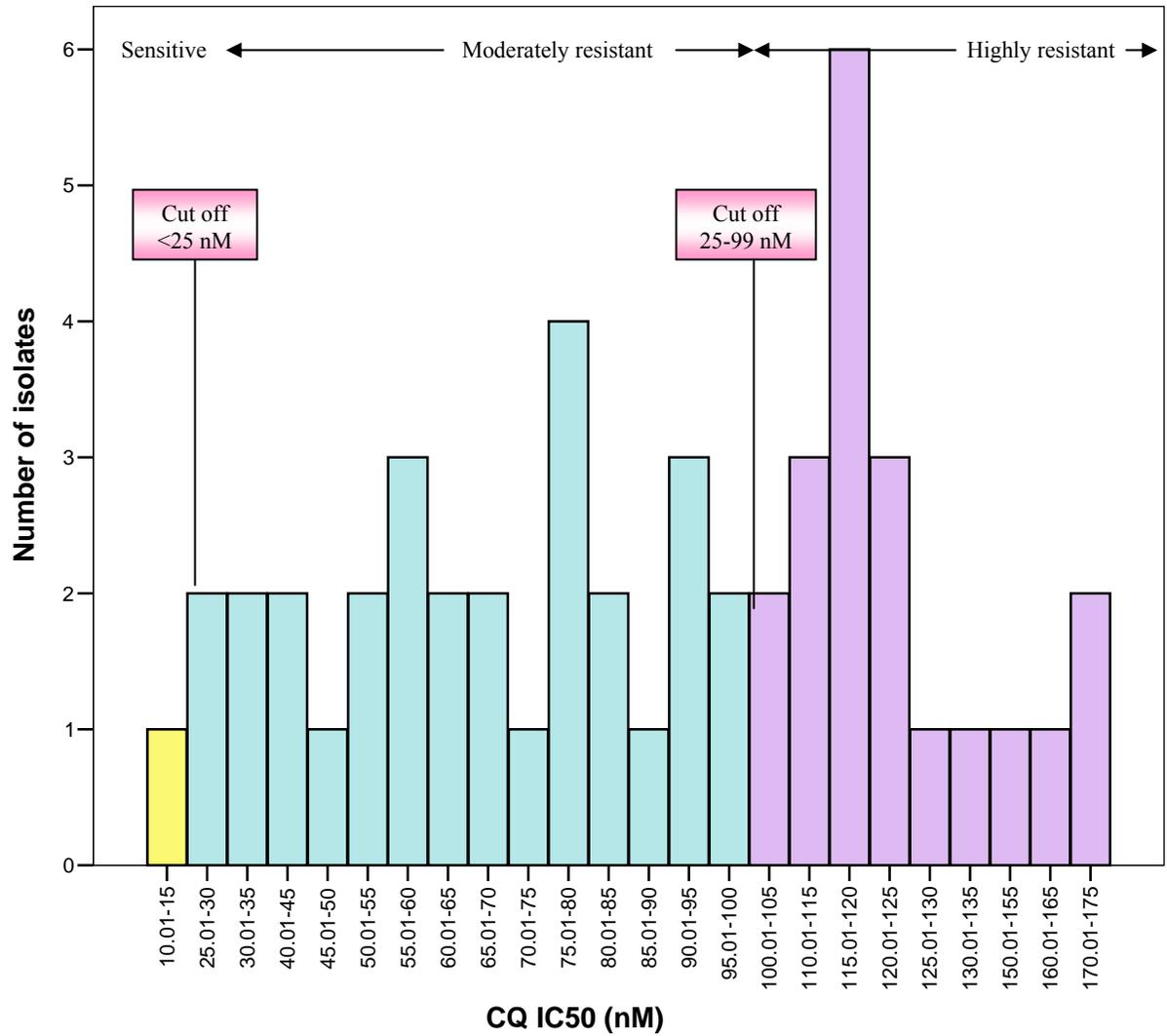


Figure 15 Distribution of parasite isolates according to their CQ IC₅₀. IC₅₀ values were categorised into 3 different levels, *i.e.*, sensitive (< 25 nM), moderately resistant (25 to 99 nM), and highly resistant (≥ 100 nM).

Table 11 Distribution of the level of CQ resistance in parasite isolates collected during different periods of time (1988-1989, 1991-1993, 2003); data are presented as number and percentage value in parenthesis.

Period of time (years)	Sensitive	Moderately resistant	Highly resistant
1988-1989	0	12 (63%)	7 (37%)
1991-1993	0	6 (38%)	10 (63%)
2003	1 (7%)	11 (73%)	3 (20%)

NS (No statistically significant difference among the these periods of time by Chi-square test)

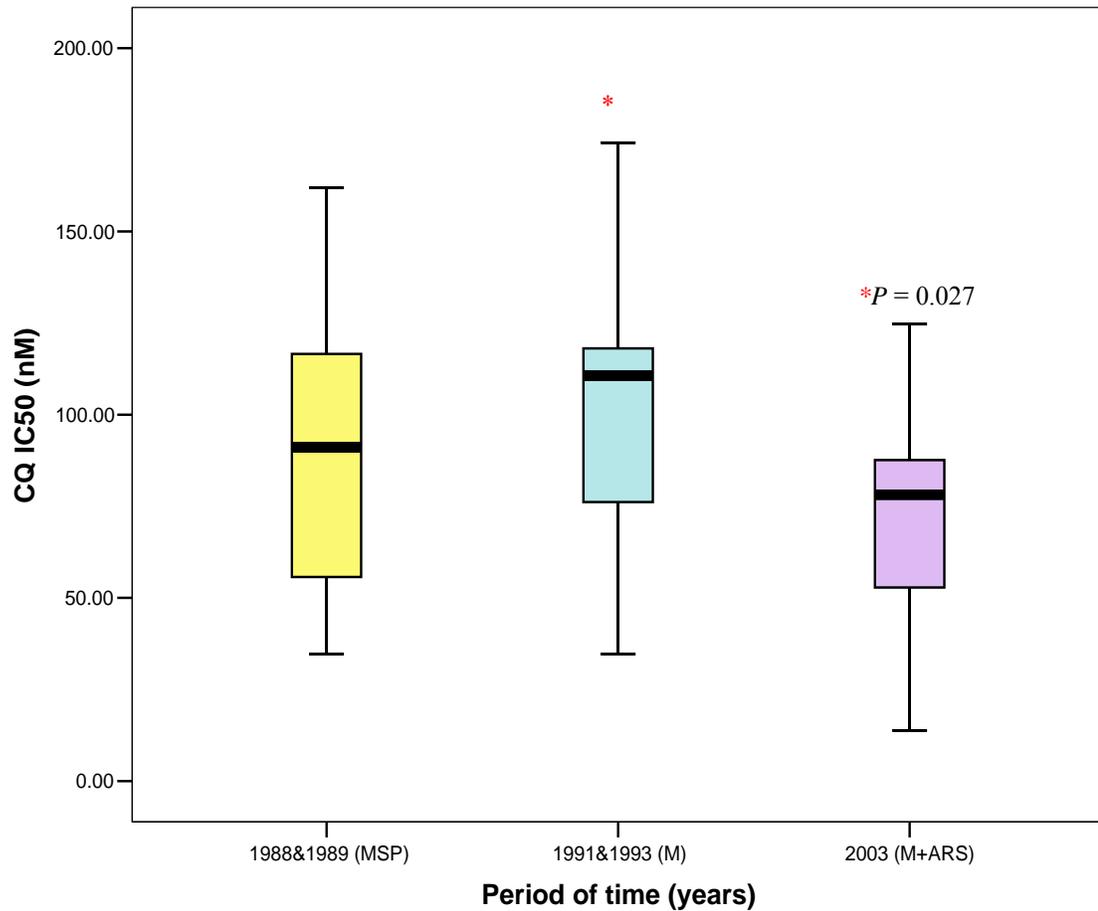


Figure 16 Box-plot of the CQ IC₅₀ values of *P. falciparum* isolates collected during different periods of time. The mean CQ IC₅₀ value of parasite isolates collected during 1991-1993 was significantly higher than those collected during 2003 (* $p = 0.027$, Mann-Whitney U test) (MSP = Mefloquine plus Sulfadoxine/Pyrimethamine; M = Mefloquine; M+ARS = Mefloquine plus Artesunate).

1.2 QN susceptibility: The IC_{50} values of QN for *P. falciparum* isolates ranged from 34.5 to 483.4 nM. Using Pickard's criteria (**Figure 17**), all 50 (100%) isolates were categorised as QN-sensitive since their IC_{50} values were less than 500 nM. The mean (95%CI) of IC_{50} values for parasite isolates collected during 1988-1989, 1991-1993 and 2003 were, 188.8 (99.6-231.2), 191.5 (66.45-281.9), and 129.48 (82.6-173.5), respectively. The mean QN IC_{50} values of isolates from different periods of time are displayed in **Figure 18**. There was no significant difference in IC_{50} values among the isolates adapted from different periods of time.

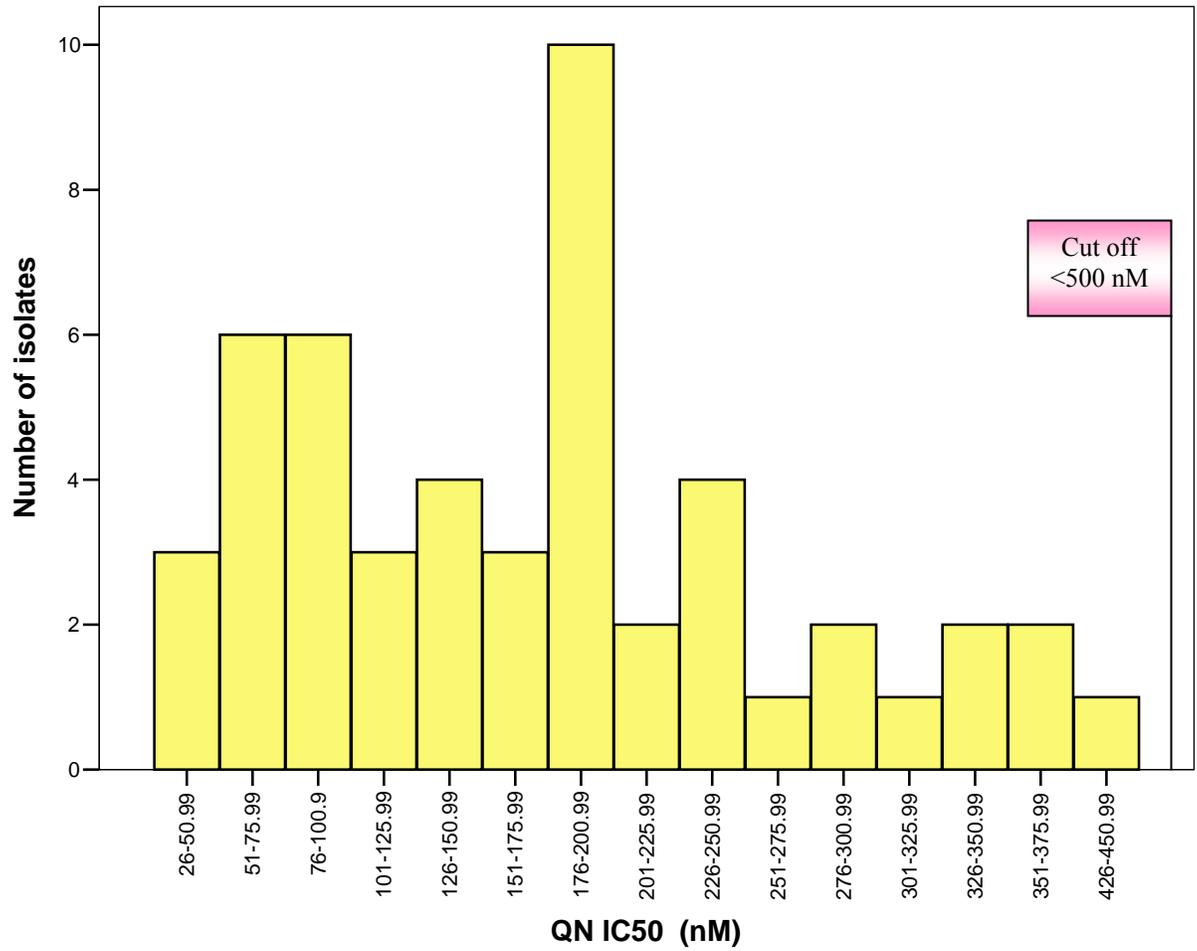


Figure 17 Distribution of the isolates according to the IC₅₀ values of QN isolates. Parasite isolates were categorised as QN sensitive and QN resistant if the IC₅₀ values were less than 500 and equal or more than 500 nM, respectively.

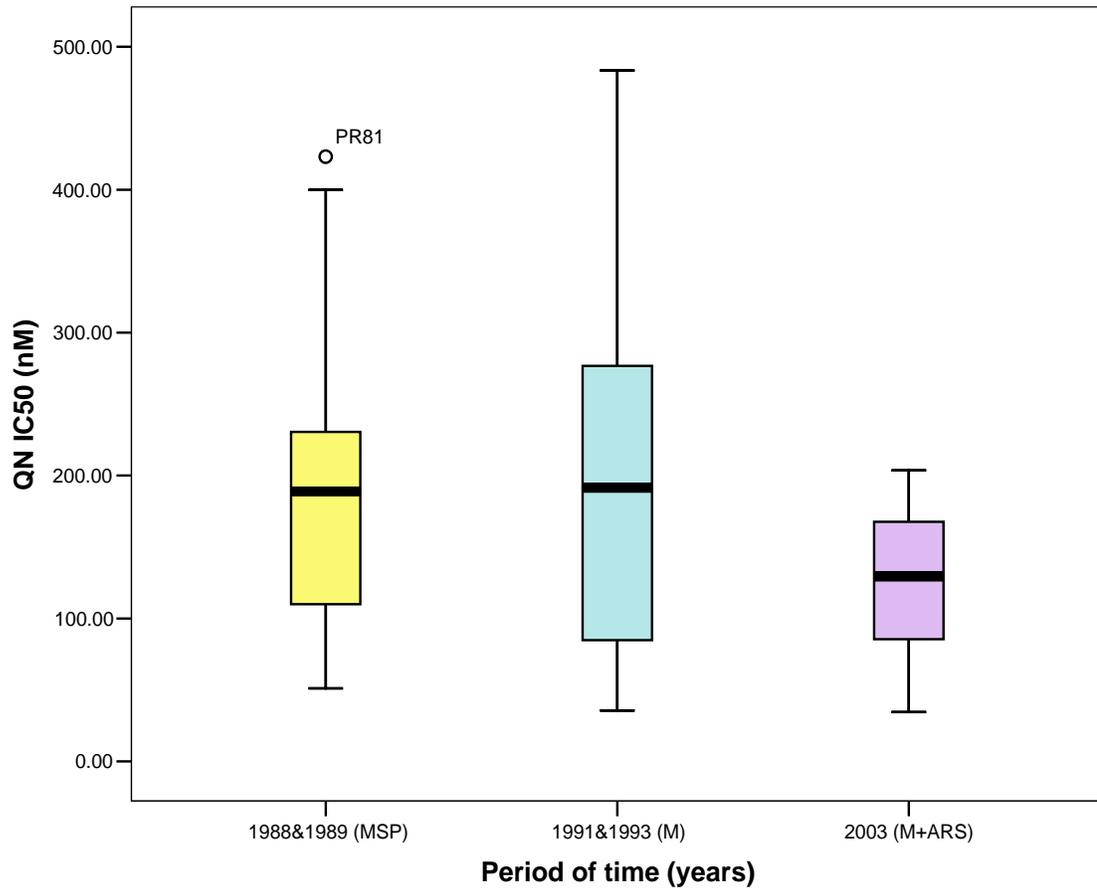


Figure 18 Box-plot of IC_{50} values of QN for *P. falciparum* isolates collected from different periods of time. There was no significant of parasite isolates collected from different period of time (Mann-Whitney U test) (MSP = Mefloquine plus Sulfadoxine/Pyrimethamine; M = Mefloquine; M+ARS = Mefloquine plus Artesunate). The parasite isolate name PR81 was outlier from range of box-plot.

1.3 MQ susceptibility: The IC₅₀ values of MQ for *P. falciparum* isolates ranged from 1.74 to 110.44 nM. The isolates were categorised as MQ-resistant and sensitive isolates if their IC₅₀ values were >20 nM and ≤ 20 nM, respectively (**Figure 19**). Based on these criteria, 34 (68%), and 16 (32%) isolates were categorised as MQ-sensitive and MQ-resistant, respectively. The mean (95% CI) IC₅₀ values of parasite isolates adapted during 1988-1989, 1991-1993 and 2003 were 7.86 (5.26-13.7), 14.4 (8.8-57.1), and 15.6 (10.22-25.1) nM, respectively (**Figure 20**). The mean MQ IC₅₀ values of parasite collected during from 1991-1993 and 2003 were significantly higher than of those parasite isolated in 1988-1989 ($p = 0.027$ and $p = 0.042$, respectively).

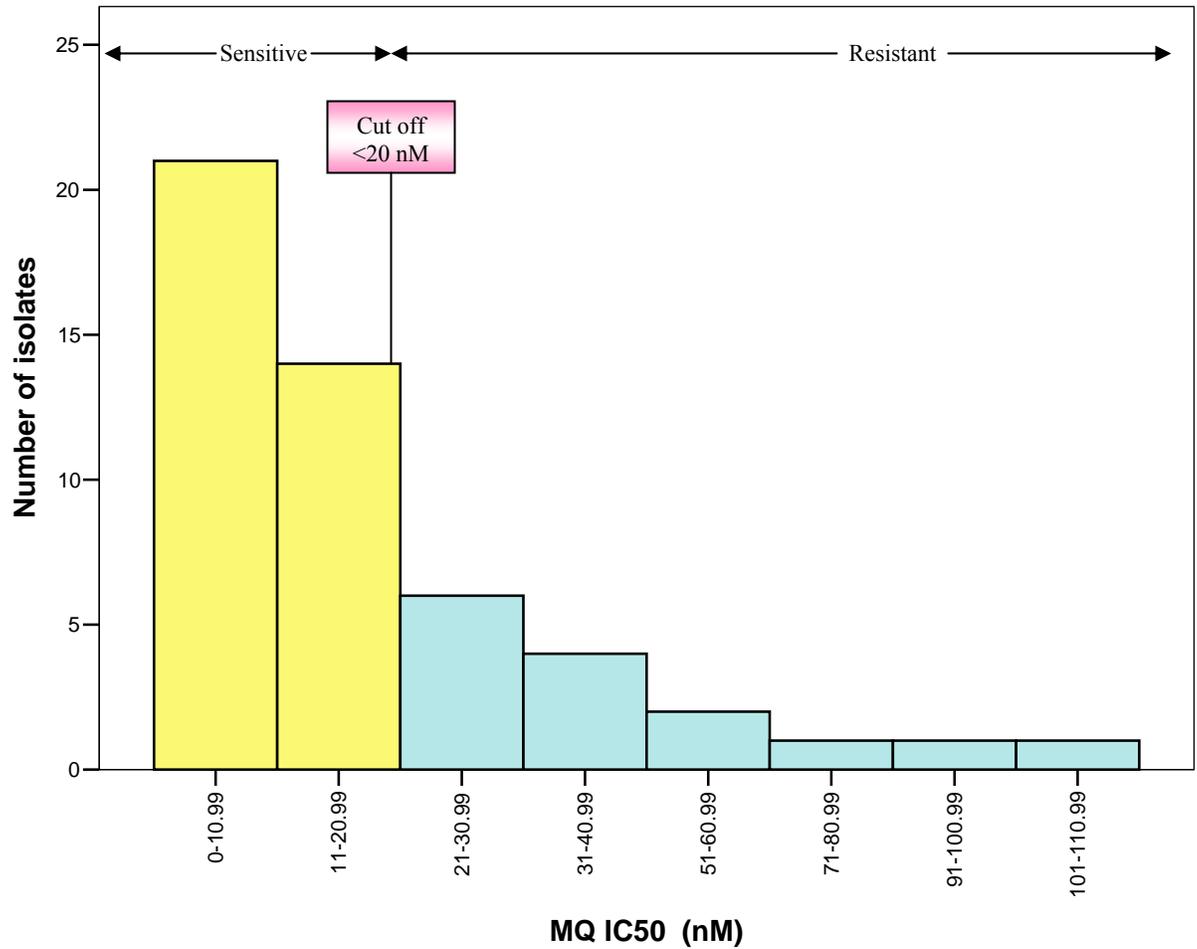


Figure 19 Distribution of *P. falciparum* isolates according to the IC₅₀ values of MQ. Parasite isolates were categorised as MQ-sensitive and MQ-resistant if its IC₅₀ values were less than 20 nM, and equal or more than 20 nM, respectively.

Table 12 Distribution of the level of MQ susceptibility in parasite isolates collected during different periods of time (1988-1989, 1991-1993, 2003); data are presented as number and percentage values in parenthesis.

Period of time (years)	Sensitive	Resistant
1988-1989	16 (84%)	3 (16%)
1991-1993	10 (63%)	6 (37%)
2003	8 (53%)	7 (47%)

NS (No statistically significant difference among the these periods of time by Chi-square test)

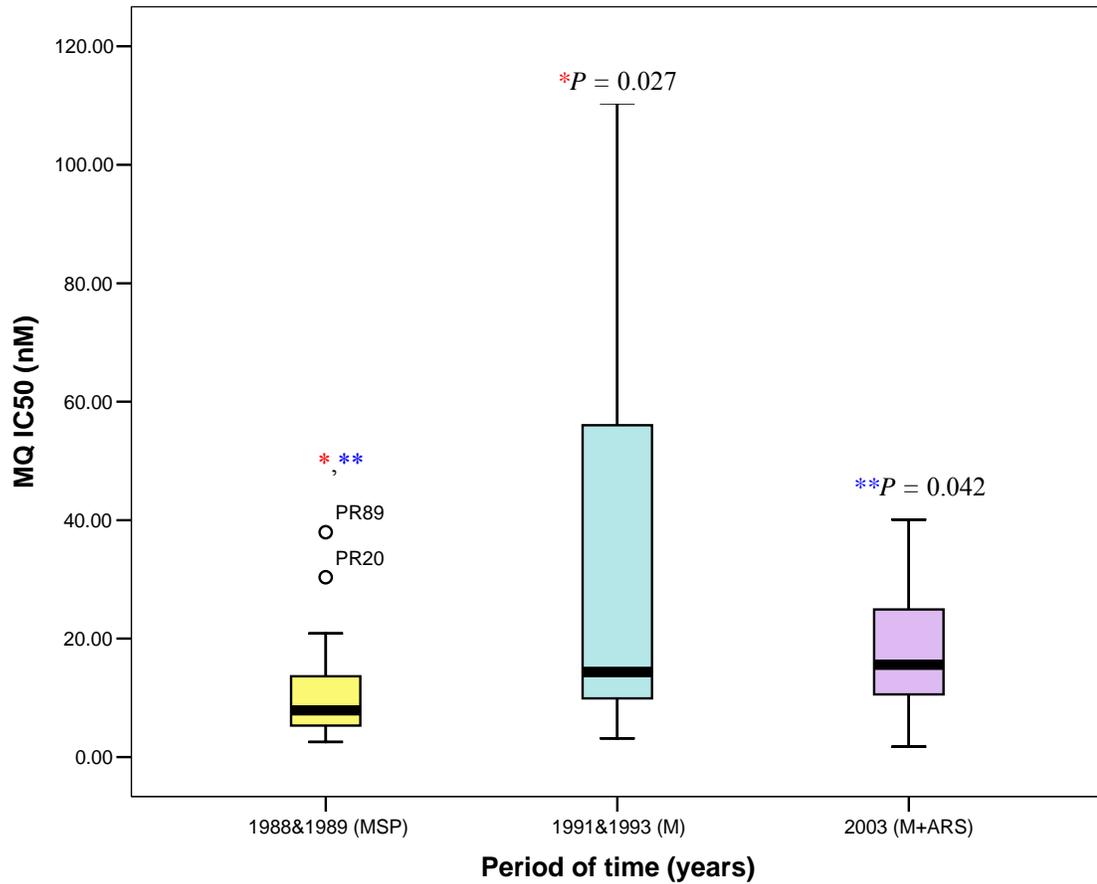


Figure 20 Box-plot of MQ IC₅₀ values of *P. falciparum* isolates from different periods of time. The mean IC₅₀ values of MQ for parasite isolates collected during 1988-1989 and 1991-1993, 1988-1989 and 2003 were significantly different (**p* = 0.027, ***p* = 0.042, respectively, Mann-Whitney U test) (MSP = Mefloquine plus Sulfadoxine/Pyrimethamine; M = Mefloquine; M+ARS = Mefloquine plus Artesunate). The parasite isolates name PR20 and PR 89 were outlier from range of box-plot.

1.4 ARS susceptibility: The IC₅₀ values of ARS for *P. falciparum* isolates ranged from 0.51 to 5.8 nM. The mean (95% CI) IC₅₀ values of parasite isolates adapted during 1988-1989, 1991-1993 and 2003 were 3.05 (2-4.15), 1.9 (1.25-2.8), and 2.12 (1.61-2.87) nM, respectively. The distribution of isolates according to ARS IC₅₀ values is shown in **Figure 21**. The mean IC₅₀ values of ARS were significantly different among isolates collected from 1988-1989 comparing to 1991-1993 ($p = 0.04$) and 2003 ($p = 0.018$) (**Figure 22**)

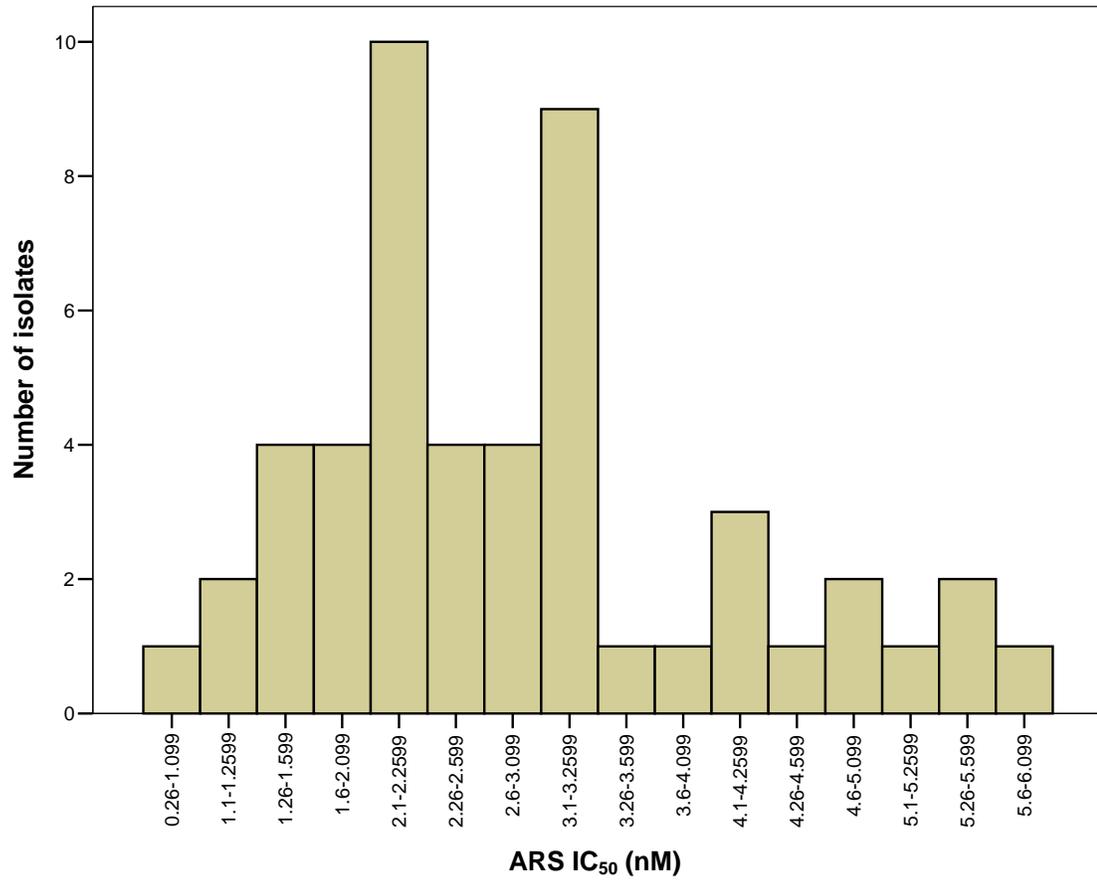


Figure 21 Distribution of the isolates according to their ARS IC₅₀ values.

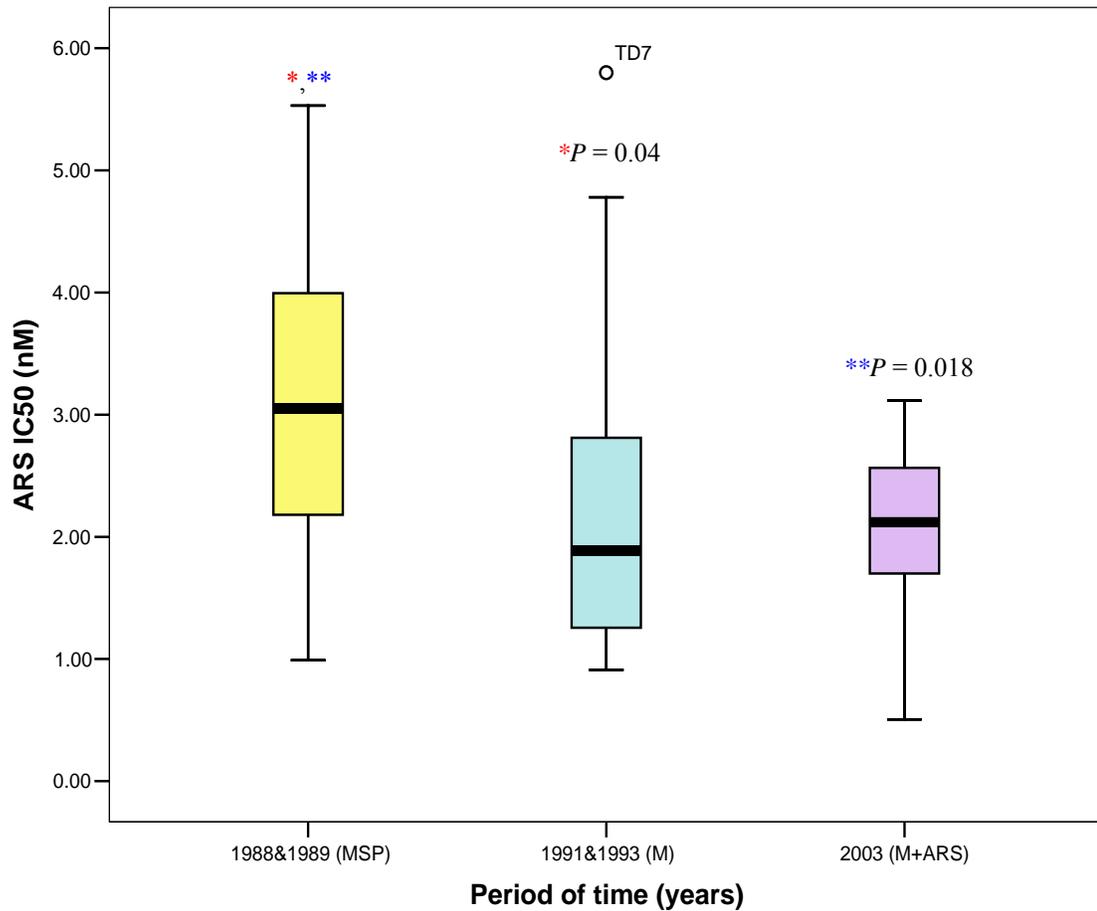


Figure 22 Box-plot of ARS IC₅₀ values of *P. falciparum* isolates from different periods of time. The mean IC₅₀ values of ARS for parasite isolates collected during 1988-1989 and 1991-1993, 1988-1989 and 2003 were significantly different ($*p = 0.04$, $**p = 0.018$, respectively, Mann-Whitney U test) (MSP = Mefloquine plus Sulfadoxine/Pyrimethamine; M = Mefloquine; M+ARS = Mefloquine plus Artesunate). The parasite isolate name TD7 was outlier from range of box-plot.

2. *In vitro* cross resistance

The correlations between the *in vitro* sensitivity of *P. falciparum* isolates to CQ, QN, MQ and ARS were determined using Spearman's correlation test (**Figure 23**). There was significantly positive but weak correlation between the IC₅₀ values of CQ and QN ($r^2 = 0.133$, $p = 0.008$) and between the IC₅₀ values of QN and MQ ($r^2 = 0.191$, $p = 0.041$) (**Figure 24**).

3. Genetic polymorphisms of *P. falciparum* isolates

3.1 *Pfcr*t polymorphism

The mutations in the *pfcr*t gene (**Figure 25 to 30**) were common. Prevalences of the mutations at codons A220S, Q271E, N326S, and R371I were virtually 100% (50 of 50). Summary of *pfcr*t polymorphism profile among parasite isolates collected from difference time period is shown in **Table 13**. Almost all isolates contained the mutant-type, only 2 (4%) and 3 (6%) isolates contained wild type at codon 76 and 356, respectively.

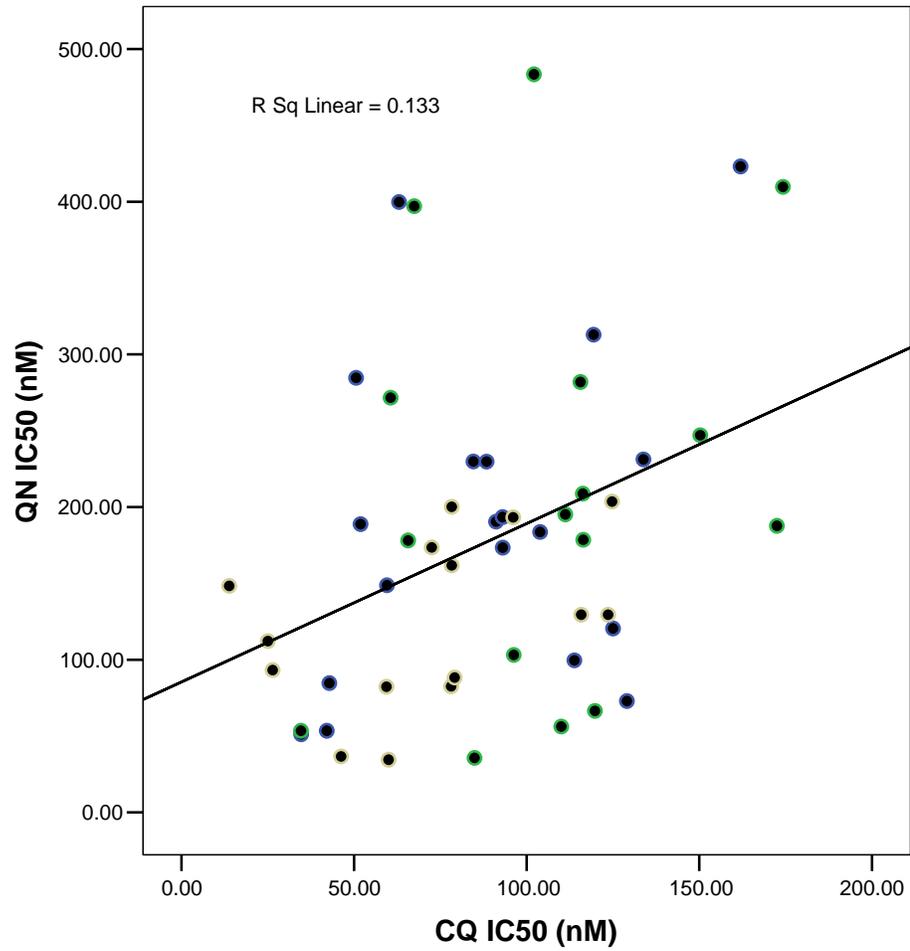


Figure 23 The scatter diagram and regression line representing the relationship between IC_{50} (nM) values of CQ and QN (Spearman's correlation test).

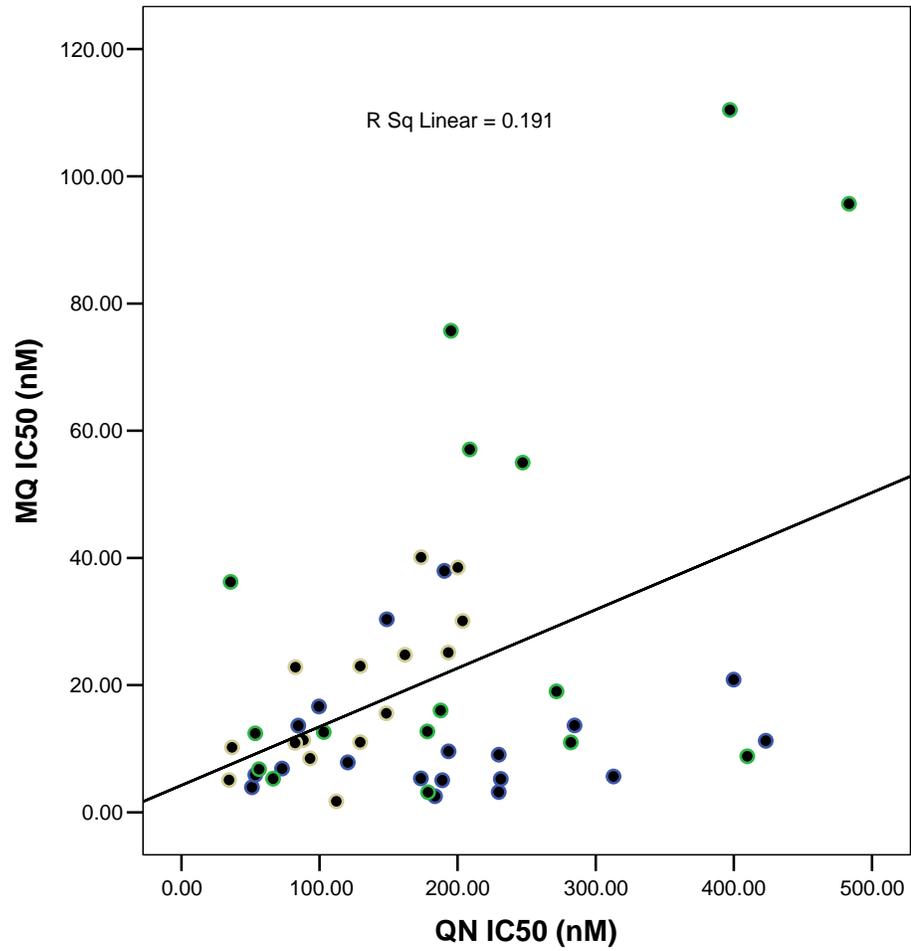


Figure 24 The scatter diagram and the regression line representing the relationship between IC₅₀ (nM) values of QN and MQ (Spearman's correlation test).

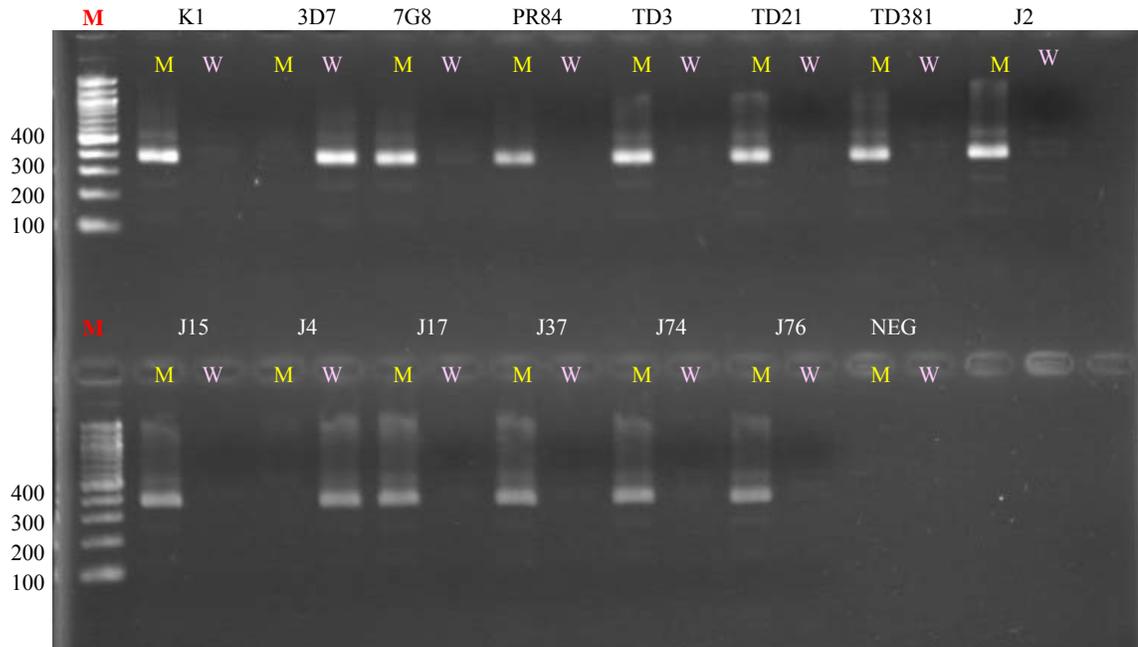


Figure 25 Detection of *pfcrt* at codon 76 by specific primer; M: 100 bp marker; K1 is mutant-type positive control and 3D7 is wild-type positive control. M (yellow): Mutation and W (pink): wild-type.

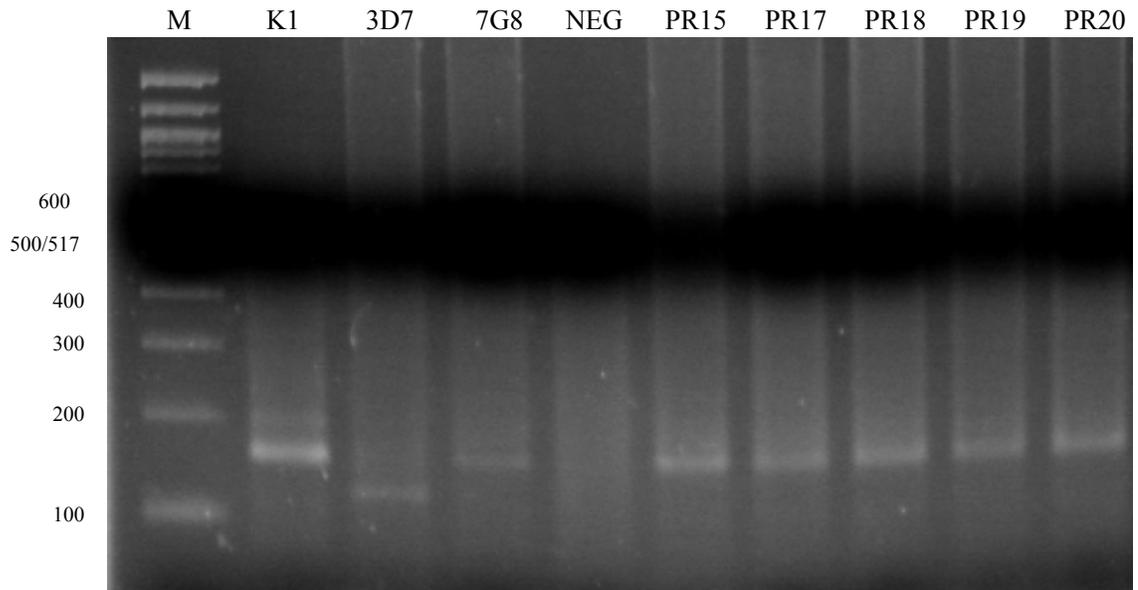


Figure 26 Detection of *pfcr1* polymorphism at codon 220, which was digested by *Bgl* I restriction enzyme. M: 100 bp marker; K1: is mutant-type positive control and 3D7 is wild-type positive control.

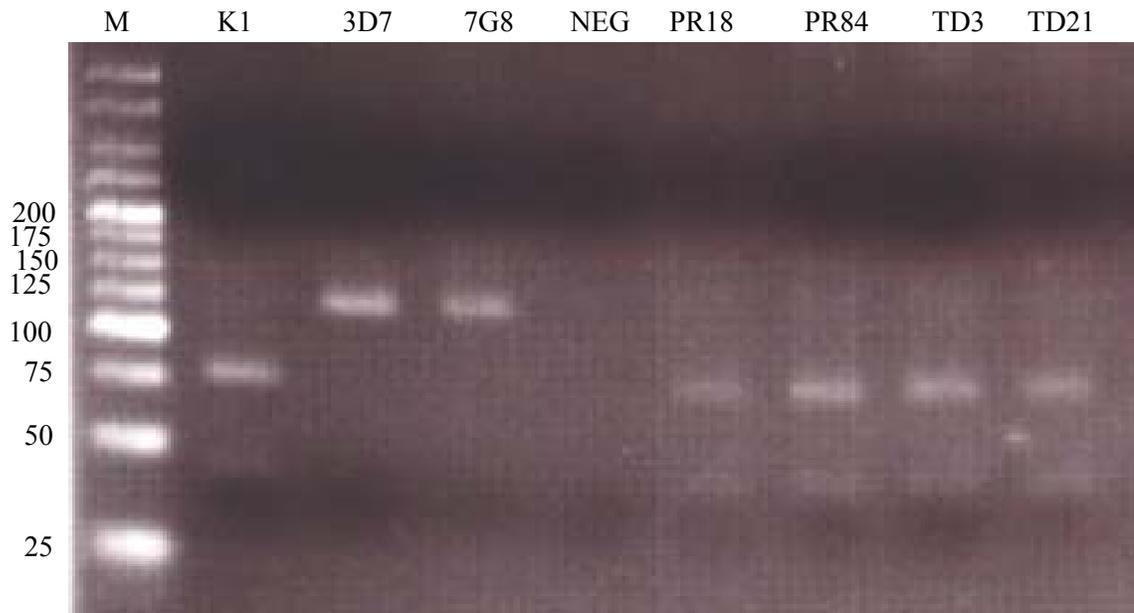


Figure 27 Detection of *pfprt* polymorphism at codon 271, which was digested by *Xmn* I restriction enzyme. M: Hyper Ladder V marker; K1 is mutant-type positive control and 3D7 is wild-type positive control.

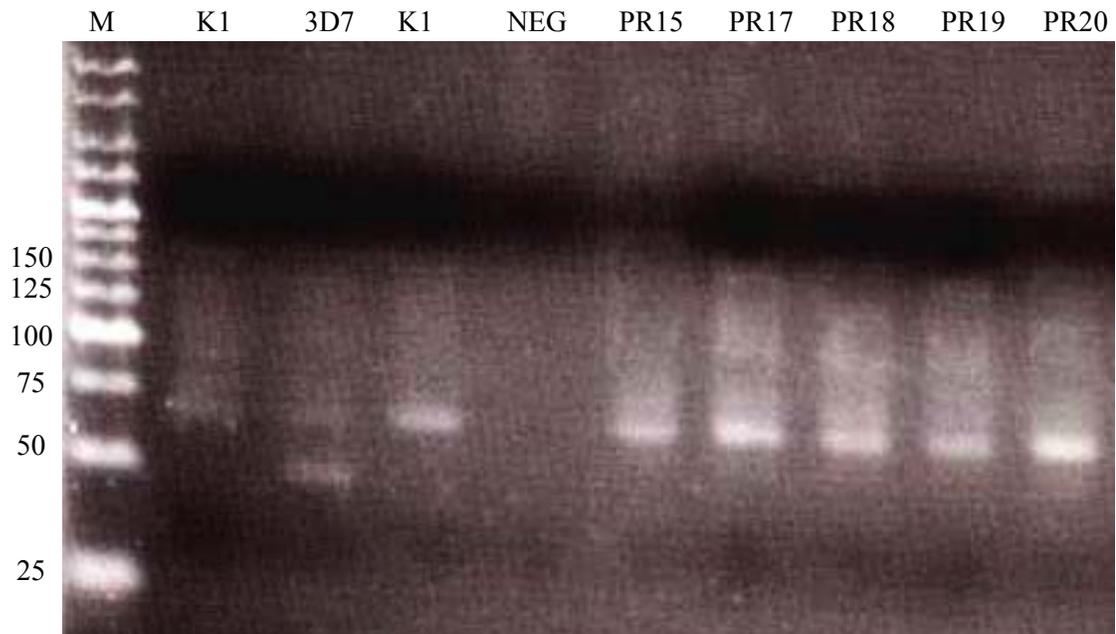


Figure 28 Detection of *pfert* polymorphism at codon 326, which was digested by *MseI* restriction enzyme. M: Hyper Ladder V marker; K1 is mutant-type positive control and 3D7 is wild-type positive control.

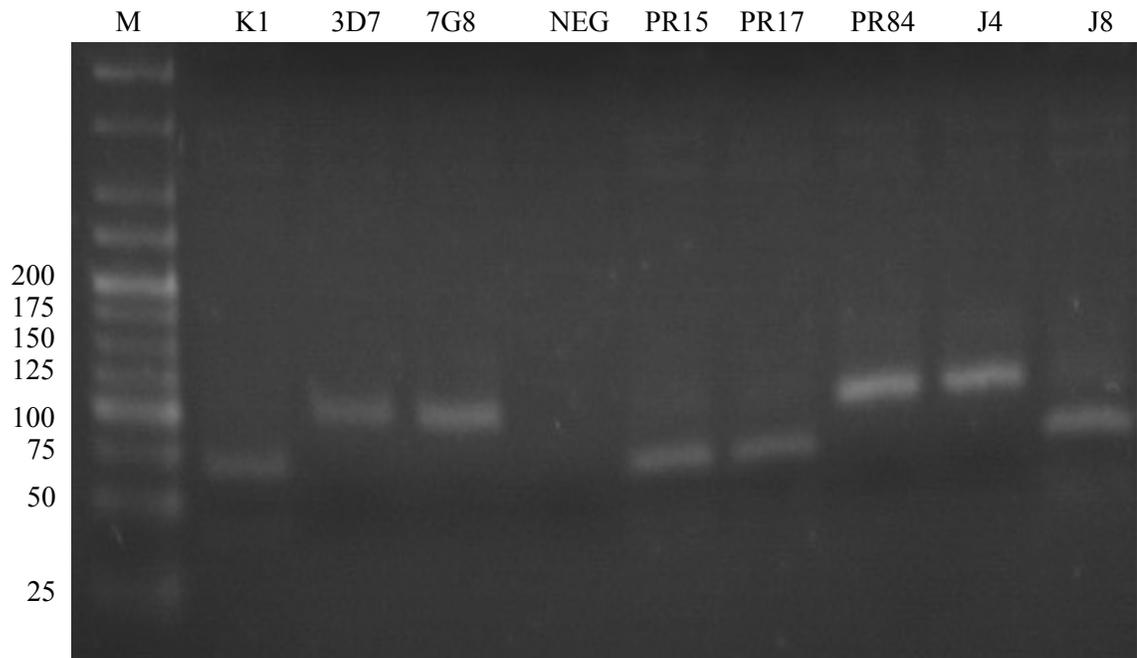


Figure 29 Detection of *pfcrt* polymorphism at codon 356, which was digested by *Acl* NI restriction enzyme. M: Hyper Ladder V marker; K1 is mutant-type positive control and 3D7 is wild-type positive control.

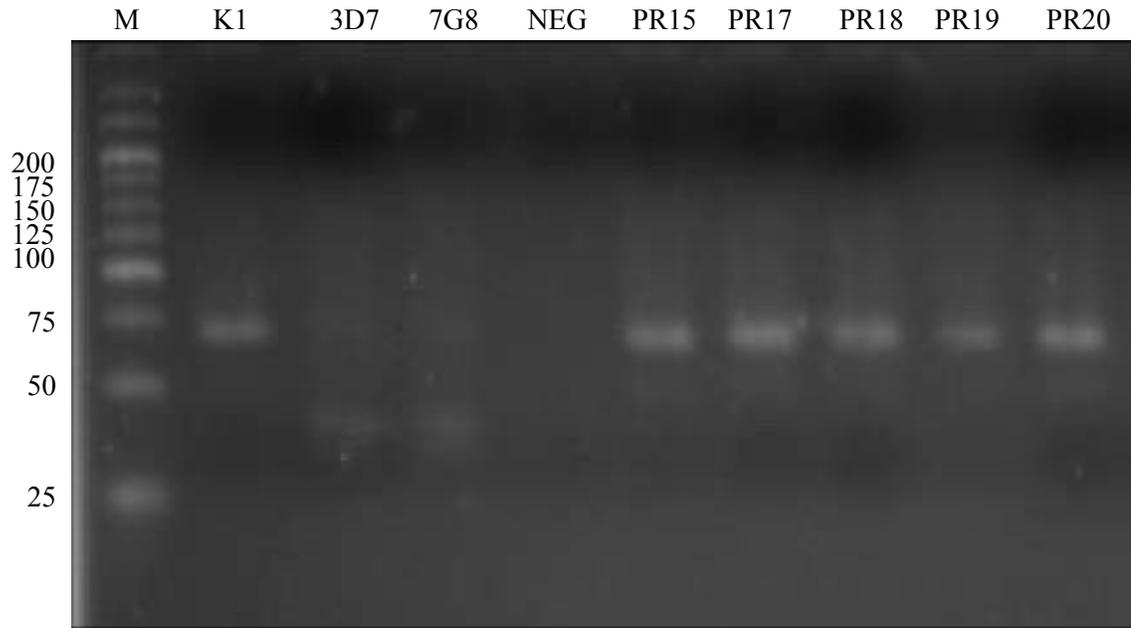


Figure 30 Detection of *pfprt* polymorphism at codon 371, which was digested by *Afl* II restriction enzyme. M: Hyper Ladder V marker; K1 is mutant-type positive control and 3D7 is wild-type positive control.

Table 13 Summary of *pfcr*t polymorphism profile of parasite isolates collected from different time periods; data are presented as member of cases and percentage value in parenthesis.

<i>pfcr</i> t/codon	Number of isolates	Wild type				Mutant type			
		1988-1989	1991-1993	2003	Total	1988-1989	1991-1993	2003	Total
K76T	50	0	0	2 (13.3)	2 (4)	19 (100)	16 (100)	13 (86.7)	48 (96)
A220S	50	0	0	0	0	19 (100)	16 (100)	15 (100)	50 (100)
Q271E	50	0	0	0	0	19 (100)	16 (100)	15 (100)	50 (100)
N326S	50	0	0	0	0	19 (100)	16 (100)	15 (100)	50 (100)
I356T	50	1 (5.3)	0	2 (13.3)	3 (6)	18 (94.7)	16 (100)	13 (86.7)	47 (94)
R371I	50	0	0	0	0	19 (100)	16 (100)	15 (100)	50(100)

NS (No statistically significant difference among *P. falciparum* isolate collected during different time periods by Chi-square test)

3.2 *Pfmdr1* polymorphism

The mutations in the *pfmdr1* gene (**Figure 31 to 35**) were common. Prevalence of the mutation codons of N86Y, Y184E, S1034C, and N1042D were 6 (12%), 41 (82%), 10 (20%), and 15 (30%), respectively (**Table 14**). Only wild type of *pfmdr1* at codon 1246 was found in these 50 isolates.

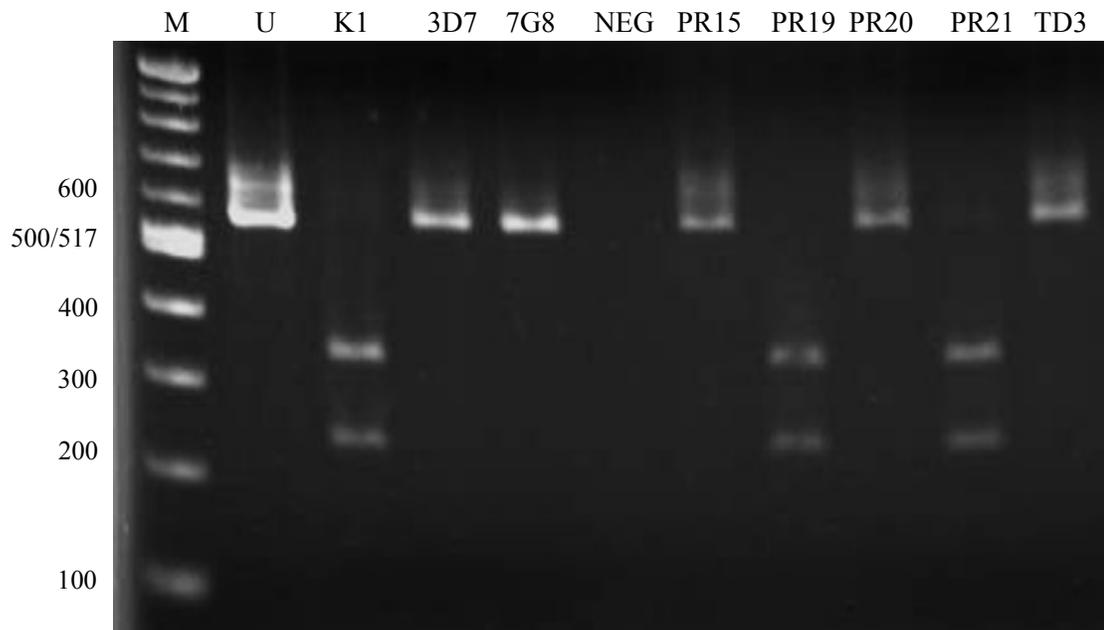


Figure 31 Detection of *pfmdr1* polymorphism at codon 86, which was digested by *Afl* III restriction enzyme. M: 100 bp marker; U: uncut secondary PCR product; K1 is mutant-type positive control and 3D7, 7G8 are wild-type positive control.

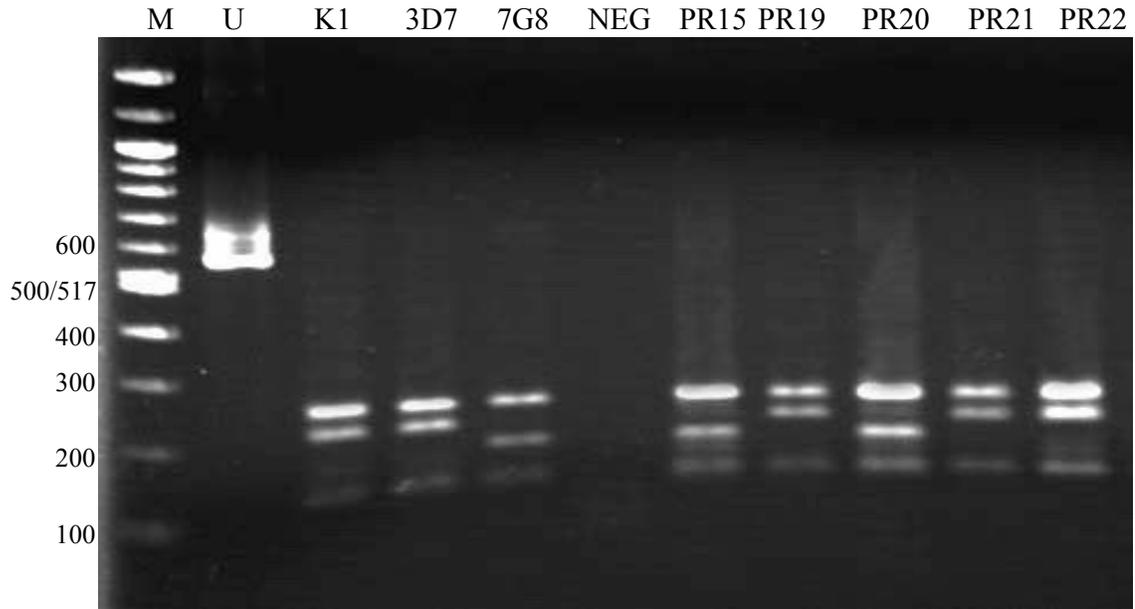


Figure 32 Detection of *pfmdr1* polymorphism at codon 184, which was digested by *Dra* I restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 and 3D7 are wild-type positive control, while 7G8 is mutant-type positive control.

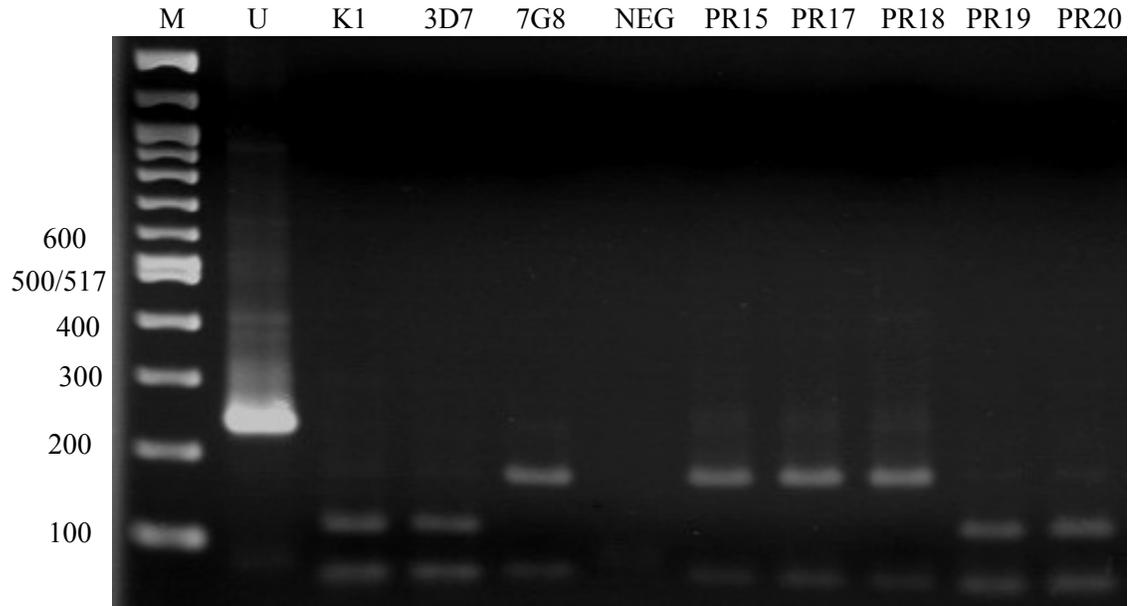


Figure 33 Detection of *pfmdr1* polymorphism at codon 1034, which was digested by *Dde* I restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 and 3D7 are wild-type positive control, while 7G8 is mutant-type positive control.

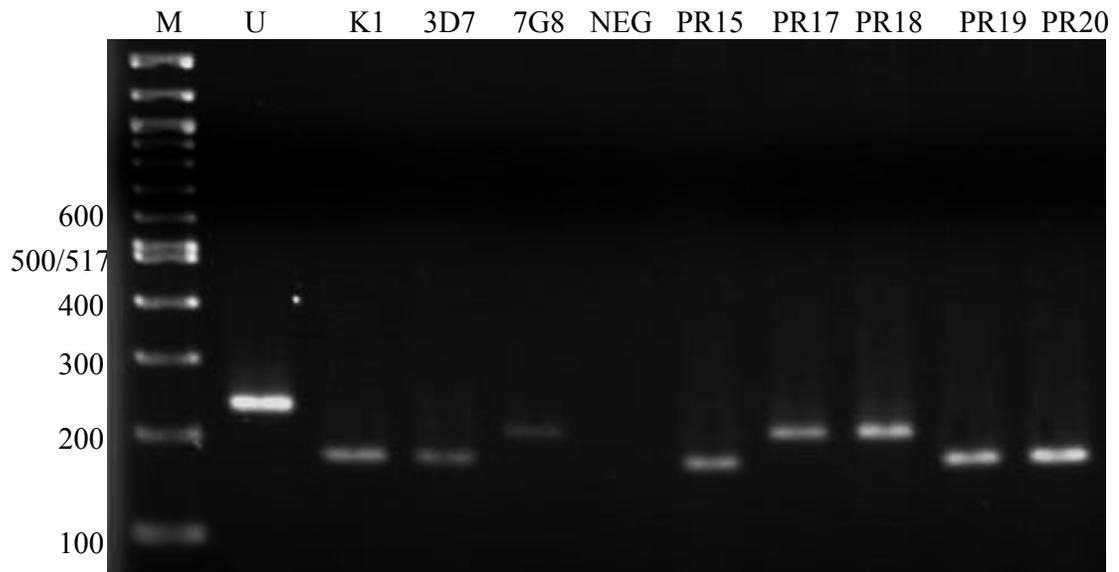


Figure 34 Detection of *pfmdr1* polymorphism at codon 1042, which was digested by *VspI* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 and 3D7 are wild-type positive control, while 7G8 is mutant-type positive control.

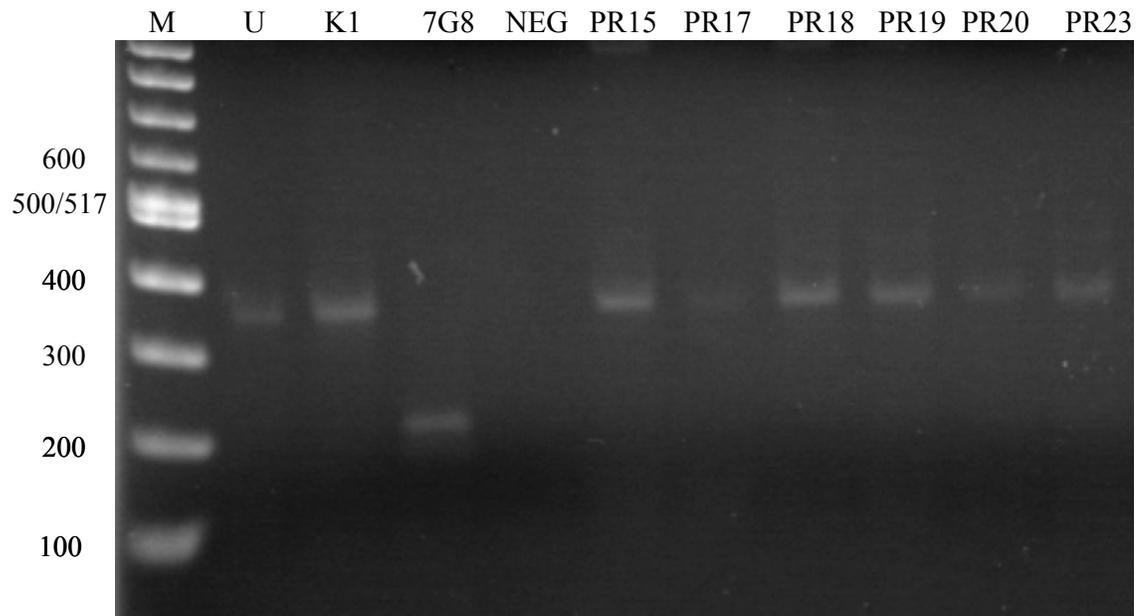


Figure 35 Detection of *pfmdr1* polymorphism at codon 1246, which was digested by *EcoRV* restriction enzyme. M: 100 bp marker; U: uncut of secondary PCR product; K1 are wild-type positive control, while 7G8 is mutant-type positive control.

Table 14 Summary of *pfmdr1* polymorphism profile of parasite isolates collected from different time periods; data are presented as member of cases and percentage value in parenthesis.

<i>pfmdr1</i> /codon	Number of isolates	Wild type				Mutant type			
		1988-1989	1991-1993	2003	Total	1988-1989	1991-1993	2003	Total
N86Y	50	15 (79)	16 (100)	13 (86.7)	44 (88)	4 (21)	0	2 (13.3)	6(12)
Y184F	50	4 (21)	3 (18.75)	2 (13.3)	9 (18)	15 (79)	13 (81.25)	13 (86.7)	41 (82)
S1034C	50	12 (63)	13 (81.25)	15 (100)*	40 (80)	7 (37)	3 (18.75)	0	10 (20)
N1042D	50	8 (42.1)	12 (75)	15 (100)**/**	35 (70)	11 (57.9)	4 (25)	0	15 (30)
D1246Y	50	19 (100)	16 (100)	15 (100)	50 (100)	0	0	0	0

* Significant difference between 1988-1989 and 2003 ($p = 0.009$; Chi-square test)

** Significant difference between 1988-1989 and 2003 ($p < 0.0001$; Chi-square test)

*** Significant difference between 1991-1993 and 2003 ($p = 0.041$; Chi-square test)

4. Association between polymorphisms of *pfcr* and *pfmdr1* genes and time periods of isolates collection

4.1 Polymorphisms of *pfcr* gene

Association between time periods of isolates collection and the prevalence of the polymorphisms in the *pfcr* gene was investigated and results showed no statistically significant association.

4.2 Polymorphisms of *pfmdr1* gene

Association between time periods of isolates collection and the prevalence of the polymorphisms in the *pfmdr1* gene was investigated. Results showed statistically significant association only two point mutations of the *pfmdr1* gene. The prevalence of S1034C and N1042D polymorphisms of the isolates collected during 1988-1989 and 2003 were significantly different ($p = 0.009$ and $p < 0.0001$, respectively). In addition, the prevalence of N1042D polymorphisms of the isolates collected during 1991-1993 and 2003 were also significantly different ($p = 0.041$) (**Table 14**).

5. Association between gene copy numbers of the *pfmdr1* gene and time periods

Association between time periods of isolates collection and the *pfmdr1* gene copy number was investigated. Results showed no statistically significant association (**Figure 36**) (independent t-test).

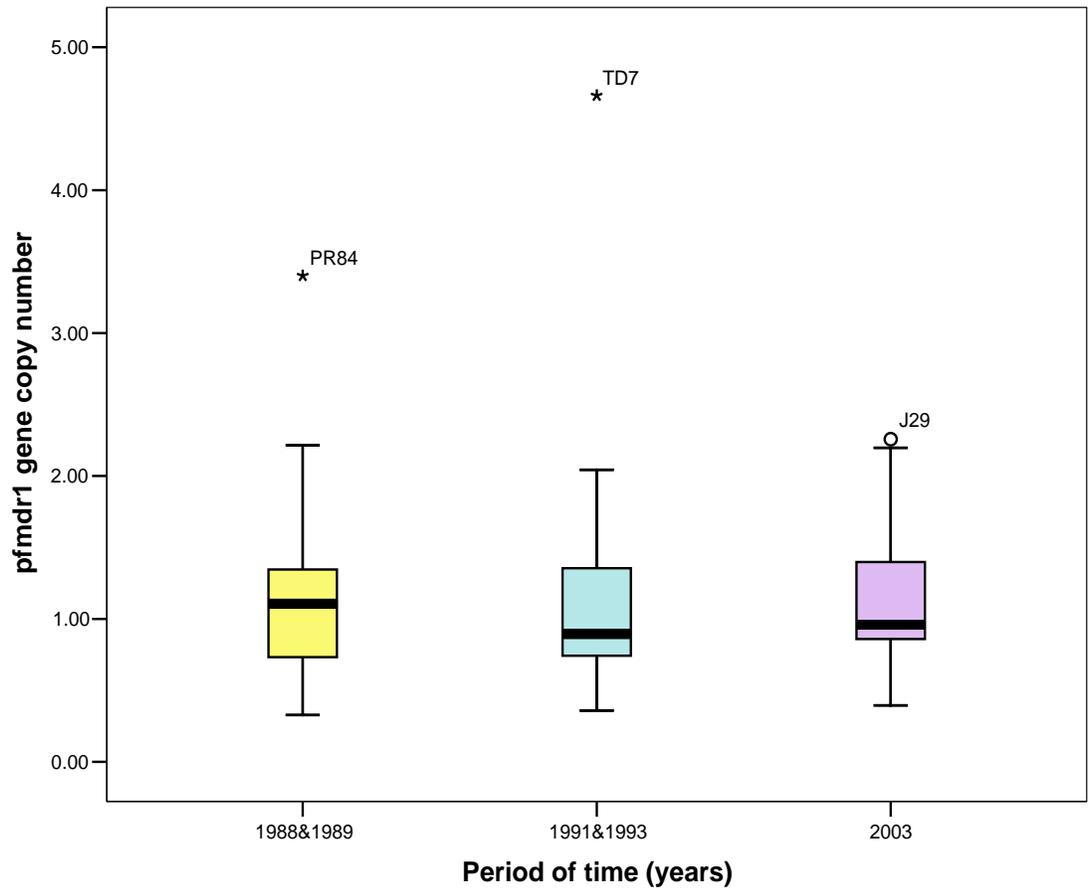


Figure 36 Box-plot of association between time period of parasite isolate selection and the *pfmdr1* gene copy number. The parasite isolate name PR84, TD7 and J29 was outlier from rang of box-plot.

6. Association between gene copy numbers of the *pfmdr1* gene and the *in vitro* susceptibility

The influence of the copy number of the *pfmdr1* gene on the *in vitro* sensitivities of CQ, QN, MQ and ARS IC₅₀ values were investigated use independent *t*-test (**Table 15**). Only the CQ IC₅₀ values significantly depended on the copy number of the *pfmdr1* gene. The parasite isolates containing more than one copy number of the *pfmdr1* gene exhibited lower CQ resistance than those containing one copy number.

6.1 Association between the gene copy numbers and allelic polymorphisms of the *pfmdr1* gene and *in vitro* drug susceptibility.

6.1.1 Codon 86

CQ: **Table 16** shows the influence of a single point mutation at codon 86 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to CQ. Improved sensitivity to CQ was observed in isolates carrying mutant genotype at codon 86. The IC₅₀ value was trend to a lower in isolates carrying mutant genotype with *pfmdr1* gene copy more than one [Mean (95%C.I.): 47 (13.9-80.2) nM] compared with those carrying wild-type genotype [84.9 (72.3-97.6) nM].

Relationship between the sensitivity of *P. falciparum* isolates to CQ, gene copy number and mutation at codon 86 of the *pfmdr1* gene was not determined due to the only one of isolate with mutant genotype in the group with one gene copy number. Sensitivity of the parasite isolates carrying mutant genotype with gene copy number more than one was trend to a higher than wild-type genotype with one gene copy [IC₅₀: 47 (13.9-80.2) nM vs 99.2 (81.6-116.7) nM].

QN, MQ and ARS: **Table 16** shows the influence of a single point mutation at codon 86 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to QN, MQ and ARS. There was no significant relationship between the sensitivity of *P. falciparum* isolates to QN, MQ and ARS, gene copy number and mutation at codon 86 of the *pfmdr1* gene ($p = 0.295$, $p = 0.465$ and $p = 0.834$, respectively).

Table 15 Association between the gene copy numbers of the *pfmdr1* gene and the *in vitro* susceptibility; data are presented as mean, 95%CI, minimum, maximum and *p*-value of IC₅₀ values to CQ, QN, MQ, and ARS following copy number.

Drug	Copy number	N	Mean	95% CI		Min	Max	<i>p</i> value
				Lower	Upper			
IC ₅₀ CQ*	1	26	100.3	83.3	117.4	25.1	174.2	0.031
	>1	24	77	64.3	89.8	13.9	125	
	total	50	89.1	78.2	100	13.9	174.2	
IC ₅₀ QN	1	26	187.9	146.7	229.2	56.2	423.4	0.503
	>1	24	167	117.3	216.7	34.5	483.4	
	total	50	177.9	146.8	208.9	34.5	483.4	
IC ₅₀ MQ	1	26	23.8	13.3	34.3	1.74	110.4	0.314
	>1	24	17.2	9.1	25.3	3.2	95.7	
	total	50	20.6	14.1	27.1	1.7	110.4	
IC ₅₀ ARS	1	26	2.4	1.9	2.8	0.9	5.3	0.348
	>1	24	2.7	2.1	3.4	0.5	5.8	
	total	50	2.6	2.2	2.9	0.5	5.8	

*Mean difference is significant at the 0.05 level (student *t*-test)

Table 16 Association between the gene copy numbers of the *pfmdr1* gene and the *in vitro* susceptibility with codon 86 mutation polymorphism; data are presented as mean, 95%CI, and *p*-value of IC₅₀ values to CQ, QN, MQ, and ARS following mutation and copy number.

Drugs	CQ*				QN				MQ				ARS			
	1		>1		1		>1		1		>1		1		>1	
Copy number																
Polymorphisms	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M	W
No.	1	25	5	19	1	25	5	19	1	25	5	19	1	25	5	19
Mean	129	99.2	47	84.9	72.9	192.6	103.5	183.7	6.89	24.5	7.9	19.6	2.38	2.4	2.76	2.73
95% C.I.	-	81.6	13.9	72.3	-	150.7	39.2	125.3	-	13.6	2.1	9.6	-	1.94	1.68	1.94
	-	116.7	80.2	97.6	-	234.4	207.3	242.2	-	35.4	13.7	29.7	-	2.84	3.84	3.51
<i>p</i> -value	0.023				0.295				0.465				0.834			

*Mean difference is significant at the 0.05 level (One-way ANOVA test). M = Mutant type, W = Wild type

6.1.2 Codon 184

CQ: **Table 17** shows the influence of a single point mutation at codon 184 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to CQ. There was a trend of decreasing sensitivity of isolates carrying single mutation at codon 184, although no statistically significant difference was found in the group with more than one copy of *pfmdr1* gene [IC₅₀: 85.8 (73.9-97.8) vs 55.7 (22.4-88.9) nM].

There was no significant relationship between the sensitivity of *P. falciparum* isolates to CQ, gene copy number and mutation at codon 184 of the *pfmdr1* gene in either the group of isolates with one or more than one gene copy number. In Addition, sensitivity of the parasite isolates carrying mutant genotype with one gene copy number was trend to a lower than wild-type genotype with more than one gene copy [IC₅₀: 98.7 (80.4-117) nM vs 55.7 (22.4-88.9) nM].

QN: **Table 17** shows the influence of a single point mutation at codon 184 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to QN. There was trend to decrease in sensitivity of isolates carrying single mutation at codon 184 in either the group with more than one copy of *pfmdr1* gene [IC₅₀: 198.3 (136.6-260) vs 91 (24.8-157.2) nM] and that with one copy of *pfmdr1* gene [IC₅₀: 198.2 (156.3-240.2) vs 64.6 (41.7-170.9) nM].

There was no significant relationship between the sensitivity of *P. falciparum* isolates to QN, gene copy number and mutation at codon 184 of the *pfmdr1* gene. However, sensitivity of the parasite isolates carrying mutant genotypes with one gene copy number was significantly lower than those carrying wild-type genotype with more than one gene copy number [IC₅₀: 198.2 (156.3-240.2) nM vs 91 (24.8-157.2) nM].

MQ and ARS: **Table 17** shows the influence of a single point mutation at codon 184 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to MQ and ARS. There was no significant relationship between the sensitivity of *P. falciparum* isolates to MQ and ARS, and gene copy number, as well as mutation at codon 184 of the *pfmdr1* gene ($p = 0.298$ and $p = 0.511$, respectively).

Table 17 Association between the gene copy numbers of the *pfmdr1* gene and the *in vitro* susceptibility with codon 184 mutation polymorphism; data are presented as mean, 95%CI, and *p*-value of IC₅₀ values to CQ, QN, MQ, and ARS following mutation and copy number.

Drugs	CQ*				QN*				MQ				ARS			
	1		>1		1		>1		1		>1		1		>1	
Genotype	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M	W
No.	24	2	17	7	24	2	17	7	24	2	17	7	24	2	17	7
Mean	98.7	119.5	85.8	55.7	198.2	64.6	198.3	91	25.2	6.8	20.9	8.2	2.44	1.83	2.9	2.31
95% C.I.	80.4	1.2	73.9	22.4	156.3	41.7	136.6	24.8	14	6.2	9.7	4.2	1.97	5.22	2.1	1.34
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	117.0	240.3	97.8	88.9	240.2	170.9	260	157.2	36.5	7.5	32	12.2	2.9	8.9	3.7	3.3
<i>p</i> -value	0.036				0.040				0.298				0.511			

*Mean difference is significant at the 0.05 level (One-way ANOVA test). No significant difference between combination of *pfmdr1* genotype and copy number [One-way ANOVA test (Scheffé)]. M = Mutant type, W = Wild type

6.1.3 Codon 1034

CQ: Table 18 shows the influence of a single point mutation at codon 1034 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to CQ. There was a trend of decreasing sensitivity of isolates carrying single mutation at codon 1034, although no statistically significant difference was found in the group with at least one copy of *pfmdr1* gene [IC₅₀: 131.4 (106.4-156.3) vs 88.8 (68.9-108.7) nM].

There was significant relationship between the sensitivity of *P. falciparum* isolates to CQ, and gene copy number including the mutation at codon 1034 of the *pfmdr1* gene. The IC₅₀ value for CQ was significantly lower in the isolates carrying mutant genotypes with one copy of *pfmdr1* gene compared to that with the isolates carrying wild type as well as more than one gene copy [IC₅₀: 131.4 (106.4-156.3) vs 74.9 (60.5-189.5) nM].

QN: Table 18 shows the influence of a single point mutation at codon 1034 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to QN. There was no significant relationship between the sensitivity of *P. falciparum* isolates QN, and gene copy number, as well as mutation at codon 1034 of the *pfmdr1* gene ($p = 0.212$).

MQ: Table 18 shows the influence of a single point mutation at codon 1034 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to MQ. There was a trend of increasing sensitivity of isolates carrying single mutation at codon 1034, although no statistically significant difference but was found in the group with one copy of *pfmdr1* gene [IC₅₀: 6.2 (3.4-9.0) vs 30.3 (16.9-43.7) nM]. There was no significant relationship between the sensitivity of *P. falciparum* isolates to MQ, and gene copy number including mutation at codon 1034 of the *pfmdr1* gene in either the group of isolates with one or more than one gene copy number.

ARS: Table 18 shows the influence of a single point mutation at codon 1034 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to ARS. There was no significant relationship between the sensitivity of *P.*

falciparum isolates to ARS, and gene copy number including mutation at codon 1034 of the *pfmdr1* gene ($p = 0.793$).

Table 18 Association between the gene copy numbers of the *pfmdr1* gene and the *in vitro* susceptibility with codon 1034 mutation polymorphism; data are presented as mean, 95%CI, and *p*-value of IC₅₀ values to CQ, QN, MQ, and ARS following mutation and copy number.

Drugs	CQ*				QN				MQ				ARS			
	1		>1		1		>1		1		>1		1		>1	
Genotype	M ¹	W	M	W ¹	M	W	M	W	M	W	M	W	M	W	M	W
No.	7	19	3	21	7	19	3	21	7	19	3	21	7	19	3	21
Mean	131.4	88.8	91.5	74.9	256.5	162.7	198.9	162.5	6.2	30.3	6	18.8	2.22	2.45	2.65	2.74
95% C.I.	106.4	68.9	84.8	60.5	133.2	125.3	127.9	105.5	3.4	16.9	2	9.7	0.72	2.04	0.15	2.04
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	156.3	108.7	98.2	89.5	379.7	200.2	269.9	219.4	9	43.7	14.1	27.9	3.71	2.86	5.46	3.45
<i>p</i> -value	0.006				0.212				0.053				0.793			

*Mean difference is significant at the 0.05 level (One-way ANOVA test). ¹ Significant difference between combination of *pfmdr1* genotype and copy number [*p* = 0.006, One-way ANOVA test (Scheffe)]. M = Mutant type, W = Wild type

6.1.4 Codon 1042

CQ: Table 19 shows the influence of a single point mutation at codon 1042 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to CQ. There was a trend of decreasing sensitivity of isolates carrying single mutation at codon 1042 but statistically significant difference was not achieved.

There was no significant relationship between the sensitivity of *P. falciparum* isolates to CQ, and gene copy number including mutation at codon 1042 of the *pfmdr1* gene. Sensitivity of the parasite isolates carrying mutant genotype with one gene copy number was however, significantly lower than wild-type genotype with more than one gene copy [IC₅₀: 121.4 (90-152.8) nM vs 71.8 (55.9-87.6) nM].

QN: Table 19 shows the influence of a single point mutation at codon 1042 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to QN. There was a trend of decreasing sensitivity of isolates carrying single mutation at codon 1042, although no statistically significant difference was found in the group with more than one copy of *pfmdr1* gene [IC₅₀: 240.5 (155.8-325.2) vs 136.8 (77.2-196.3) nM].

There was no significant relationship between the sensitivity of *P. falciparum* isolates to QN, and gene copy number including mutation at codon 1042 of the *pfmdr1* gene. However, sensitivity of the parasite isolates carrying mutant genotype with one gene copy number was trend to a lower than wild-type genotype with gene copy number of more than copy [IC₅₀: 248 (142.9-353.1) nM vs 136.8 (77.2-196.3) nM].

MQ: Table 19 shows the influence of a single point mutation at codon 1042 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to CQ. There was a trend of increasing sensitivity of isolates carrying single mutation at codon 1042, although no statistically significant difference was found in the group with at one copy of *pfmdr1* gene [IC₅₀: 6.1 (3.7-8.5) vs 31.7 (17.8-45.6) nM]. In addition, sensitivity of the parasite isolates carrying mutant genotype with one gene

copy number was higher than wild-type genotype with gene copy number of more than one [IC₅₀: 20.1 (8.7-31.4) nM].

There was no significant relationship between the sensitivity of *P. falciparum* isolates to MQ, gene copy number and mutation at codon 1042 of the *pfmdr1* gene in either the group of isolates with one or more than one gene copy. Sensitivity of the parasite isolates carrying wild-type genotype with one gene copy number was lower than those carrying mutant genotype with gene copy number of more than one [IC₅₀: 31.7 (17.8-45.6) nM vs 10.2 (4.8-15.6) nM].

ARS: **Table 19** shows the influence of a single point mutation at codon 1042 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to ARS. There was no significant relationship between the sensitivity of *P. falciparum* isolates to ARS, and gene copy number including mutation at codon 1034 of the *pfmdr1* gene ($p = 0.129$).

Table 19 Association between the gene copy numbers of the *pfmdr1* gene and the *in vitro* susceptibility with codon 1042 mutation polymorphism; data are presented as mean, 95%CI, and *p*-value of IC₅₀ values to CQ, QN, MQ, and ARS following mutation and copy number.

Drugs	CQ*				QN*				MQ*				ARS			
Copy number	1		>1		1		>1		1		>1		1		>1	
Genotype	M ¹	W	M	W ¹	M	W	M	W	M	W	M	W	M	W	M	W
No.	8	18	7	17	8	18	7	17	8	18	7	17	8	18	7	17
Mean	121.4	90.9	89.8	71.8	248	161.3	240.5	136.8	6.1	31.7	10.2	20.1	2.15	2.5	3.58	2.38
95% C.I.	90	70.3	65.4	55.9	142.9	121.6	155.8	77.2	3.7	17.8	4.8	8.7	0.88	2.07	2.05	1.71
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>p</i> -value	152.8	111.5	114.2	87.6	353.1	200.9	325.2	196.3	8.5	45.6	15.6	31.4	3.42	2.91	5.11	3.05
<i>p</i> -value	0.022				0.032				0.026				0.129			

*Mean difference is significant at the 0.05 level (One-way ANOVA test). ¹ Significant difference between combination of *pfmdr1* genotype and copy number [*p* = 0.022, One-way ANOVA test (Scheffe)]. M = Mutant type, W = Wild type

6.1.5 Codon 1246

CQ: Table 20 shows the influence of a single point mutation at codon 1246 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P.falciparum* isolates to CQ. Comparison of sensitivity of isolates carrying single mutation at codon 1246 and wild-type was not performed due to the absence of isolate with mutant genotype at this codon.

There was significant relationship between the sensitivity of *P. falciparum* isolates to CQ, and gene copy number including mutation at codon 1246 of the *pfmdr1* gene. The IC₅₀ value for CQ was significantly lower in the isolates carrying wild-type genotype with more than copy of *pfmdr1* gene compared to that with one gene copy [IC₅₀: 77 (64.3-89.8) vs 100.3 (83.3-117.3) nM]. Sensitivity of the parasite isolates carrying wild-type genotype with one gene copy number was significantly lower than wild-type genotype with gene copy number of more than one [IC₅₀: 100.3 (83.3-117.3) nM vs 77 (64.3-89.8) nM].

QN, MQ and ARS: Table 20 shows the influence of a single point mutation at codon 1246 and *pfmdr1* gene copy number (1 and > 1 copy number) on the sensitivity of *P. falciparum* isolates to QN, MQ and ARS. There was no significant relationship between the sensitivity of *P. falciparum* isolates to QN, MQ and ARS, and gene copy number including mutation at codon 1246 of the *pfmdr1* gene ($p = 0.503$, $p = 0.314$ and $p = 0.348$, respectively).

Table 20 Association between the gene copy numbers of the *pfmdr1* gene and the *in vitro* susceptibility with codon 1246 mutation polymorphism; data are presented as mean, 95%CI, and *p*-value of IC₅₀ values to CQ, QN, MQ, and ARS following mutation and copy number.

Drugs	CQ*				QN				MQ				ARS			
	1		>1		1		>1		1		>1		1		>1	
Genotype	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M	W
No.	-	26	-	24	-	26	-	24	-	26	-	24	-	26	-	24
Mean	-	100.3	-	77	-	188	-	167	-	23.8	-	17.2	-	2.4	-	2.73
95% C.I.	-	83.3	-	64.3	-	146.8	-	117.3	-	13.3	-	9.1	-	1.95	-	2.1
	-	117.3	-	89.8	-	229.2	-	216.7	-	34.3	-	25.3	-	2.82	-	3.36
<i>p</i> -value	0.031				0.503				0.314				0.348			

*Mean difference is significant at the 0.05 level (One-way ANOVA test). M = Mutant type, W = Wild type

6.2 Multiple point mutation of *Pfmdr1* gene

Association between time periods of isolates collection and the multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 is summarized in **Table 21**. The prevalence of gene mutation was observed with isolates carrying wMwww pattern (27 isolates). In addition, a trend of increasing prevalence was observed towards the time periods of collection.

CQ: **Table 22** shows the influence of multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P. falciparum* isolates to CQ. There was no significant difference in the sensitivity of *P. falciparum* isolates carrying wild-type genotype at all the five codons compared with those carrying at least one point mutation.

QN: **Table 22** shows the influence of multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P. falciparum* isolates to QN. There was a trend of higher sensitivity of *P. falciparum* isolates carrying wild-type genotype at all the five codons compared with those carrying at least one point mutation. However, statistically significant difference was found only with the isolates carrying a single point mutation at codon 184 [IC₅₀: 167.3 (127.8-206.8) nM] and combination of point mutation at codon 184, 1034 and 1042 [IC₅₀: 259.5 (183.6-335.4) nM].

MQ: **Table 22** shows the influence of multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P. falciparum* isolates to MQ. The sensitivity of isolates carrying a single point mutation at codon 184 appears to be lower than that carrying wild-type genotype or that carrying mutation of at least one codon (double, triple mutation). However, statistically significant difference was found only with the isolates carrying a single point mutation at codon 86 [IC₅₀: 7.7 (3.3-12.2) nM], triple point mutation at codon 184, 1034 and 1042 [IC₅₀: 6.1 (3.7-8.4) nM] and double point mutation at codon 184 and 1042 [IC₅₀: 11.7(4.2-19.2) nM].

ARS: **Table 22** shows the influence of multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P. falciparum* isolates to ARS. There was no significant difference in the sensitivity of *P. falciparum* isolates

carrying wild-type genotype at all the five codons compared with those carrying at least one point mutation.

Table 21 Association between pattern of *pfmdr1* gene polymorphism and time period of isolates collection

Codons 86,184,1034,1042,1246	Time periods			Total
	1988-1989	1991-1993	2003	
wwwww	0	2	0	2
Mwwww	4	0	2	6
wMMMw	7	2	0	9
wwMMw	0	1	0	1
wMwMw	4	1	0	5
wMwww	4	10	13	27
Total	19	16	15	50

Table 22 The influence of multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P.falciparum* isolates to CQ, QN, MQ and ARS; data are presented as mean, 95%CI, and *p*-value of IC₅₀ values.

Drugs	CQ				QN*				MQ*				ARS			
	Codons 86,184,1034,1042,1246	No.	95% C.I.	Mean	<i>p</i> value	No.	95% C.I.	Mean	<i>p</i> value	No.	95% C.I.	Mean	<i>p</i> value	No.	95% C.I.	Mean
wwwww	2	34.6- 119.8	77.2	0.062	2	22.4- 142.4	59.9	0.007	2	36.4- 54.1	8.9	0.015	2	0.43- 1.95	1.19	0.159
Mwwww	6	17.6- 103.9	60.7		6	18.8- 177.9	98.4		6	3.3- 12.2	7.7		6	1.86- 3.53	2.69	
wMMMw	9	96.7- 144.2	120.5		9	183.6- 335.4	259.5		9	3.7- 8.4	6.1		9	1.33- 3.6	2.46	
wwMMw	1	-	110		1	-	56.2		1	-	6.8		1	-	1.27	
wMwMw	5	36.3- 126.1	81.2		5	123.4- 386.9	255.1		5	4.2- 19.2	11.7		5	1.37- 6.14	3.76	
wMwww	27	72.7- 100.5	86.6		27	127.8- 206.8	167.3		27	20.8- 41.9	31.4		27	2.03- 2.91	2.47	
Total	50	78.2- 100	89.1		50	146.7- 208.9	177.9		50	14.1- 27.2	20.6		50	2.18- 2.91	2.55	

*Mean difference is significant at the 0.05 level (One-way ANOVA test).

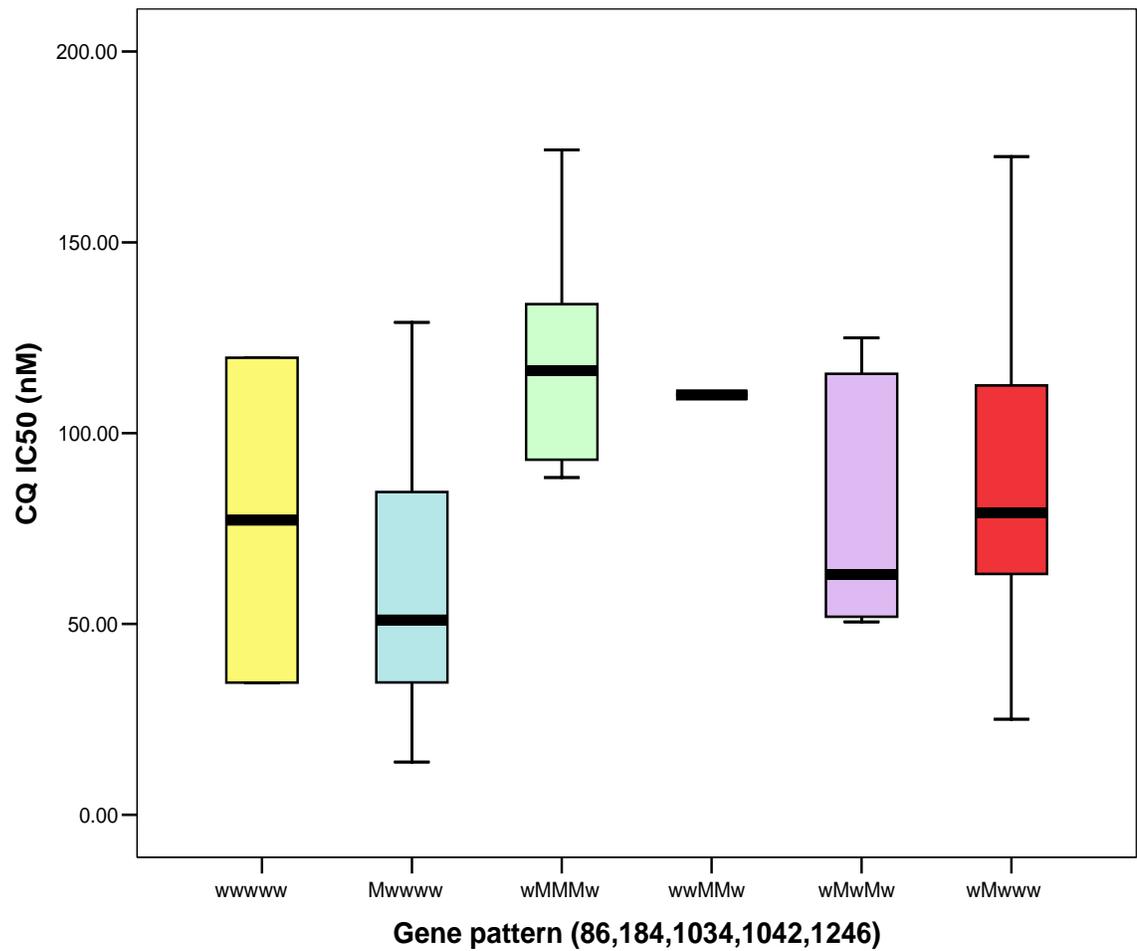


Figure 37 Box-plot presented association between multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P.falciparum* isolates to CQ; data are presented as mean of IC₅₀ values [One-way ANOVA test (Scheffé)]. M = Mutant type, W = Wild type

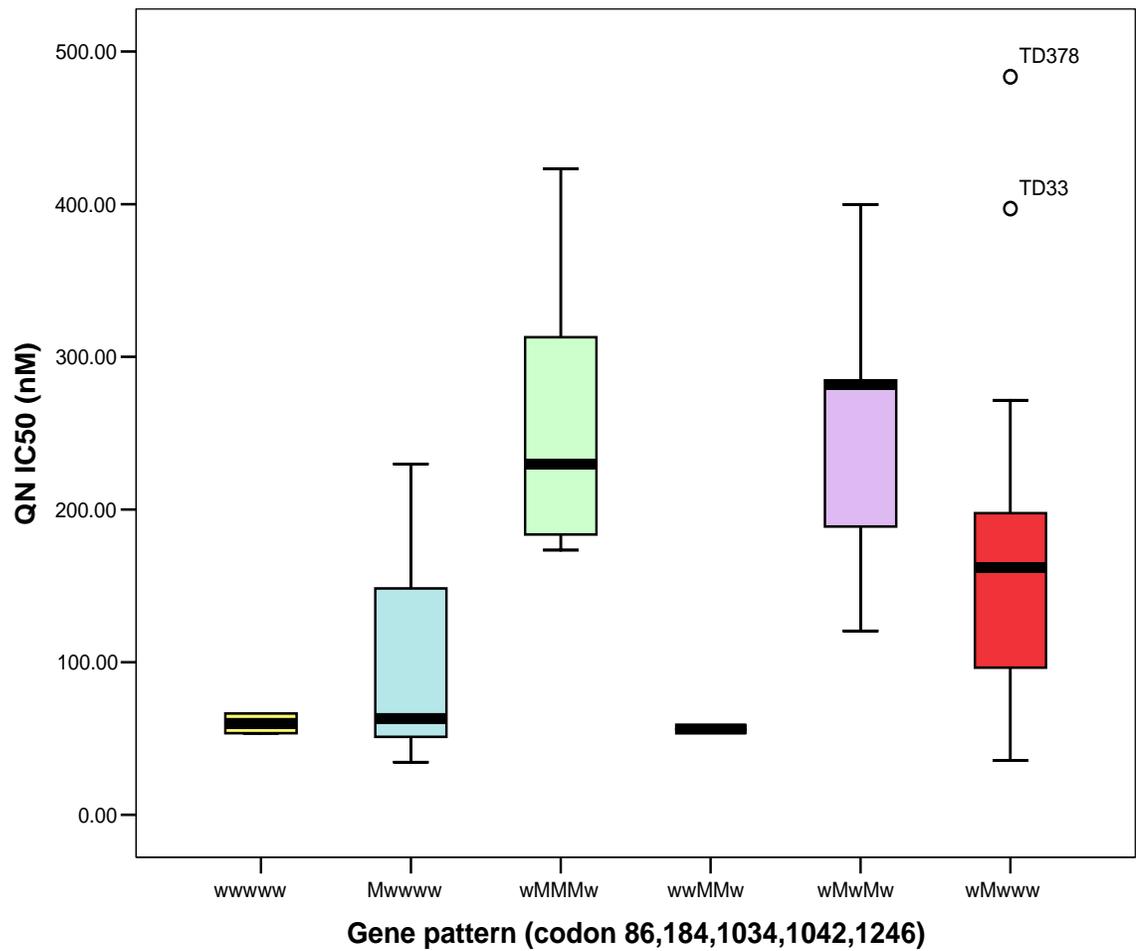


Figure 38 Box-plot presented association between multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P.falciparum* isolates to QN; data are presented as mean of IC₅₀ values [One-way ANOVA test (Scheffé)]. The parasite isolate name TD33 and TD378 were outlier from rang of box-plot. M = Mutant type, W = Wild type

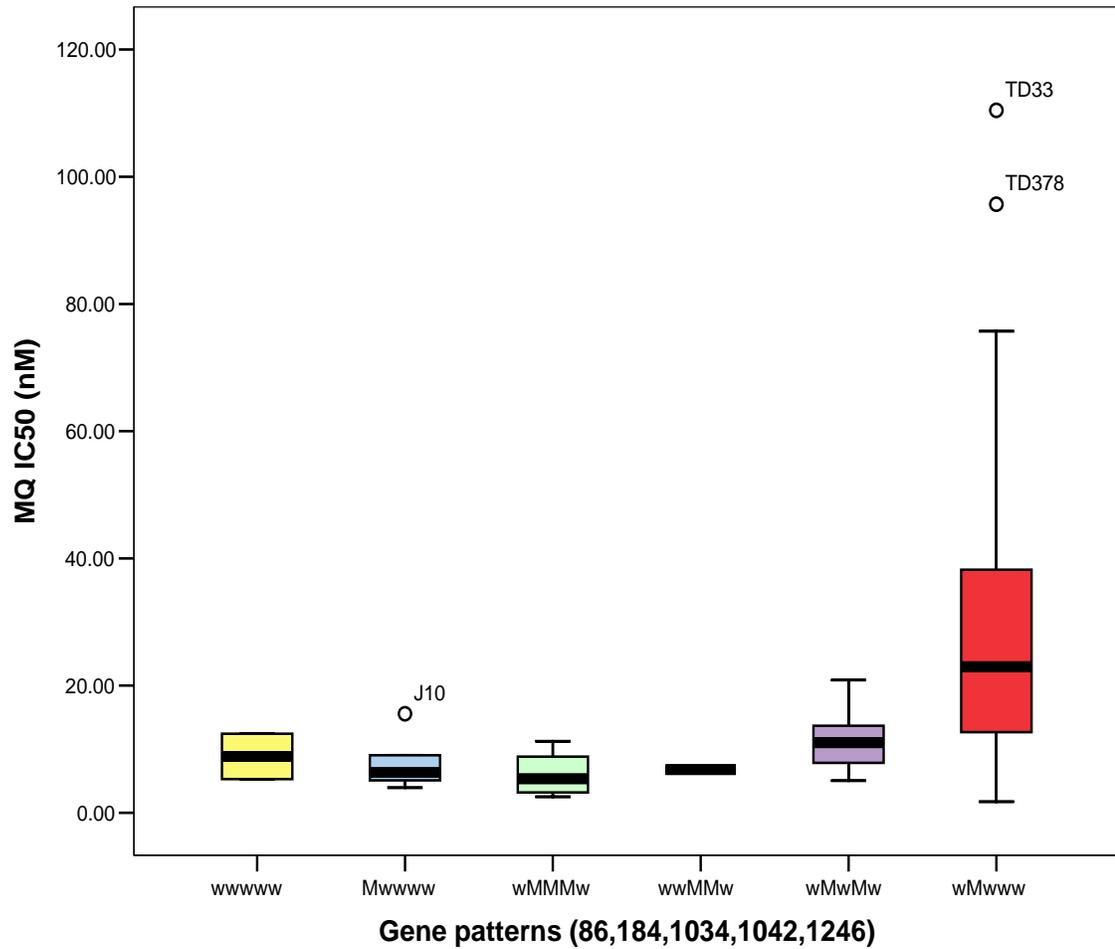


Figure 39 Box-plot presented association between multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P.falciparum* isolates to MQ; data are presented as mean of IC₅₀ values [One-way ANOVA test (Scheffé)]. The parasite isolate name J10, TD33 and TD378 were outlier from rang of box-plot. M = Mutant type, W = Wild type

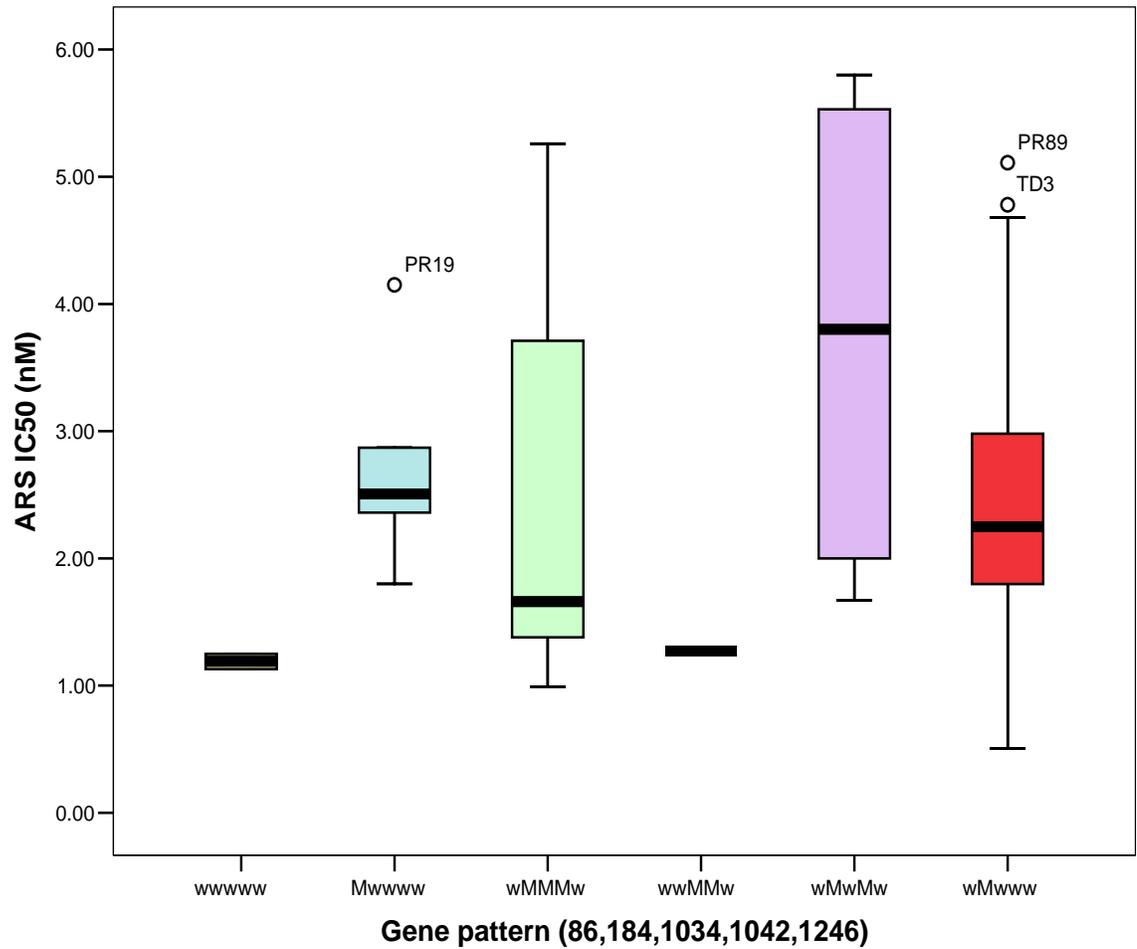


Figure 40 Box-plot presented association between multiple point mutation patterns of *pfmdr1* gene at codon 86, 184, 1034, 1042 and 1246 on the sensitivity of *P.falciparum* isolates to ARS; data are presented as mean of IC₅₀ values [One-way ANOVA test (Scheffe)]. The parasite isolate name PR 19, PR 89 and TD3 were outlier from rang of box-plot. M = Mutant type, W = Wild type

6.3 Determination of the molecular markers influencing *in vitro* drug susceptibility level.

Univariate and multivariate analysis were used to determine the predictor of CQ and MQ resistance. The level of CQ resistance was categorised into moderately resistant (25-99 nM) and highly resistant (≥ 100 nM) according to Pickard's criteria. Multivariate analysis showed that the copy number of the *pfmdr1* gene was the only predictor of the level of CQ resistance (**Table 23**). Those parasites containing one copy number of the *pfmdr1* gene had 5.6 times more likely to be highly CQ-resistant parasites. We also categorized parasites into 2 groups according to their responding to MQ *i.e.*, MQ sensitive (<20 nM) and MQ resistance (≥ 20 nM). In contrast to CQ resistance, only the mutation at 1042 was the predictor for mefloquine resistance (**Table 24**). The parasites containing 1042D were approximately 16 times more likely to be MQ-sensitive parasites.

Table 23 Association of *pfmdr1* copy number and *pfert* and *pfmdr1* gene polymorphisms with CQ susceptibility; data are presented as crude odds ratio, 95% CI, *p* value and adjust odds ratio.

Characteristic	Moderately resistant (%)	Highly resistant (%)	Crude Odds ratio	95% CI	<i>p</i> value	Adjust Odds ratio	95% CI	<i>p</i> value
K76T								
K76	1 (100)	0	0.7	0.9-1.0	0.4	0.9	-	1
76T	28 (58)	20 (42)						
I356T								
I356	2 (100)	0	1.5	0.8-1.0	0.2	0	-	0.9
356T	27 (57)	20 (43)						
N86Y								
N86	25 (57)	19 (43)	0.3	0.03-3.1	0.3	8.1	0.3-168.1	0.1
86Y	4 (80)	1 (20)						
Y184F								
Y184	5 (63)	3 (37)	1.2	0.2-5.6	0.8	2.8	0.3-22.3	0.3
184F	24 (59)	17 (41)						
S1034C								
S1034	26 (67)	13 (33)	4.6	1.0-21.1	0.03	0.4	0.03-3.05	0.6
1034C	3 (30)	7 (70)						
N1042D								
N1042	23 (68)	11 (32)	3.1	0.8-11.0	0.07	0.2	0.06-1.05	0.06
1042D	6 (40)	9 (60)						
Copy number								
>1	18 (78)	5 (22)	4.9	0.05-0.7	0.01	5.6	1.4-21.9	0.012
1	11 (42)	15 (58)						

Table 24 Association of *pfmdr1* copy number and *pfprt* and *pfmdr1* gene polymorphisms with MQ susceptibility; data are presented as crude odds ratio, 95% CI, *p* value and adjust odds ratio.

Characteristic	Sensitive (%)	Resistant (%)	Crude Odds ratio	95% CI	<i>p</i> value	Adjust Odds ratio	95% CI	<i>p</i> value
K76T								
K76	2 (100)	0	1	0.8-1.02	0.3	1.08	-	1
76T	32 (67)	16 (33)						
I356T								
I356	3 (100)	0	1.5	0.8-1.01	0.2	1.0	-	1
356T	31 (66)	16 (34)						
N86Y								
N86	28 (64)	16(36)	0.3	0.7-0.9	0.07	1.9	-	1
86Y	6 (100)	0						
Y184F								
Y184	9 (100)	0	5.7	0.6-9.0	0.02	0	-	0.9
184F	25 (61)	16 (39)						
S1034C								
S1034	24 (60)	16 (40)	0.7	0.5-0.8	0.01	0.008	1.8-142.4	0.9
1034C	10 (100)	0						
N1042D								
N1042	20 (57)	15 (43)	0.09	0.01-0.8	0.01	16.25	0.2-5.5	0.012
1042D	14 (93)	1 (7)						
Copy number								
>1	18 (75)	6 (25)	2	0.1-1.7	0.3	1.2	0.15-1.7	0.78
1	16 (62)	10 (38)						

|

