CHAPTER V
RESULTS

1. Effect of temperature on malarial growth and development

To investigate the influence of mimicking febrile temperature on parasite growth and development, the culture of 5% parasites with highly synchronous ring stage *P. falciparum* (synchronized 2–4 hours post-invasion) was subjected to a temperature shift pattern at 41°C (phase A) for 2 hours, followed by incubation at 37°C (phase B) for an additional 18 hours. The culture was then shifted to the temperature of 41°C (phase C) for 4 hours before turning down to 37°C (phase D) for 24 hours. This group was used as heat-shock group (HS). The experiments performed under the temperature of 37°C of all phases at different incubation time of 2, 20, 24 and 48 h (phase A, B, C and D) was used as non-heat shock group (non-HS) or control group (**Figure 10**). After incubation, the number of parasites was examined under light microscope following Giemsa staining. The parasite development at the end of each phase (A, B, C and D) was measured and the comparison of parasite number was made in order to examine the effect of temperature on overall asexual development in cloning strain, K1 and 3D7, and five field isolated strains.

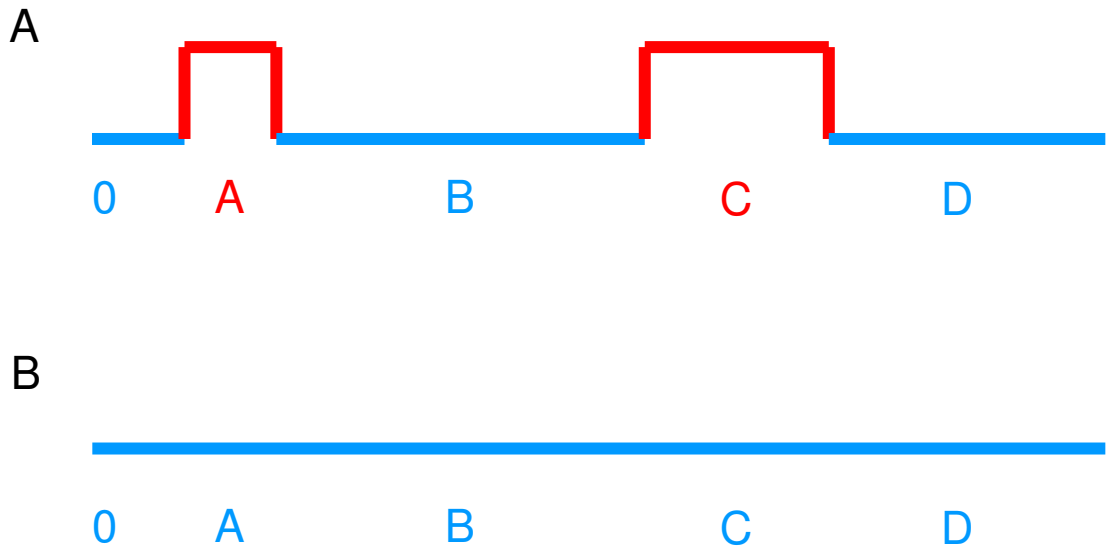


Figure 10 The schematic pattern of temperature for heat shock (10A) and non-heat shock group (10B). The blue line represented the culture temperature at 37°C and the red line represented the culture temperature at 41°C.

1.1 Effect of temperature on malarial growth and development on *P. falciparum* standard strains

The standard strains used in this experiment were K1 and 3D7 strain. The K1 is chloroquine (CQ) resistant strain and 3D7 is CQ sensitive strain. The K1 and 3D7 strains used in this study contained only young intraerythrocytic parasites (ring) as shown in **Figure 12A.** The non-HS group was cultured at 37°C and the pattern of temperature shift in HS group was ranged from 37°C to 41°C at ring stage (Phase A) and trophozoite stage (Phase C) for 2 and 4 hours, respectively.

The results showed that at phase 0, the parasites were at the same stage even in the HS group after incubation at 41°C for 2 hours. The parasite number, development and morphology were examined at the end of Phase A by thin smear and Giemsa staining under light microscope. The parasite number of K1 and 3D7 strains was not decrease. At phase 0, the number of K1 and 3D7 parasites in HS group after incubation at 41°C strains was 5 x 10^5 parasite infected red blood cell (PIRBC)/1µl pack red blood cell (PRBC), which was the same as in non-HS group. Both groups (HS and non-HS group) of parasites were also the same (**Figure 12**).

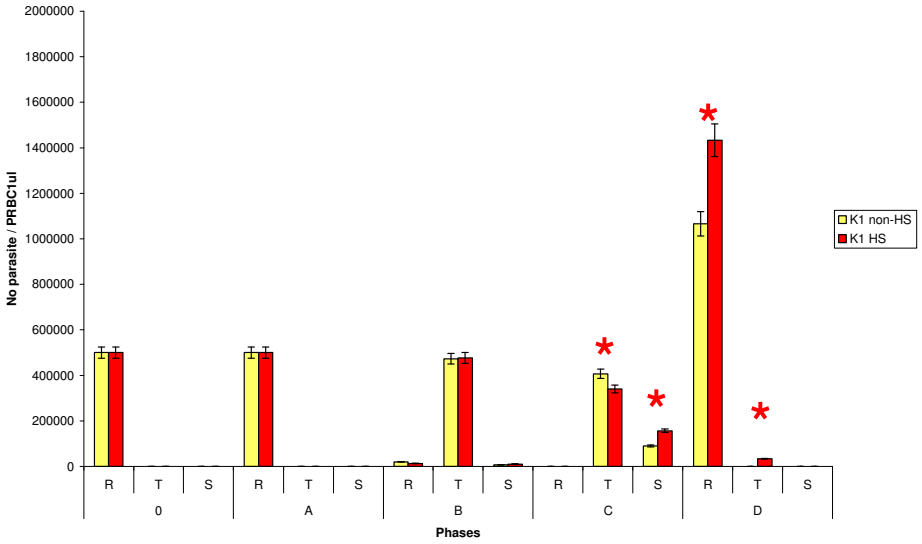
When the temperature was down from 41°C to 37 °C for 18 hours (Phase B), the K1 strain of HS group and non-HS group could maintain the total number of parasites at 5 x 10^5 PIRBC/ 1µl PRBC (p = 0.423 at 95% CI). The total number of parasites of 3D7 HS group was decreased that is the number of parasite of non-HS group was 5 x 10^5 PIRBC/ 1µl PRBC whereas in the HS group was 4.9 x 10^5 PIRBC/ 1µl PRBC. The mean value of PIRBC of 3D7 non-HS and 3D7 HS group was not significant different (p = 0.184 at 95% CI). In addition, the morphology of HS and non-HS group of K1 strain at Phase B was the same whereas some parasites of strain 3D7 died (**Figure 11 and Figure 12**).

At Phase C, the HS group was exposed to 41°C for 4 hours after medium changing at the end of Phase B. During Phase C, the parasites were trophozoite stage. At the end of Phase C, the parasite development and morphology were examined. The results demonstrated that HS group of K1 grew almost as the same as control (non-HS). It was caused firstly by the total number of parasite of K1 strain in both groups (non-HS and HS group) are not different (4.97 x 10^5 PIRBC/ 1µl PRBC) whereas the

total number of parasite in 3D7 non-HS and HS group was 4.97 x 10^5 and 3.90 x 10^5 PIRBC/ 1µl PRBC, respectively. The total number of parasites between 3D7 non-HS and HS group are significant different (p = 0.001 at 95% CI). Secondly, K1 and 3D7 showed the same results between each stage. Non-HS and HS group at trophozoite stage showed higher number of parasite when compared to that of schizonts stage and the number of parasite at schizont stage of HS was significantly higher than that of non-HS (p_{K1} = 0.032, p_{3D7} = 0.06 at 95% CI) (**Table 6**). Finally, the morphology of K1 and 3D7 parasite of non-HS and HS were not different (**Figure 13**)

At the end of phase C, the parasite was cultured at 37°C for 24 hours (Phase D) and the rate of reinfection and development was determined as shown in **Table 6**. The parasite of HS group of both strains developed to trophozoite while the parasite of non-HS group did not develop to tropozoite (**Figure 11**).

A



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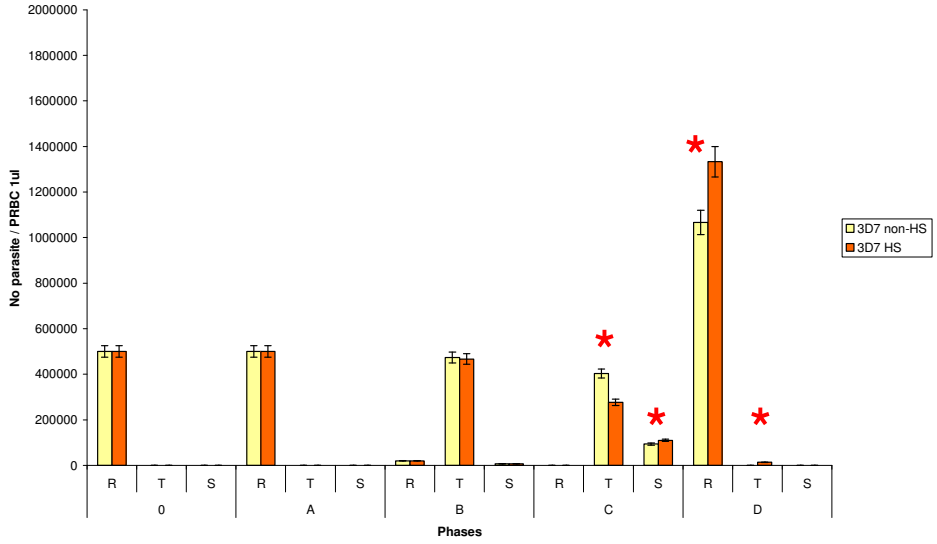


Figure 11 The graph shows the number of parasite in different stages of each phase. Figure A and B show the parasite number of K1 and 3D7 strain, respectively. The red star indicates that the parasite numbers are significantly different at 95%CI.

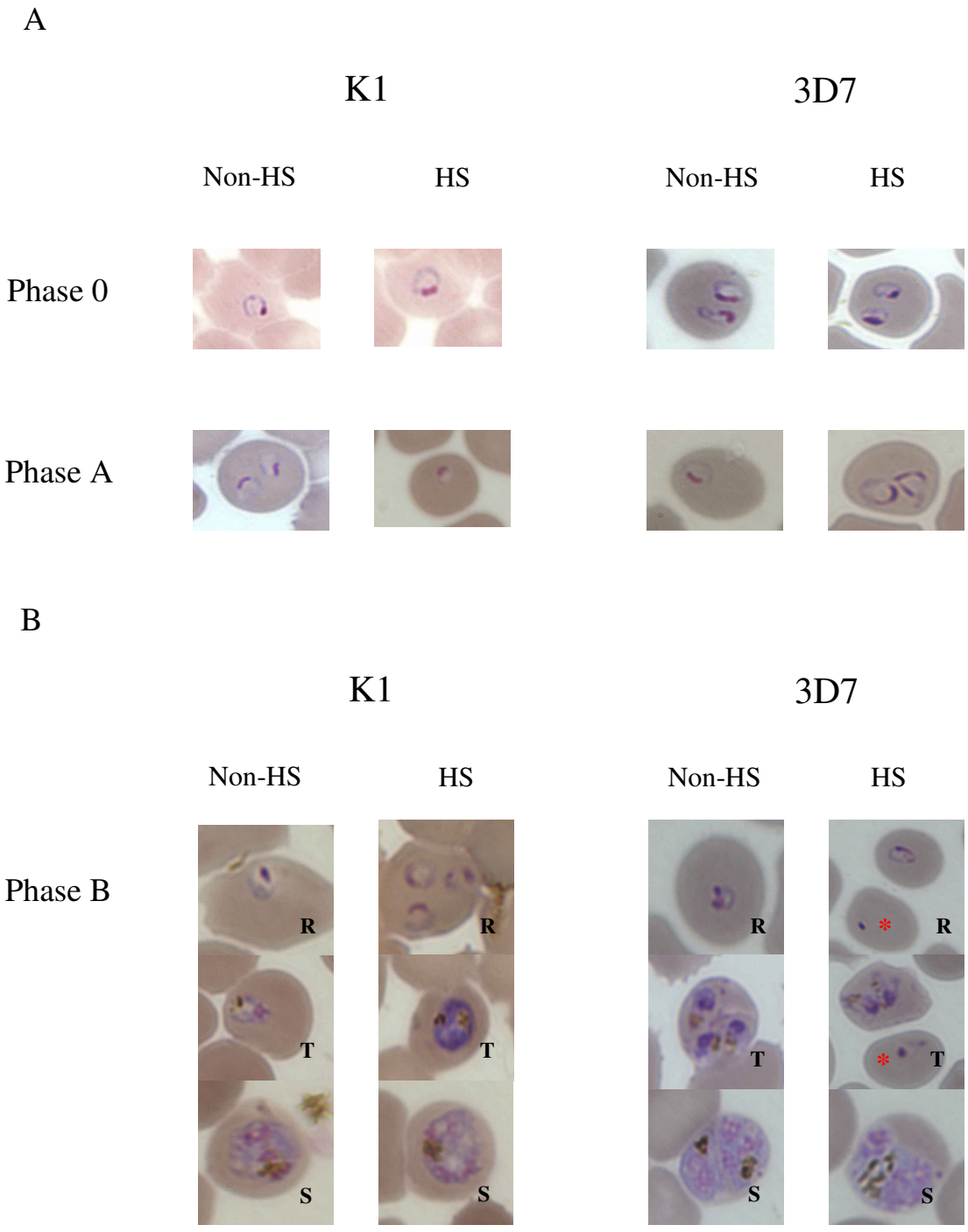


Figure 12 The morphology of K1 and 3D7 strain at Phase 0, Phase A and Phase B. Figure 12A shows the parasite at ring stage of Phase 0 and Phase A. Figure 12B shows the parasite development in three different stage which are ring (R), trophozoite (T), schizonts (S). The red star indicates died parasite.

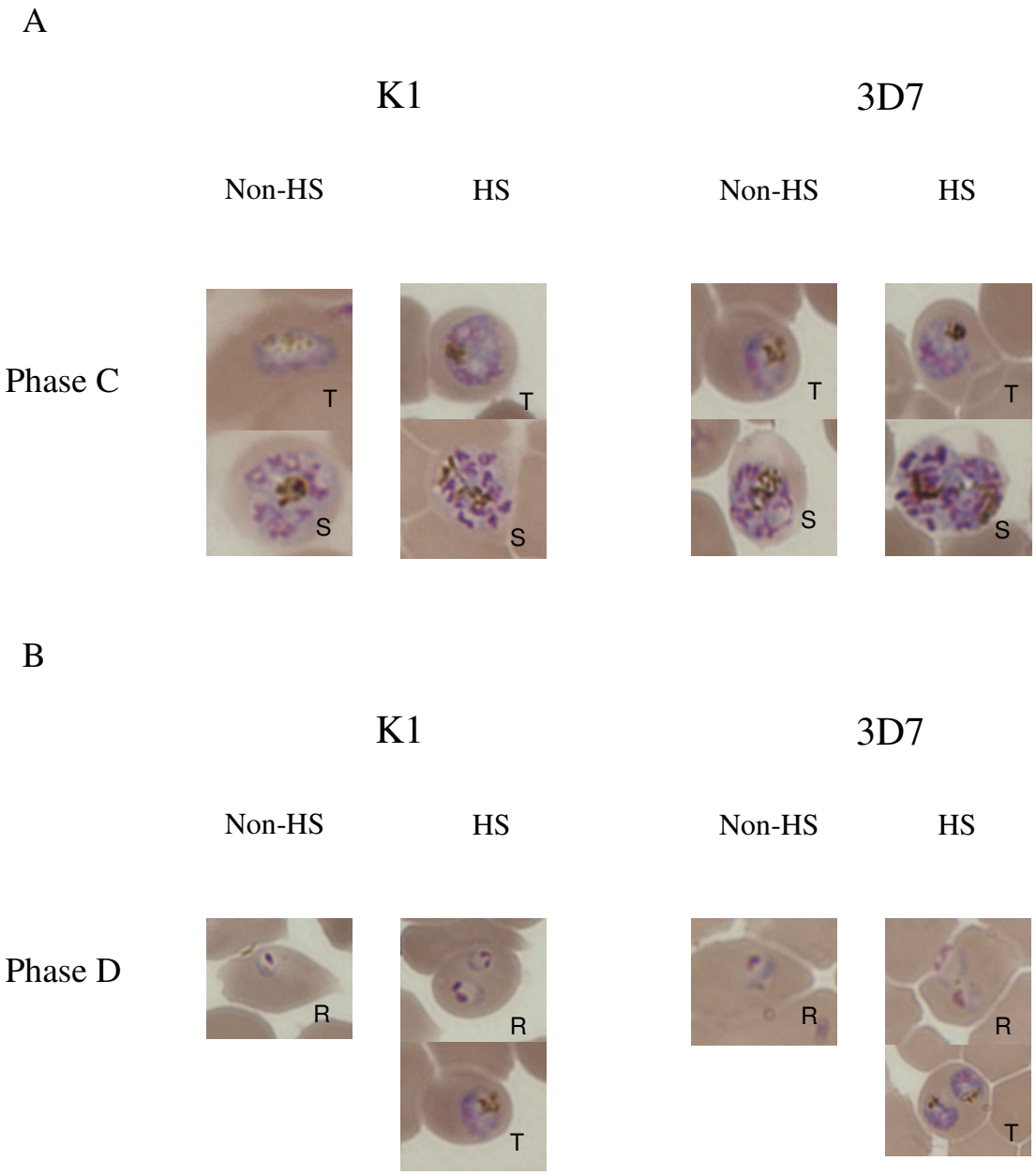


Figure 13 The morphology of K1 and 3D7 strain at Phase C (A) and Phase D (B). The parasite at trophozoite and schizonts stage are shown in Phase C and the parasite reinfection and development in new cycle which are ring (R) and trophozoite (T) are shown in Phase D.

Table 6 The reinfection ratio of K1 and 3D7 strain of both non-HS and HS group. The *p* value represents the correlation between non-HS and HS which is significantly different.

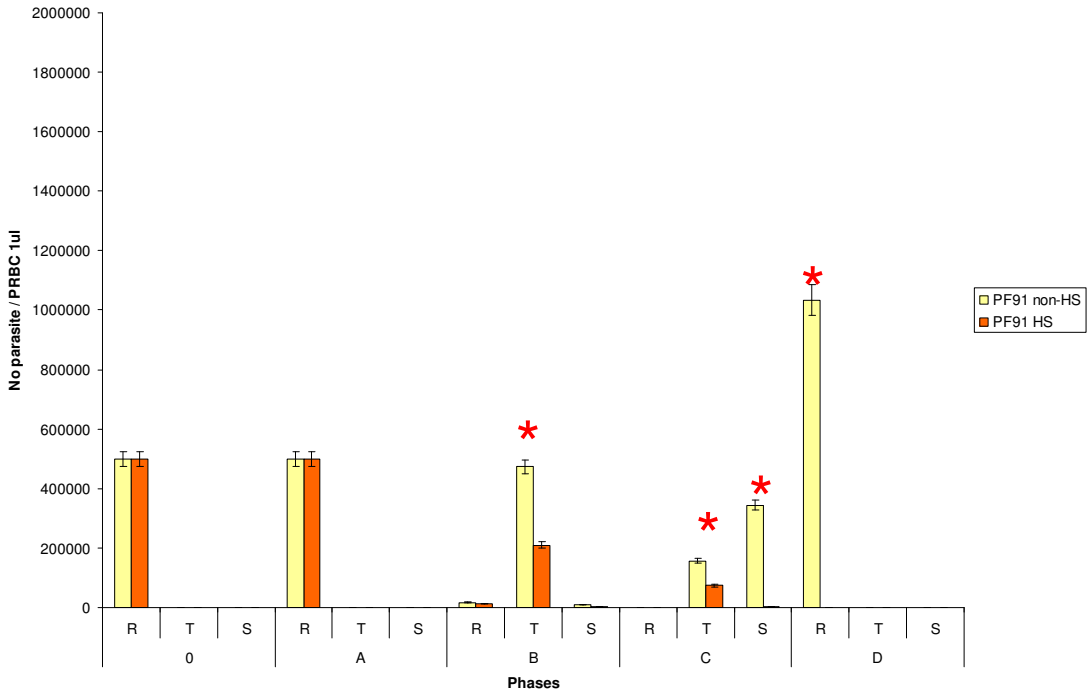
	Reinfection ratio	*p* value at 95% CI between non-HS and HS
K1 non-HS	2.1456	
K1 HS	3.4870	0.04
3D7 non-HS	2.1483	
3D7 HS	2.9863	0.05

1.2 Effect of temperature on malarial growth and development on *P. falciparum* filed isolates

In order to determine the effect of temperature on field isolated strains, five isolates namely PF91, PF112, PF235, TMPF224 and TMPF 225 from Tak province, Thailand were used. The young intraerythrocytic parasites (ring) of five isolates were prepared and then cultured at 37°C (non-HS). The HS group was cultured under the same pattern of temperature shift including Phase A, B and C as shown in **Figure 9**. The results showed that at phase 0, all parasites were at the same stage. At the end of Phase A of HS group, the, parasite development and morphology were examined by thin smear and Giemsa staining under light microscope. Fives isolates showed the same results and similar to those of standard strains (K1 and 3D7) that is the parasite number was not decrease after incubation at 41°C for 2 hours in HS group and the number of parasite at phase 0 of all isolates were 5 x 10^5 PIRBC/ 1μl PRBC. The parasite number of all isolates (HS and non-HS) was 5 x 10^5 PIRBC/1μl PRBC and the morphology of parasite from HS was identical to that of non-HS. The parasite number of all phases and parasite morphology of Phase 0 and Phase A are shown in **Figures 14-16**.

After incubation at 37°C for 18 hours (Phase B), the total parasite of HS group of all isolates was decreased when compared to that of non-HS group. The total parasite of HS group of isolate PF91, PF112, PF235, TMPF224 and TMPF225 was equal to 2.26x10^5, 1.96x10^5, 1.96x10^5, 2.47x10^5, and 2.27x10^5 PIRBC/ 1μl PRBC, respectively whereas the parasite number of non-HS of all isolates was equal to 5 x 10^5 PIRBC/1μl PRBC. At Phase B, the morphology of ring stage non-HS group was different to that of Phase A. The ring stage in both groups, non-HS and HS, showed very thin rim of cytoplasm (**Figures 17-19**). However, non-HS group at Phase B showed prominent central vacuole but thicker rim of cytoplasm and HS group showed formed pyknotic ring. The results are the same in all five isolates.

A



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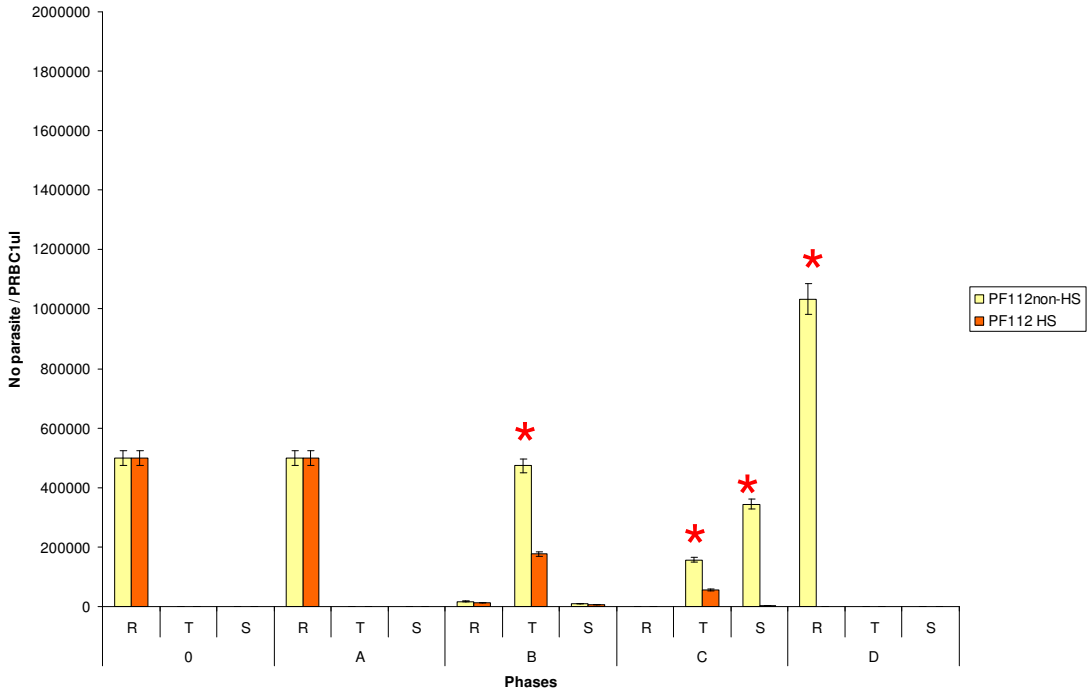
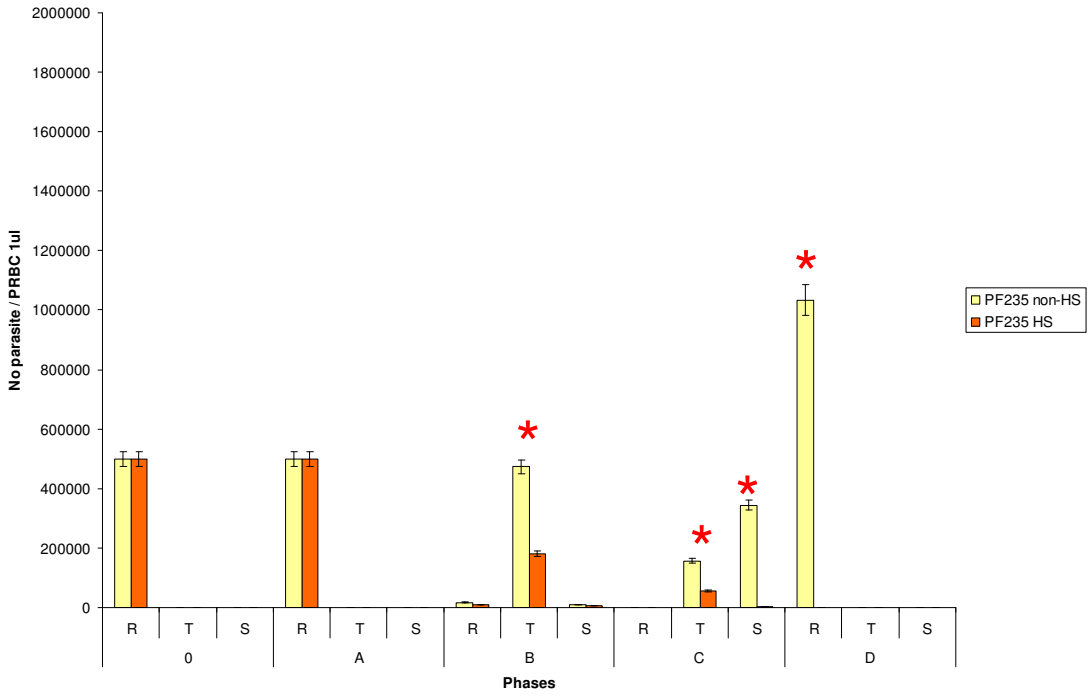


Figure 14 The graph shows the number of parasite in different stage at each phase of isolate PF91 (A) and PF112 (B). The red star indicates the significantly different in the number of parasite at 95%CI.

A



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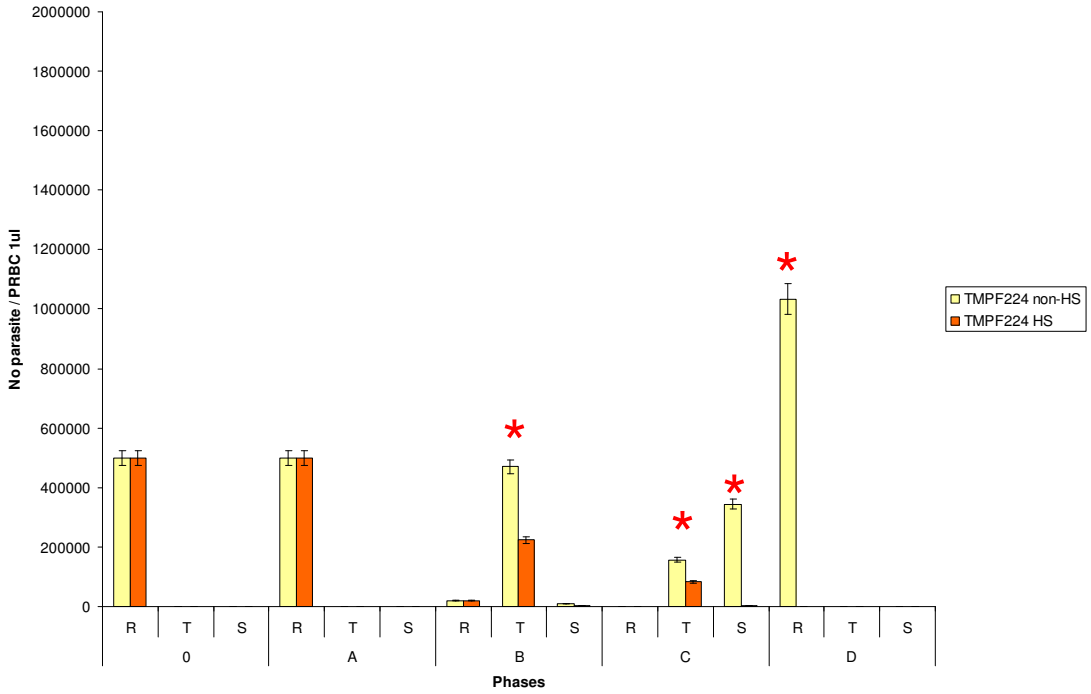


Figure 15 The graph shows the number of parasite in different stage at each phase of isolate PF235 (A) and TMPF224 (B). The red star indicates the significantly different in the number of parasite at 95% CI.

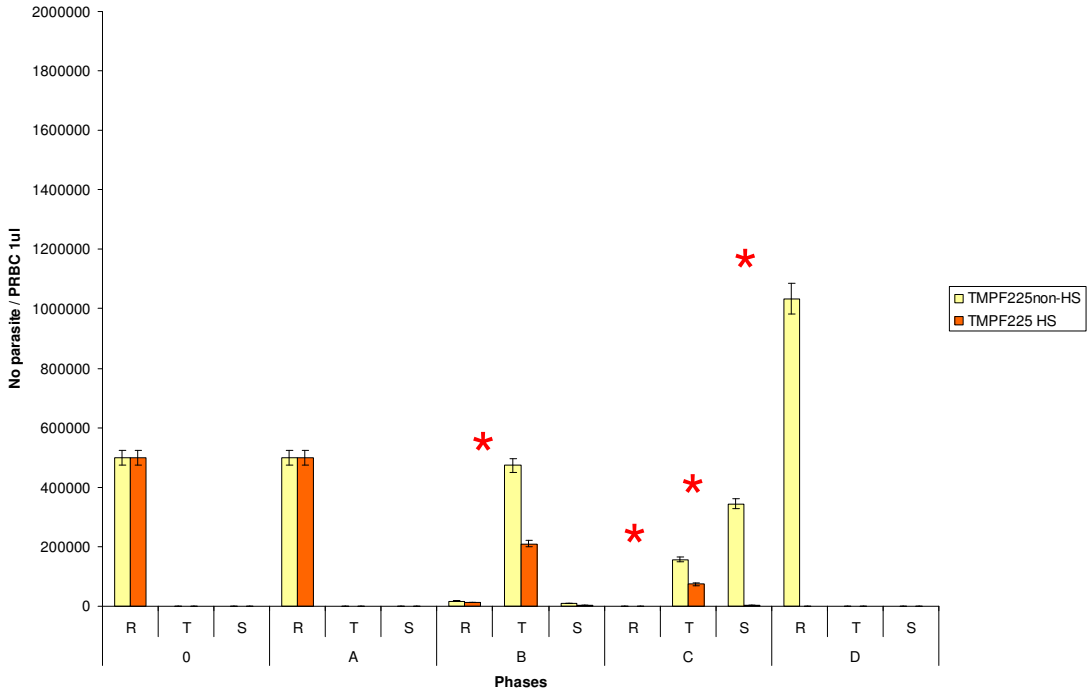


Figure 16 The graph shows the number of parasite in different stage at each phase of isolate TMPF225. The red star indicates the significantly different in the number of parasite at 95% CI.

After exposure to 41°C for 4 hours during the second half of cycle (Phase C), trophozoite stage developed to schizonts and schizonts developed further to ring. The morphology of these parasites formed pyknotic trophozoite and hyposegmented schizonts. Parasitaemia in the HS cultures remained markedly depressed after a further 18 hours of incubation at 37°C and 4 hours at 40°C as shown in **Table 7** and **Figures 17-19**. Whereas non-HS showed healthy trophozoite and schizonts. The total parasite number was similar when compared among isolates and cloning strains (K1 and 3D7).

When the parasite of both group was cultured to 37°C (Phase D), non-HS group formed new ring infection while HS group were diminished of new ring and almost parasites did not propagate. The non-HS showed higher rate of infection than HS in every isolates (**Table 8** and **figures 17** and **19**)

Table 7 The total parasite number of isolate PF91, PF112, PF235, TMPF224 and TMPF 225 (non-HS and HS). The *p* value represents the correlation between non-HS and HS group which is significantly different.

	PF91		PF112		PF235		TMPF224		TMPF225	
	non-HS	HS	non-HS	HS	non-HS	HS	non-HS	HS	non-HS	HS
Phase 0	5	5	5	5	5	5	5	5	5	5
Phase A	5	5	5	5	5	5	5	5	5	5
Phase B	5	2.27	5	1.96	5	1.96	5	2.47	5	2.27
Phase C	5	0.764	5	0.59	5	0.59	5	0.864	5	0.764
Phase D	10.33	0.001	10.33	0.012	10.33	0.012	10.33	0.013	10.33	0.102

Table 8 The rate of reinfection of isolate PF91, PF112, PF235, TMPF224 and TMPF 225 (non-HS and HS). The *p* value represents the correlation between non-HS and HS group which is significantly different

rate of	PF91		PF112		PF235		TMPF224		TMPF225	
reinfection	non-HS	HS	non-HS	HS	non-HS	HS	non-HS	HS	non-HS	HS
1	2.2	0.018987	2.2	2.2	1200	0.023077	2.2	0.016393	2.2	0.018987
2	2.2	0.016438	2.2	2.2	1200	0.016644	2.2	0.016129	2.2	0.016438
3	1.8	0.013978	1.8	1.8	1300	0.024528	1.8	0.013978	1.8	0.013978
Mean	2.066667	0.016468	2.066667	2.066667	1233.333	0.021416	2.066667	0.0155	2.066667	0.016468

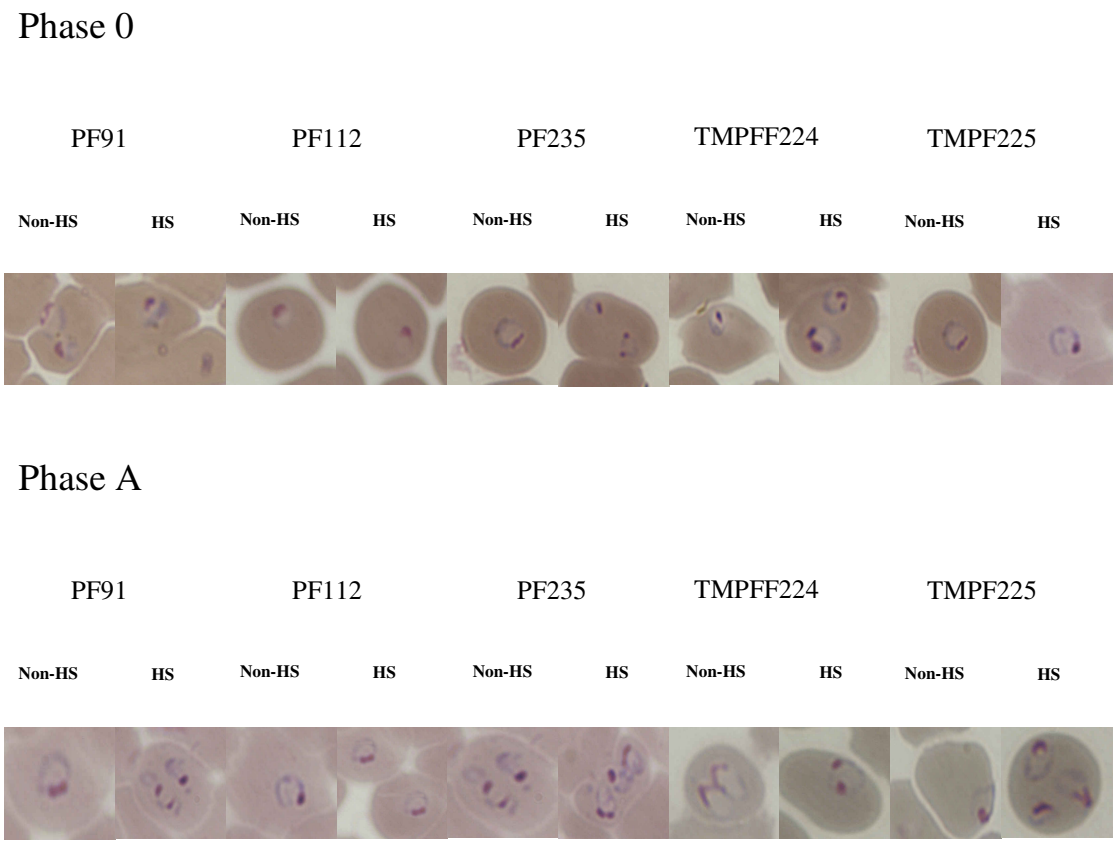
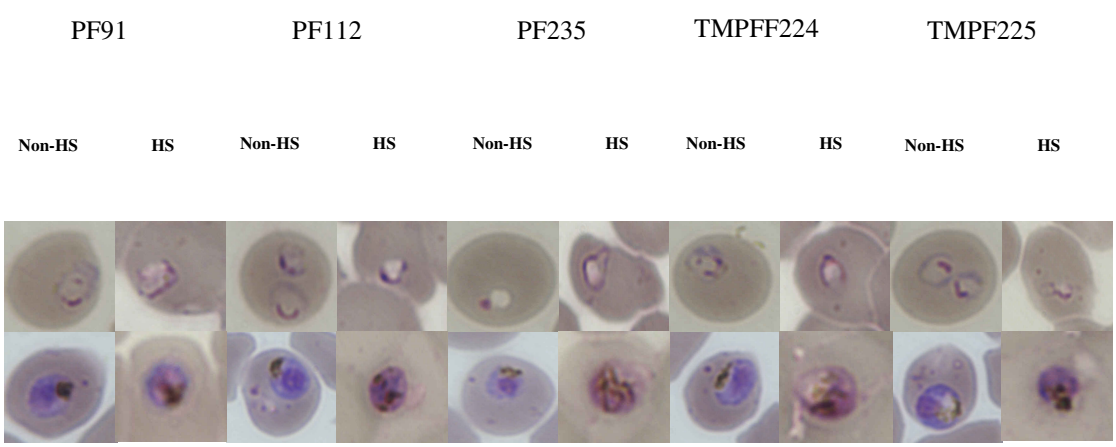


Figure 17 The morphology of ring stage isolate PF91, PF112, PF235, TMPF224 and TMPF 225 at Phase 0 and Phase A

Phase B



Phase C

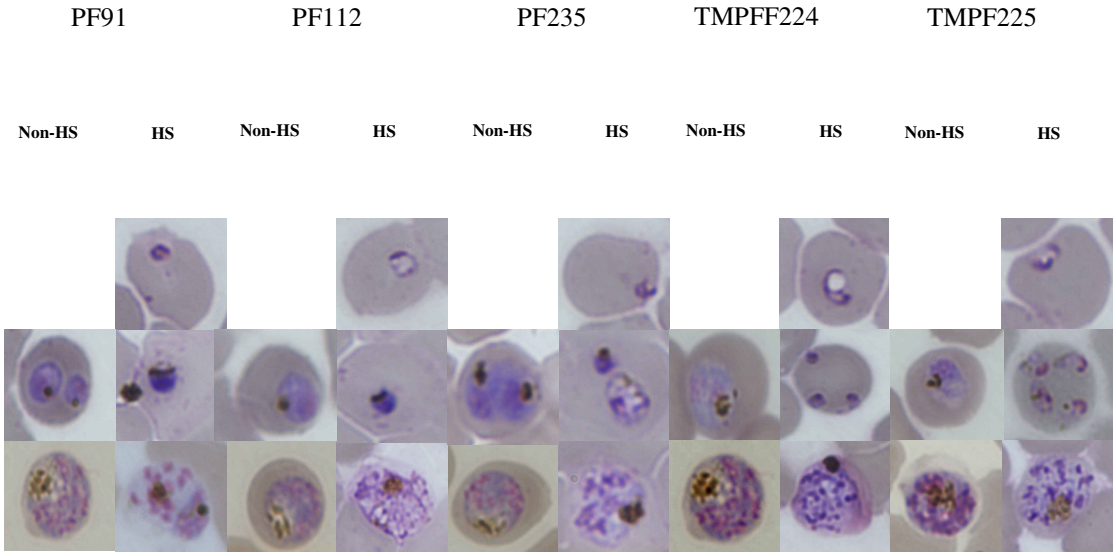


Figure 18 The morphology of isolate PF91, PF112, PF235, TMPF224 and TMPF 225 at Phase B and C

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Phase D

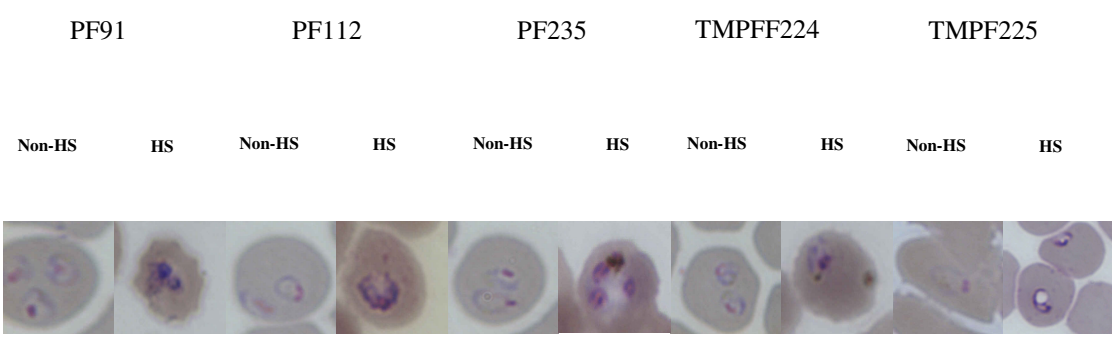

Figure 19 The morphology of isolate PF91, PF112, PF235, TMPF224 and TMPF 225 at Phase D

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2. *In vitro* drug sensitivity assay

In order to investigate the effect of temperature on drug sensibility of malarial parasites, an *in vitro* drug sensitivity assay was done. A total of 7 strains of malaria including two standard strains and five isolates were tested of their susceptibilities to chloroquine (CQ) and artesunate (ARS) when grown under temperature stress. The level of CQ and ARS susceptibility was determined (resistant and sensitive parasite isolates) by using the criteria of Pickard and colleague, (Pickard *et al.,* 2003). CQ susceptibility was categories in to three level, sensitive (S, IC_{50}<25 nM), moderate resistant (MR, IC_{50} equal to 25 to 100 nM) and resistant (R, IC_{50} <100). There was no evidence indicating ARS resistant so far therefore ARS susceptibility was not classified.

The effect of temperature on drug sensitivity (IC_{50}) after heat at phase B was investigated. The results showed that the IC_{50} value of K1 strain grown under HS and non-HS condition was not changed whereas the IC_{50} value of strain 3D7 grown under heat stress was lower than that of non-HS (**Table 9**). Five isolates were affected by temperature shifts to 41°C causing cell damage and died therefore the IC_{50} of HS condition was not be able to calculate. The effect of temperature after heat at phase B and culture back to 37°C for one cycle (new ring infection) was also tested and the results were shown in **Table 10.**

Table 9 The drug susceptibility (IC_{50} value) of standard strain (K1 and 3D7) and isolate PF91, PF112, PF235, TMPF224 and TMPF 225 after HS and non-HS condition

Parasite	Mean IC_{50} of drug susceptibility (nM)	
	CQ	**ARS**
K1 non-HS	100.5	0.4
K1 HS	104.5	0.9
3D7non-HS	11.3	0.4
3D7 HS	4.7	0.7
PE91non-HS	63.6	1.3
PE91 HS	NA	NA
PEF112non-HS	43.2	1.3
PEF112 HS	NA	NA
PE235non-HS	57.2	0.8
PE235 HS	NA	NA
TMPF224non-HS	44.8	1.7
TMPF224 HS	NA	NA
TMPF225non-HS	44.8	1.7
TMPF225	NA	NA

NA is not available

Table 10 The drug susceptibility (IC_{50} value) of standard strain (K1 and 3D7) and isolates PF91, PF112, PF235, TMPF224 and TMPF 225 after HS and non-HS condition at the end of phase B followed by one round of culture at 37^{o}C

Parasite	Mean IC_{50} of drug susceptibility (nM)	
	CQ	**ARS**
K1 non-HS	100.5	0.4
K1 HS	104.5	0.9
3D7non-HS	11.3	0.4
3D7 HS	4.7	0.7
PE91non-HS	63.6	1.3
PE91 HS	63.6	1.0
PEF112non-HS	43.2	1.3
PEF112 HS	43.2	0.8
PE235non-HS	57.2	0.8
PE235 HS	57.2	0.8
TMPF224non-HS	44.8	1.7
TMPF224 HS	44.8	1.7
TMPF225non-HS	48.9	1.4
TMPF225	46.2	1.6

3. Effect of temperature and drug stress on malarial drug development

In order to investigate the effect of temperature and drug stress on malarial drug development, the parasites of non-HS and HS groups were cultured with antimalarial drugs (CQ and ARS). This mimics the condition where malarial parasite grown in patient's body with treatment failure combined with fever. At the beginning, all cultures were grown with drug following their IC_{50} concentration but all of the parasites died. Therefore, the drug concentrations used in the following experiments are as followed: concentration of CQ and ARS are 40 and 1 nM, respectively.

3.1 Effect of temperature with chloroquine (CQ) on malarial drug development

K1 was co-cultured with 40nM chloroquine in the pattern of non-HS and HS. The results show that the total number of parasite was not different at phase 0, A and B while at phase C, the number of trophozoite stage of K1 under non-HS with CQ was higher than that of K1 under HS with CQ. During phase D, the number of schizonts is similar in all conditions whereas the number of ring stage K1 under non-HS with CQ was higher than that of K1 under HS with CQ. There was only K1 strain under HS developed to trophozoite (**Figure 20**).

The morphology of parasite in all conditions (K1 non-HS, K1 HS, K1 non-HS CQ and K1 HS CQ) was almost the same. Only the morphology of some K1 schizonts under HS with CQ at phase C showed some hyposegmented (**Figure 21**).

As shown in **Table 11**, the number of trophozoite stage at phase C in K1 under non-HS and HS without CQ was higher than that of K1 under non-HS and HS with CQ. Whereas the number of schizonts stage in K1 under non-HS and HS with CQ was higher than that of K1 under non-HS and HS without CQ.

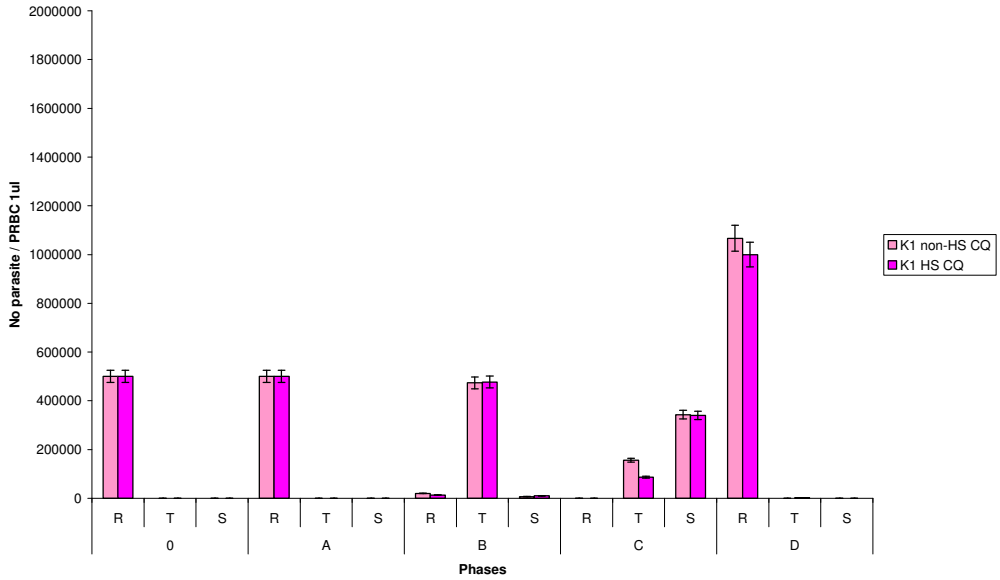


Figure 20 Number of ring (R), trophozoites (T) and schizonts (S) stages of K1 strain under treated with CQ non-heat shock and heat shock condition. Non-HS CQ, non heat shock condition treated with CQ and HS CQ, heat shock condition treated with CQ

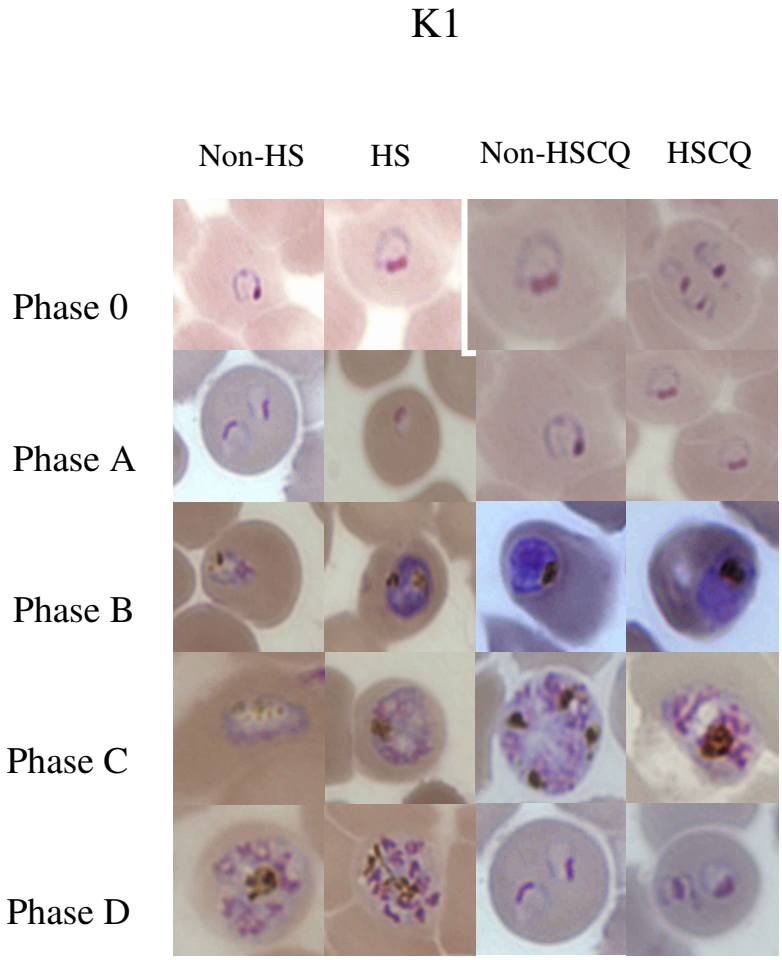


Figure 21 The morphology of malaria parasite, K1 strain treated and untreated with chloroquine under heat and non heat shock condition. Non-HS, not heat shock condition, HS, heat shock condition, non-HS CQ, non heat shock treated with CQ and HS CQ, heat shock treated with CQ

Table 11 The total number of parasite in K1 under non-heat shock (K1 non–HS), K1 under heat shock (K1 HS), K1 under non-heat shock with chloroquine (K1 non –HS CQ) and K1 under heat shock with chloroquine (K1 HS CQ). R is ring, T is trophozoite and S is schizont.

PHASE	**0**			**A**			**B**			**C**			**D**		
	R	T	S	R	T	S	R	T	S	R	T	S	R	T	S
K1 HS	500000	0	0	500000	0	0	13000	476667	10000	0	340000	156667	1433333	33333	0
K1HS CQ	500000	0	0	500000	0	0	13000	476667	10000	0	86667	340000	1000000	2667	0
K1 non-HS	500000	0	0	500000	0	0	20000	473333	6667	0	413333	83333	1066667	0	0
K1 non-HS CQ	500000	0	0	500000	0	0	20000	473333	6667	0	156667	343333	1066667	0	0

3.2 Effect of temperature with chloroquine (CQ) on 3D7 strain development

3D7 was co-cultured with 40nM chloroquine in the pattern of non-HS and HS, like K1. The results show that at phase 0 and A, the total number of parasite was not different whereas at phase B, the total number of parasite was extremely decreased. At phase C, the number of trophozoite stage in 3D7 under non-HS with CQ was higher than that of 3D7 under HS with CQ however the number of schizont was the same. At phase D, the number of ring stage in 3D7 under non-HS with CQ and 3D7 under HS with CQ was very small (**Figure 22**).

The morphology of 3D7 strain under non-HS, 3D7 under HS, 3D7 under non-HS with CQ and 3D7 under HS with CQ at phase 0 and A was almost the same. Only the morphology of 3D7 parasite under non-HS and HS with CQ showed schizonts hyposegmented (**Figure 23**)**.**

As shown in **Table 12**, the number of trophozoite at phase C in 3D7 under non-HS and HS without CQ was higher than that of 3D7 undernon-HS and HS with CQ. Whereas the number of schizonts stage in 3D7 under non-HS and HS with CQ was higher than that of 3D7 undernon-HS and HS without CQ.

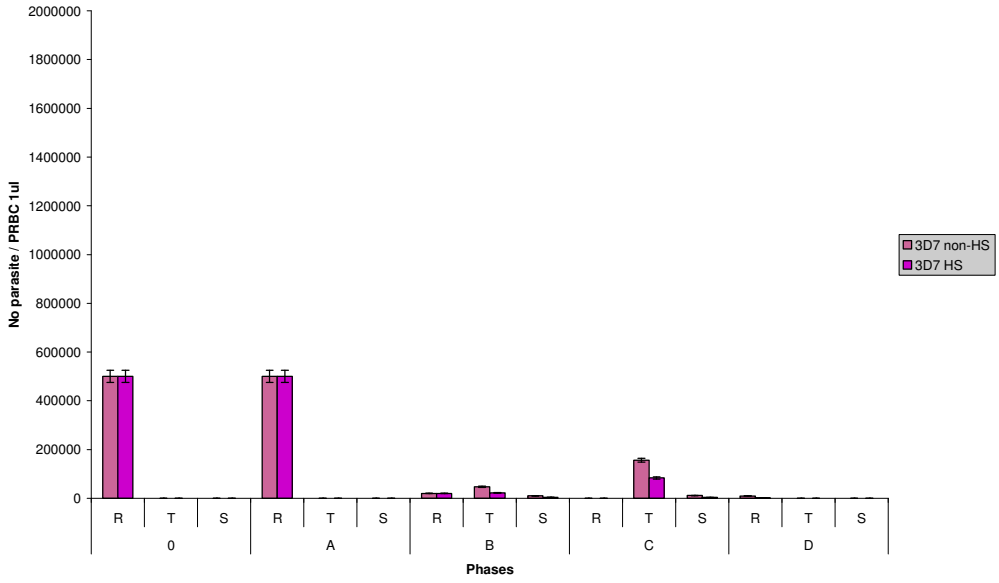


Figure 22 Number of ring (R), trophozoites (T) and schizonts (S) stages of 3D7 strain under treated with CQ non-heat shock and heat shock condition. Non-HS CQ, non heat shock condition treated with CQ and HS CQ, heat shock condition treated with CQ

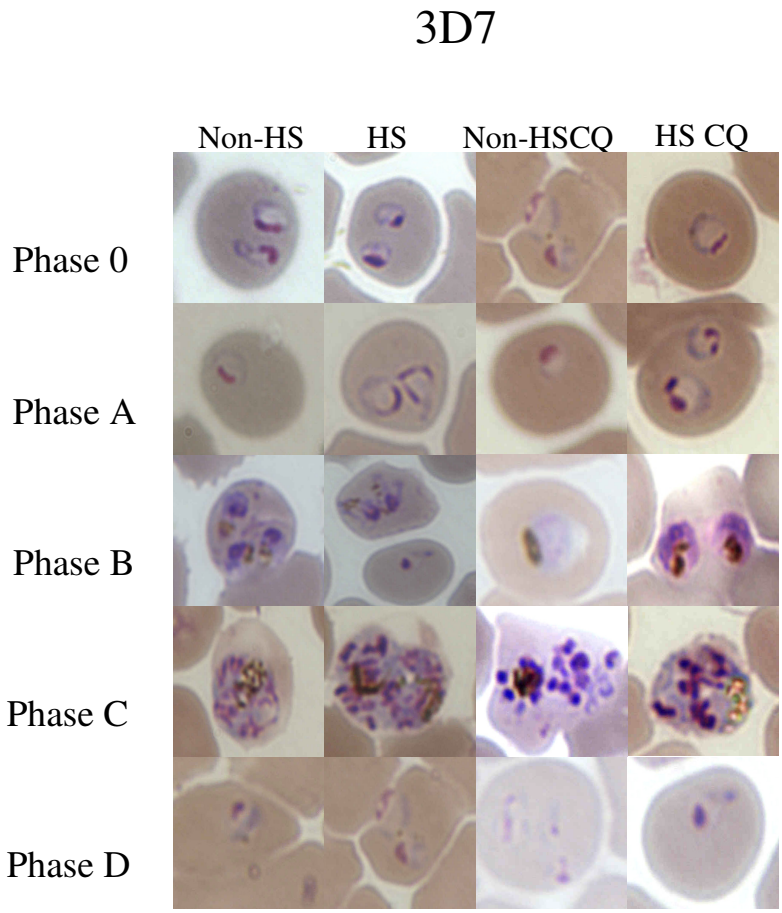


Figure 23 The morphology of malaria parasite, 3D7 strain treated and untreated with chloroquine under heat and non heat shock condition. Non-HS, not heat shock condition, HS, heat shock condition, non-HS CQ, non heat shock treated with CQ and HS CQ, heat shock treated with CQ

Table 12 The total of parasite number of 3D7 under non- heat shock (3D7 non–HS), 3D7 under heat shock (3D7 HS), 3D7 undernon-heat shock with chloroquine (3D7 non –HS-CQ) and 3D7 under heat shock with chloroquine (3D7 HS CQ). R is ring, T is trophozoite and S is schizonts.

PHASE	0			A			B			C			D		
	R	T	S	R	T	S	R	T	S	R	T	S	R	T	S
3D7 HS	500000	0	0	500000	0	0	20000	466667	6667	0	276667	110000	1333333	13333	0
3D7 HS CQ	500000	0	0	500000	0	0	20000	22333	3333	0	83333	3067	1333	0	0
3D7 non-HS	500000	0	0	500000	0	0	20000	473333	6667	0	403333	93333	1066667	0	0
3D7 non-HS CQ	500000	0	0	500000	0	0	20000	47000	10000	0	156667	11000	9333	0	0

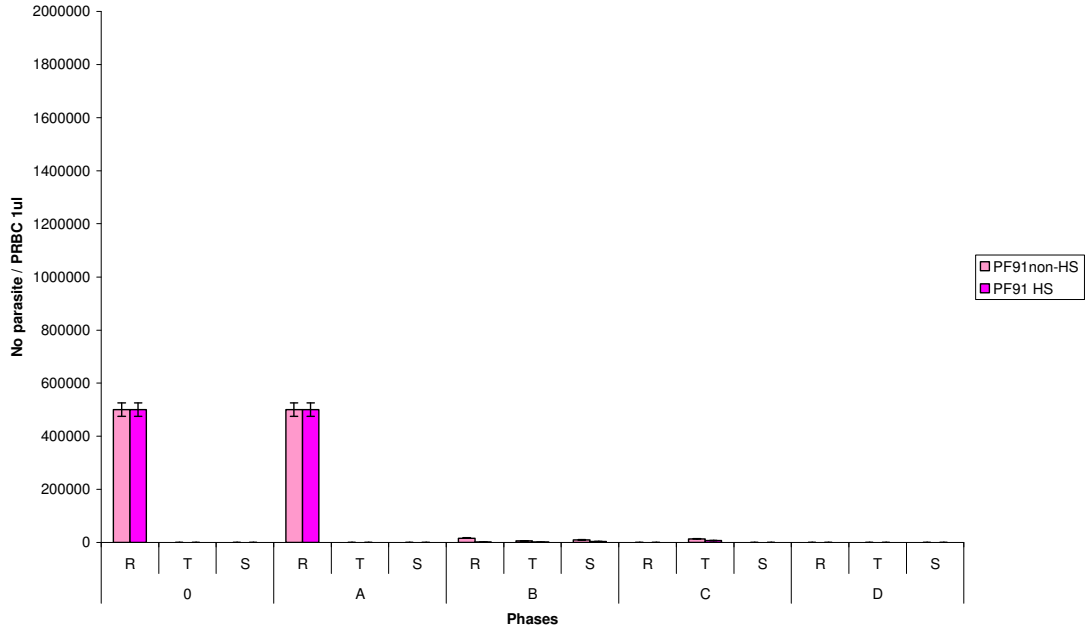
3.3 Effect of temperature with chloroquine (CQ) on fives isolates strain development.

Five isolates, PF91, PF112, PF235, TMPF224 and TMPF225 were co-cultured with 40 nM chloroquine in the pattern of non-HS and HS like standard strain. The results of all isolates are the same. At phase 0 and A, the total number of parasite was not different whereas at phase B, the total number of parasite was extremely decreased. At phase C, the number of trophozoite stage in 3 isolates, PF235, TMPF224 and TMPF225 under non-HS with CQ was higher than of these isolates under HS with CQ. The schizonts stage was found only in isolates PF91 and PF235. At phase D, the number of parasite in all groups was very low as measured by this method (**Figures 24-26**).

The morphology of all isolates under non-HS, HS, non-HS with CQ and HS with CQ at phase 0 and A was almost the same however some died parasites were found at phase B under non-HS and HS with CQ (**Figure 2**7).

As shown in **Table 13**, at phase B, C and D, the number of parasite of all isolates under non-HS and HS with CQ was lower than that of without CQ. Whereas the number of parasite at phase 0 and A was not different in all groups.

A



B

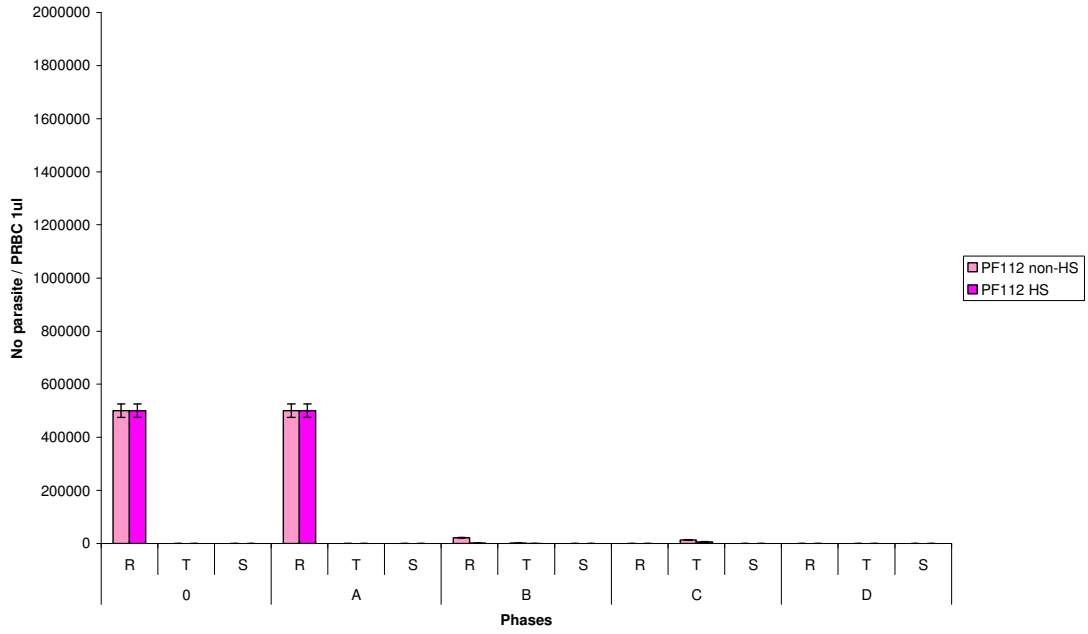
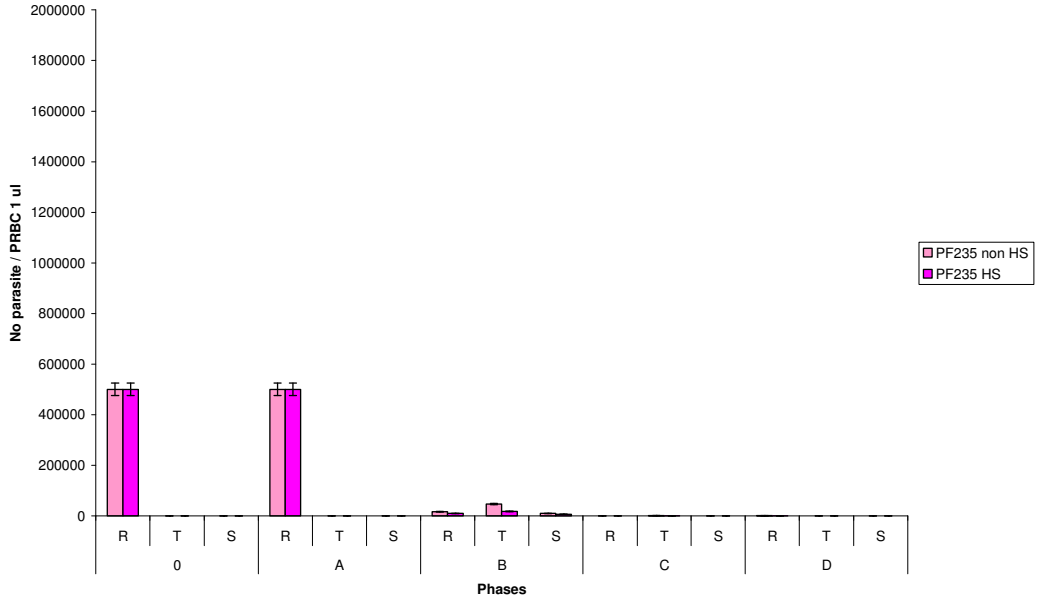


Figure 24 Number of ring (R), trophozoites (T) and schizonts (S) stages of PF91 (A) and PF112 (B) strains under treated with CQ non-heat shock and heat shock condition Non-HS CQ, non heat shock condition under treated with CQ and HS CQ, heat shock condition under treated with CQ

A



B

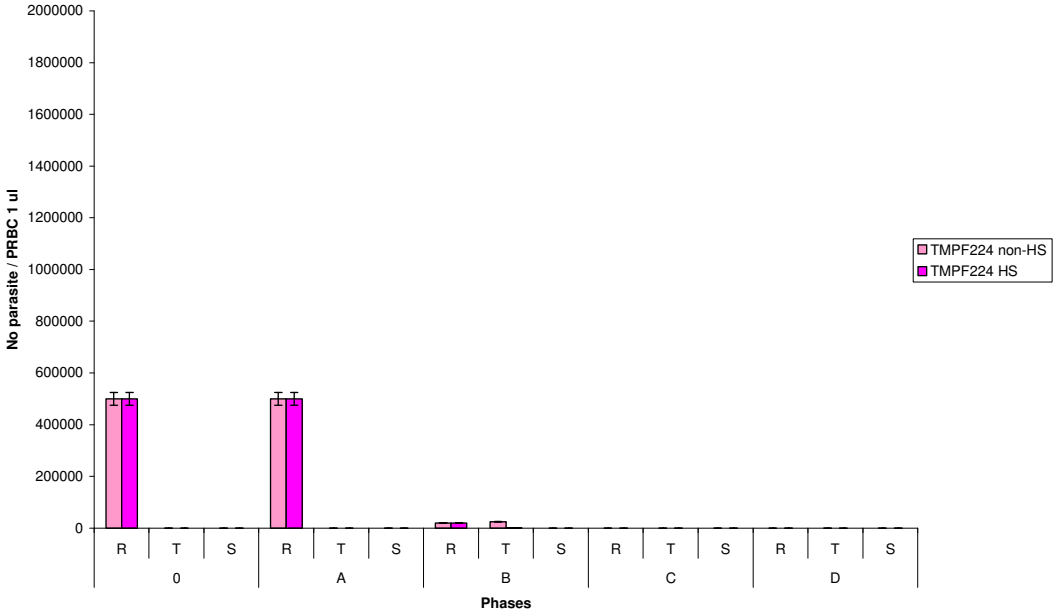


Figure 25 Number of ring (R), trophozoites (T) and schizonts (S) stages of PF235 (A) and TMPF224 (B) strains under treated with CQ non-heat shock and heat shock condition Non-HS CQ, non heat shock condition under treated with CQ and HS CQ, heat shock condition under treated with CQ

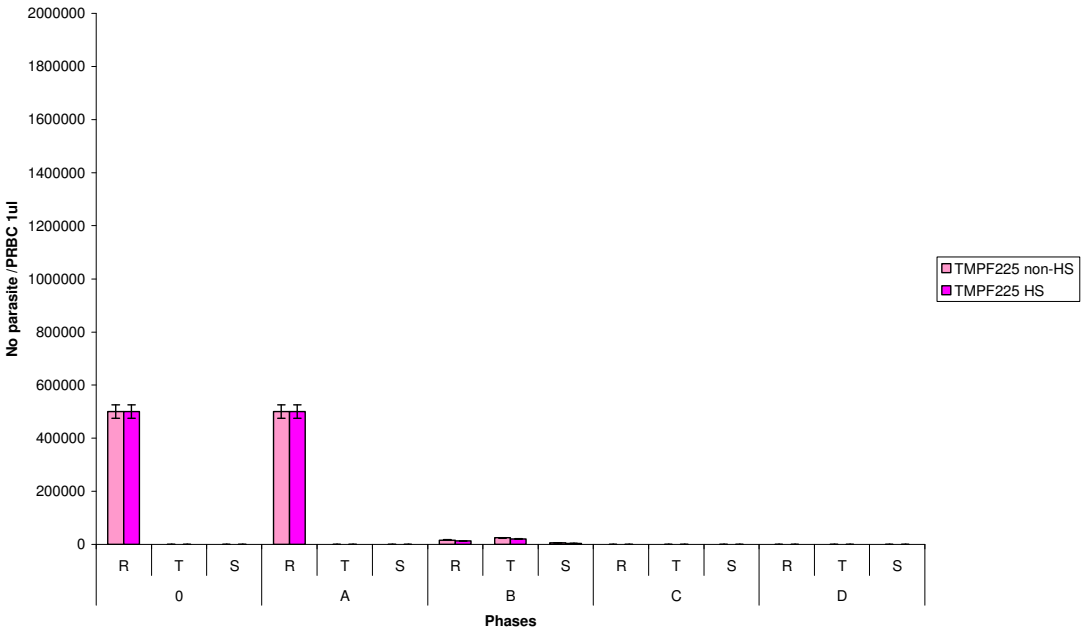


Figure 26 Number of ring (R), trophozoites (T) and schizonts (S) stages of TMPF225 strains under treated with CQ non-heat shock and heat shock condition Non-HS CQ, non heat shock condition under treated with CQ and HS CQ, heat shock condition under treated with CQ

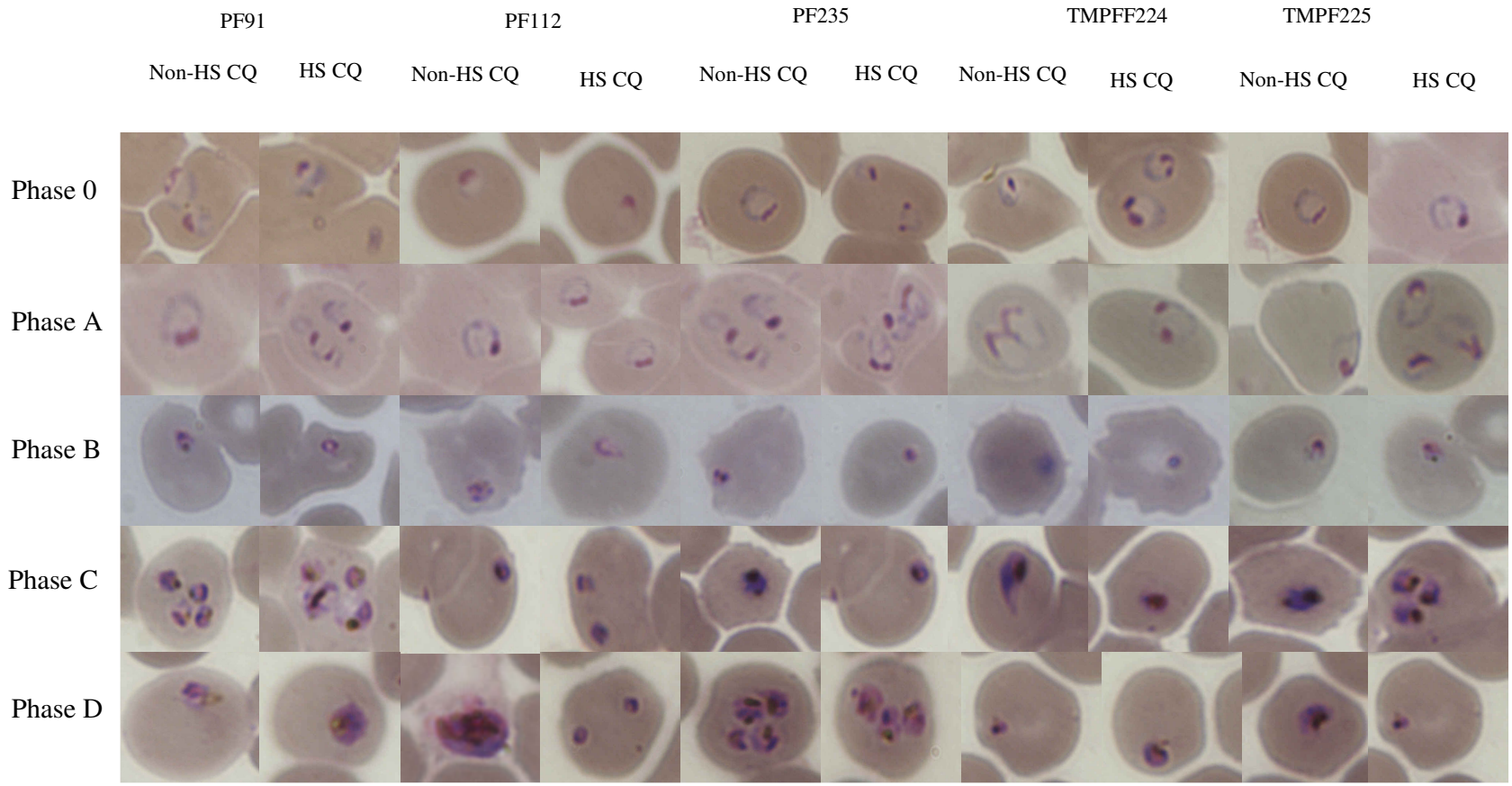


Figure 27 The morphology of malaria parasite, five strains treated and untreated with chloroquine under heat and non heat shock condition. Non-HS, not heat shock condition, HS, heat shock condition, non-HS CQ, non heat shock treated with CQ and HS CQ, heat shock treated with CQ

Table 13 The total number of parasite number of fives isolate under different conditions such as non-heat shock (non–HS), heat shock (HS), non- heat shock with chloroquine (non –HS-CQ) and heat shock with chloroquine (HS CQ). R is ring, T is trophozoite and S is schizonts.

PHASE	0			A			B			C			D		
	R	T	S	R	T	S	R	T	S	R	T	S	R	T	S
PF91 non-HS	500000	0	0	500000	0	0	16667	473333	10000	0	156667	343333	1033333	0	0
PF91 HS	500000	0	0	500000	0	0	13333	210000	3333	0	73333	3067	1233	0	0
PF91non-HS CQ	500000	0	0	500000	0	0	16667	5667	10000	0	13667	0	0	0	0
PF91 HS CQ	500000	0	0	500000	0	0	1633	1000	3333	0	7333	0	0	0	0
PF112non-HS	500000	0	0	500000	0	0	16667	473333	10000	0	156667	343333	1033333	0	0
PF112 HS	500000	0	0	500000	0	0	13333	176667	6000	0	56667	2367	1233	0	0
PF112 non-HS CQ	500000	0	0	500000	0	0	22000	1067	0	0	13667	0	0	0	0
PF112 HS CQ	500000	0	0	500000	0	0	1467	733	0	0	6667	0	0	0	0
PF235 non-HS	500000	0	0	500000	0	0	16667	473333	10000	0	156667	343333	1033333	0	0
PF235 HS	500000	0	0	500000	0	0	10000	180000	6000	0	56667	2367	1233	0	0
PF235 non HS CQ	500000	0	0	500000	0	0	16667	47333	10000	0	1067	0	1033	0	0
PF235 HS CQ	500000	0	0	500000	0	0	10000	18000	6000	0	933	0	733	0	0
TMPF224 non-HS	500000	0	0	500000	0	0	20000	470000	10000	0	156667	343333	1033333	0	0
TMPF224 HS	500000	0	0	500000	0	0	20000	223333	3333	0	83333	3067	1333	0	0
TMPF224 non-HS CQ	500000	0	0	500000	0	0	20000	24667	0	0	0	0	0	0	0
TMPF224 HS CQ	500000	0	0	500000	0	0	20000	1500	0	0	0	0	0	0	0
TMPF225non-HS	500000	0	0	500000	0	0	16667	473333	10000	0	156667	343333	1033333	0	0
TMPF225 HS	500000	0	0	500000	0	0	13333	210000	3333	0	73333	3067	1233	0	0
TMPF225 non-HS CQ	500000	0	0	500000	0	0	16667	24667	6167	0	0	0	0	0	0
TMPF225 HS CQ	500000	0	0	500000	0	0	13333	21000	3333	0	0	0	0	0	0

3.4 Effect of temperature with Artesunate (ARS) on K1 strain development

K1 was co-culture with 1nM Artesunate (ARS) in the pattern of non-HS and HS. The results show that at phase 0 and A, the total number of parasite in all conditions was not differentially whereas at phase B, the total number of parasite was decreased. At phase C and phase D showed died parasites (**Figure 28**).

The morphology of K1 under non-HS, K1 under HS was different to those of K1 under non-HS with ARS and K1 under HS with ARS at phase B, C and D. The parasite under non HS and HS with ARS died whereas the parasite without ARS could propagate (**Figure 29**).

As shown in **Table 14**, the number of parasite in non-HS and HS with ARS was very low when compared to those of groups without ARS.

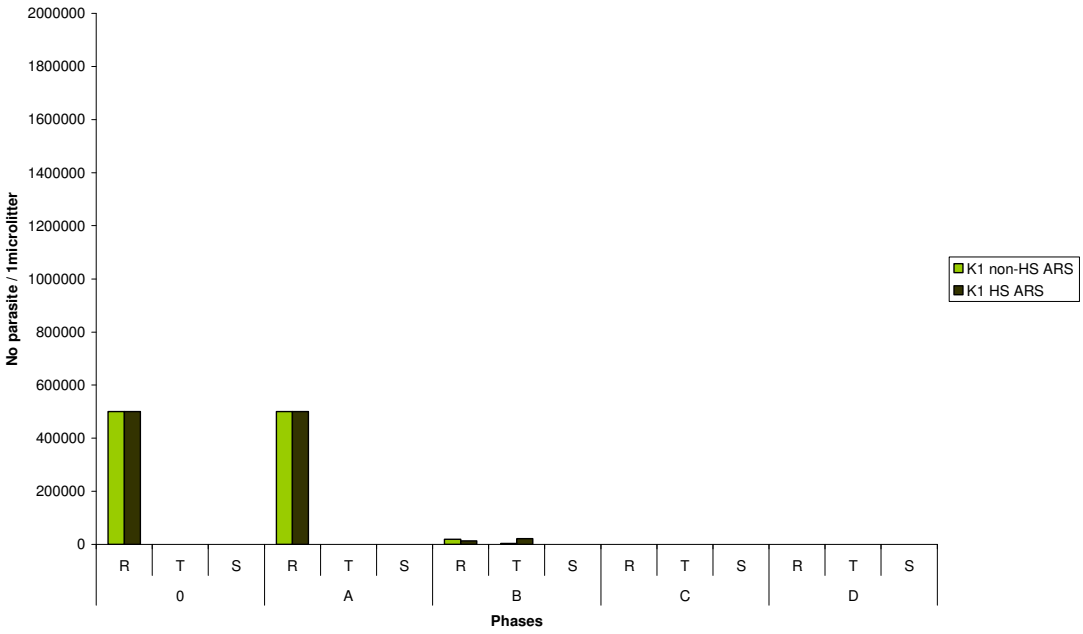


Figure 28 Number of ring (R), trophozoites (T) and schizonts (S) stages of K1 strain under treated with ARS non-heat shock and heat shock condition. Non-HS ARS, non heat shock condition under treated with ARS and HS ARS, heat shock condition under treated with ARS

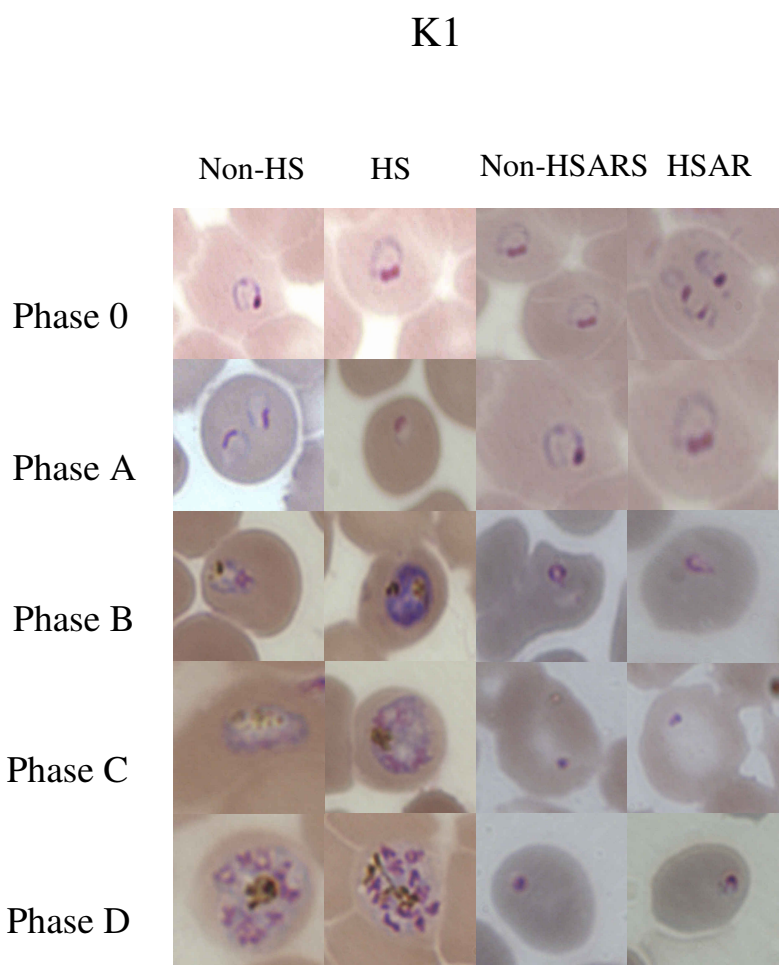


Figure 29 The morphology of malaria parasite, K1 strain treated and untreated with ARS under heat and non heat shock condition. Non-HS, not heat shock condition, HS, heat shock condition, non-HS ARS, non heat shock treated with ARS and HS ARS, heat shock treated with ARS

Table 14 The total of parasite number of K1 under non-heat shock (K1 non–HS), K1 under heat shock (K1 HS), K1 under non- heat shock with Artesunate (K1 non –HS-ARS) and K1 under heat shock with Artesunste (K1 HS ARS). R is ring, T is trophozoite and S is schizonts

PHASE	0			A			B			C			D		
	R	T	S	R	T	S	R	T	S	R	T	S	R	T	S
K1 non-HS	500000	0	0	500000	0	0	20000	473333	6667	0	406667	90000	1066667	0	0
K1 HS	500000	0	0	500000	0	0	13333	476667	10000	0	340000	156667	1433333	33333	0
K1 non-HS ARS	500000	0	0	500000	0	0	20000	4000	0	0	0	0	0	0	0
K1 HS ARS	500000	0	0	500000	0	0	13000	22333.33	0	0	0	0	0	0	0

3.5 Effect of temperature with Artesunate (ARS) on 3D7 strain development

The strain 3D7 was co-cultured with 1nM Artesunate (ARS) in the pattern of non-HS and HS. The results show that at phase 0 and A, the total number of parasite in all groups was not different whereas at phase B, the total number of parasite was decreased. Phase C and Phase D showed died parasites (**Figure 30**).

The morphology of strain 3D7 without ARS at phase B, C and D showed differences to those groups with ARS. The parasites under ARS, non HS and HS conditions died parasite whereas those grown without ARS could propagate (**Figure 31**).

As shown in **Table 15,** the number of parasite at phase B of non-HS and HS groups with ARS was very low when compared to those of without ARS.

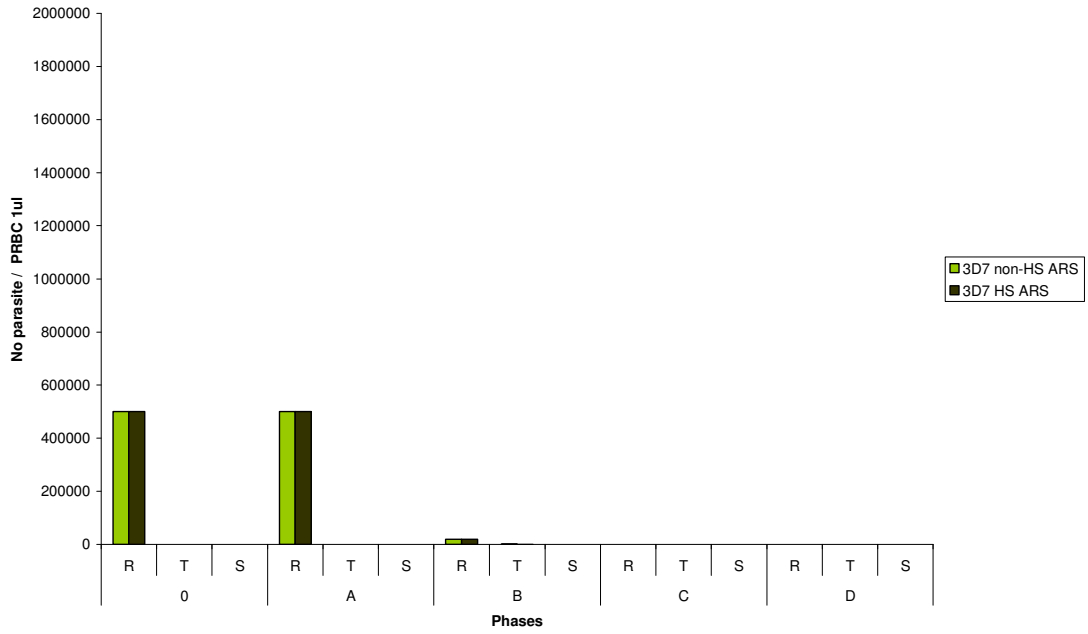


Figure 30 Number of ring (R), trophozoites (T) and schizonts (S) stages of 3D7 strain under treated with ARS non-heat shock and heat shock condition. Non-HS ARS, non heat shock condition under treated with ARS and HS ARS, heat shock condition under treated with ARS

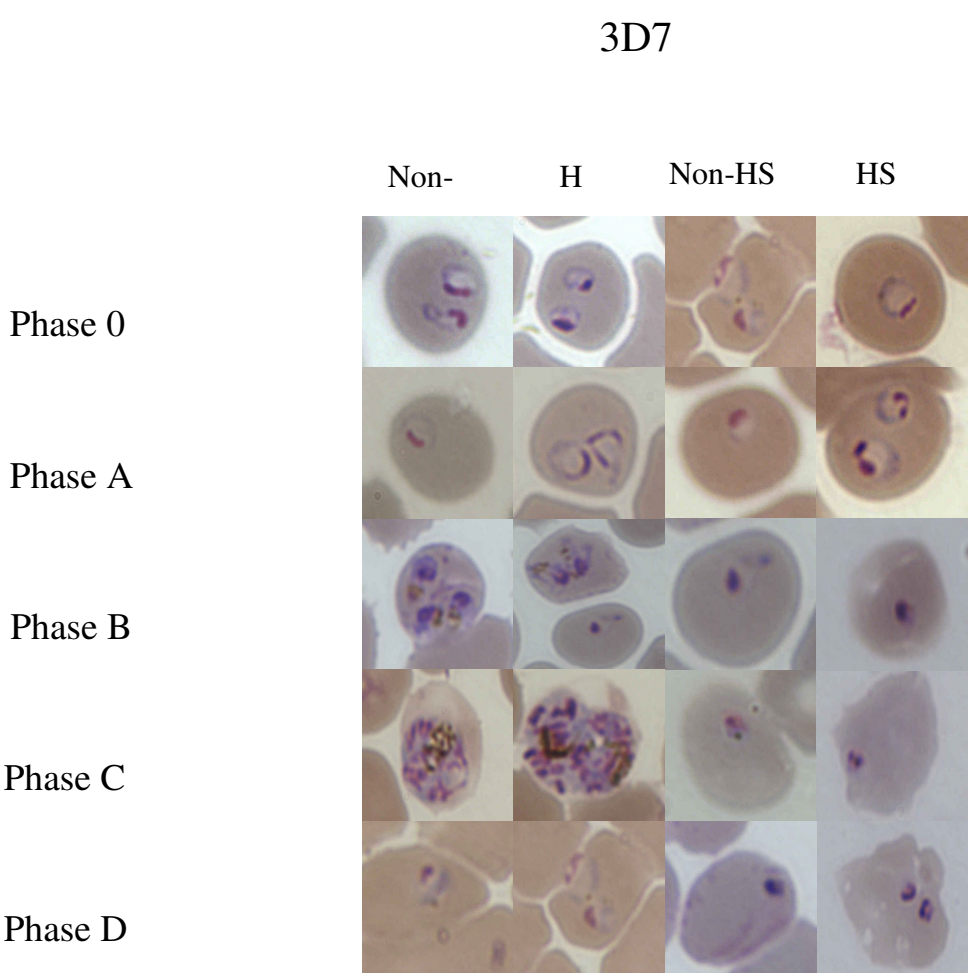


Figure 31 The morphology of malaria parasite, 3D7 strain treated and untreated with ARS under heat and non heat shock condition. Non-HS, not heat shock condition, HS, heat shock condition, non-HS ARS, non heat shock treated with ARS and HS ARS, heat shock treated with ARS

Table 15 The total number of parasite strain 3D7 under non-heat shock (3D7 non–HS), 3D7 under heat shock (3D7 HS), 3D7 under non-heat shock with Artesunate (3D7 non-HS ARS) and 3D7 under heat shock with Artesunate (3D7 HS ARS). R is ring, T is trophozoite and S is schizonts.

PHASE	0			A			B			C			D		
	R	T	S	R	T	S	R	T	S	R	T	S	R	T	S
3D7 non-HS	500000	0	0	500000	0	0	20000	473333	6667	0	403333	93333	1066667	0	0
3D7 HS	500000	0	0	500000	0	0	20000	466667	6667	0	276667	110000	1333333	13333	0
3D7 non-HS ARS	500000	0	0	500000	0	0	20000	1067	0	0	0	0	0	0	0
3D7 HS ARS	500000	0	0	500000	0	0	20000	733	0	0	0	0	0	0	0

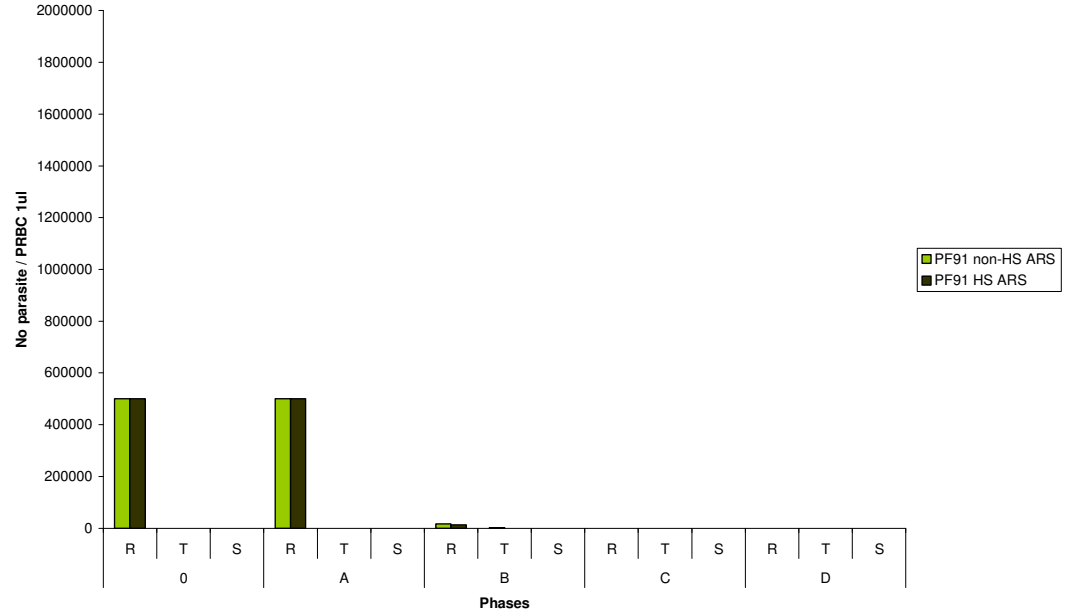
3.6 Effect of temperature with Artesunate (ARS) on five isolates strain development.

Fives isolates, PF91, PF112, PF235, TMPF224 and TMPF225, were co-cultured with 1nM Artesunate (ARS) in the pattern of non-HS and HS. The results showed that at phase 0 and A, the total number of parasite was not different whereas at phase B, the total number of parasite was decreased. The parasites died at phase C and phase D (**Figures 32-34**).

The morphology of fives isolates at phase B, C and D of groups under non-HS, HS with ARS was different to those of without ARS. The parasites under non-HS and HS with ARS died whereas those grown without ARS could propagate (**Figure 35**).

As shown in **Table 16,** the number of parasite at phase B of non-HS and HS groups with ARS was very low when compared to those of without ARS.

A



B

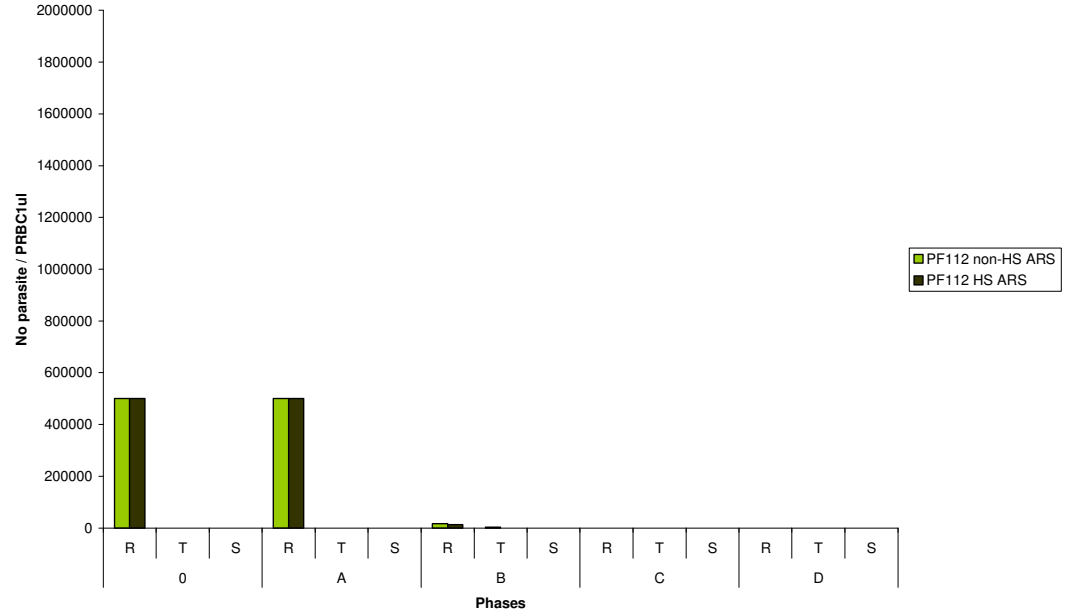
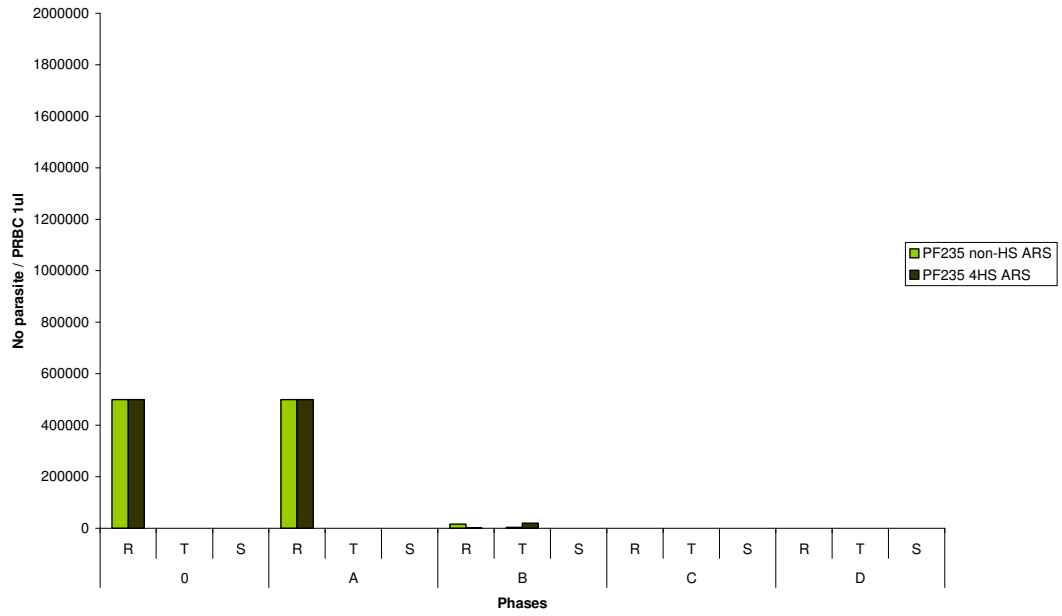


Figure 32 Number of ring (R), trophozoites (T) and schizonts (S) stages of PF91 strain (A) and PF112 strain (B) under treated with ARS non-heat shock and heat shock condition. Non-HS ARS, non heat shock condition under treated with ARS and HS ARS, heat shock condition under treated with ARS



B

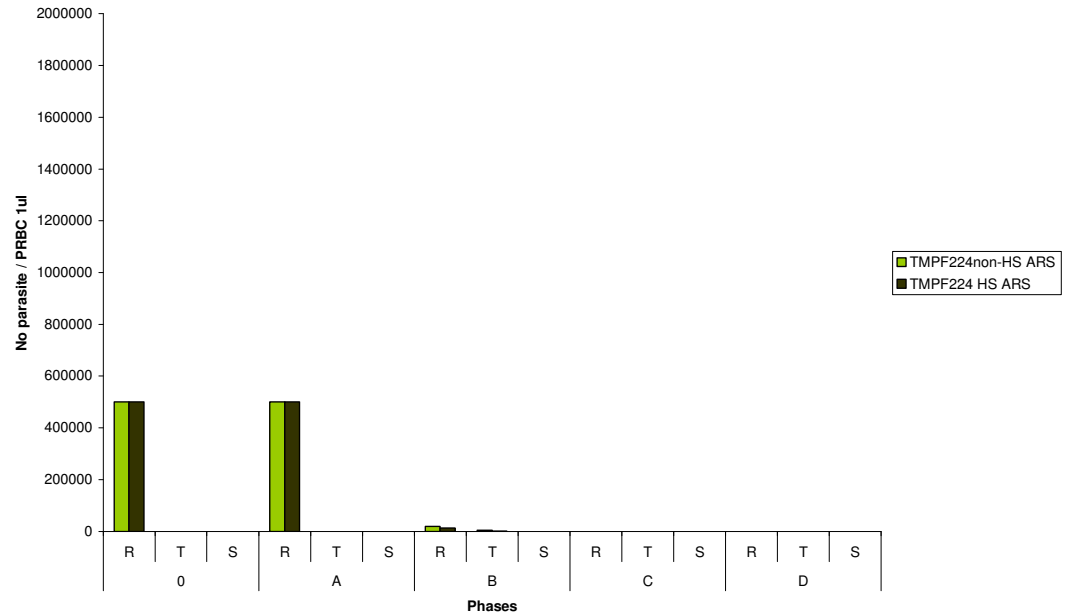


Figure 33 Number of ring (R), trophozoites (T) and schizonts (S) stages of PF235 strain (A) and TMPF224 strain (B) under treated with ARS non-heat shock and heat shock condition. Non-HS ARS, non heat shock condition under treated with ARS and HS ARS, heat shock condition under treated with ARS

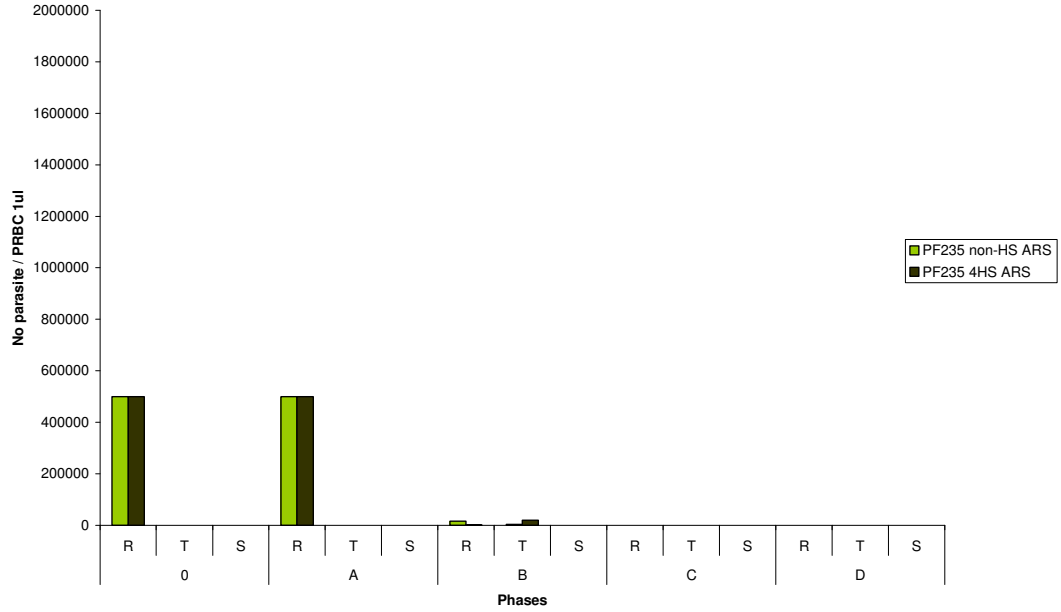


Figure 34 Number of ring (R), trophozoites (T) and schizonts (S) stages of PF235 strain under treated with ARS non-heat shock and heat shock condition. Non-HS ARS, non heat shock condition under treated with ARS and HS ARS, heat shock condition under treated with ARS

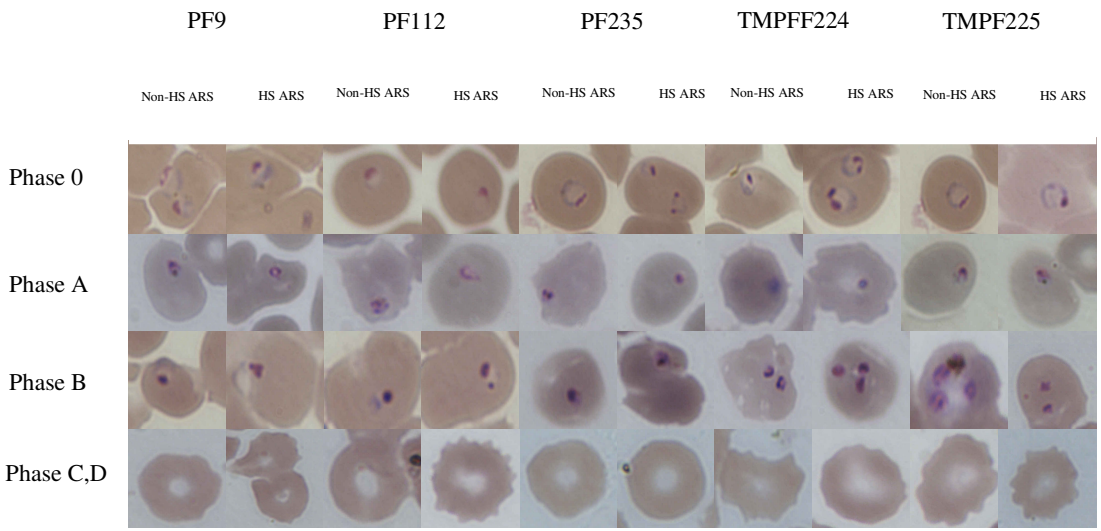


Figure 35 The morphology of malaria parasite, five strains treated and untreated with ARS under heat and non heat shock condition. Non-HS, not heat shock condition, HS, heat shock condition, non-HS ARS, non heat shock treated with ARS and HS ARS, heat shock treated with ARS

Table 16 The total of parasite number of fives isolates under non-heat shock (non-HS), heat shock (HS), non- heat shock with ARS(non-HS ARS) and heat shock with ARS (HS ARS). R is ring, T is trophozoite and S is schizonts.

PHASE	0			A			B			C			D		
	R	T	S	R	T	S	R	T	S	R	T	S	R	T	S
PF91 non-HS	500000	0	0	500000	0	0	16667	473333	10000	0	156667	343333	1033333	0	0
PF91 HS	500000	0	0	500000	0	0	13333	210000	3333	0	73333	3067	1233	0	0
PF91 non-HS ARS	500000	0	0	500000	0	0	16667	2667	0	0	0	0	0	0	0
PF91 HS ARS	500000	0	0	500000	0	0	13333	0	0	0	0	0	0	0	0
PF112non-HS	500000	0	0	500000	0	0	16667	473333	10000	0	156667	343333	1033333	0	0
PF112 HS	500000	0	0	500000	0	0	13333	176667	6000	0	56667	2367	1233	0	0
PF112 non-HS ARS	500000	0	0	500000	0	0	16667	3533	0	0	0	0	0	0	0
PF112 HS ARS	500000	0	0	500000	0	0	13333	0	0	0	0	0	0	0	0
PF235 non-HS	500000	0	0	500000	0	0	16667	473333	10000	0	156667	343333	1033333	0	0
PF235 HS	500000	0	0	500000	0	0	10000	180000	6000	0	56667	2367	1233	0	0
PF235 non-HS ARS	500000	0	0	500000	0	0	16667	3267	0	0	0	0	0	0	0
PF235 4HS ARS	500000	0	0	500000	0	0	1100	20667	0	0	0	0	0	0	0
TMPF224 non-HS	500000	0	0	500000	0	0	20000	470000	10000	0	156667	343333	1033333	0	0
TMPF224 HS	500000	0	0	500000	0	0	20000	223333	3333	0	83333	3067	1333	0	0
TMPF224non-HS ARS	500000	0	0	500000	0	0	20000	4800	0	0	0	0	0	0	0
TMPF224 HS ARS	500000	0	0	500000	0	0	13333	567	0	0	0	0	0	0	0
TMPF225non-HS	500000	0	0	500000	0	0	16667	473333	10000	0	156667	343333	1033333	0	0
TMPF225 HS	500000	0	0	500000	0	0	13333	210000	3333	0	73333	3067	1233	0	0
TMPF225 non-HS ARS	500000	0	0	500000	0	0	16667	10167	0	0	1167	0	0	0	0
TMPF225 HS ARS	500000	0	0	500000	0	0	13333	3333	0	0	0	0	0	0	0

4. Effect of temperature and drug stress on *P. falciparum* heat shock protein 70 (*Pf*HSP70s)

4.1 Protein expression pattern assayed by SDS PAGE

The total protein of *P. falciparum* strain K1, 3D7 and five isolates grown under different conditions was analyzed by SDS PAGE by loading boiled 2.5 x 10^6 cells with loading buffer per well and the gel was stained with colloidal coomassie blue. As shown in **Figure 36**, the pattern of protein expression of *P. falciparum* grown under heat shock (HS) and non heat shock (non-HS) with or without CQ and ARS was similar that is no specific band was observed.

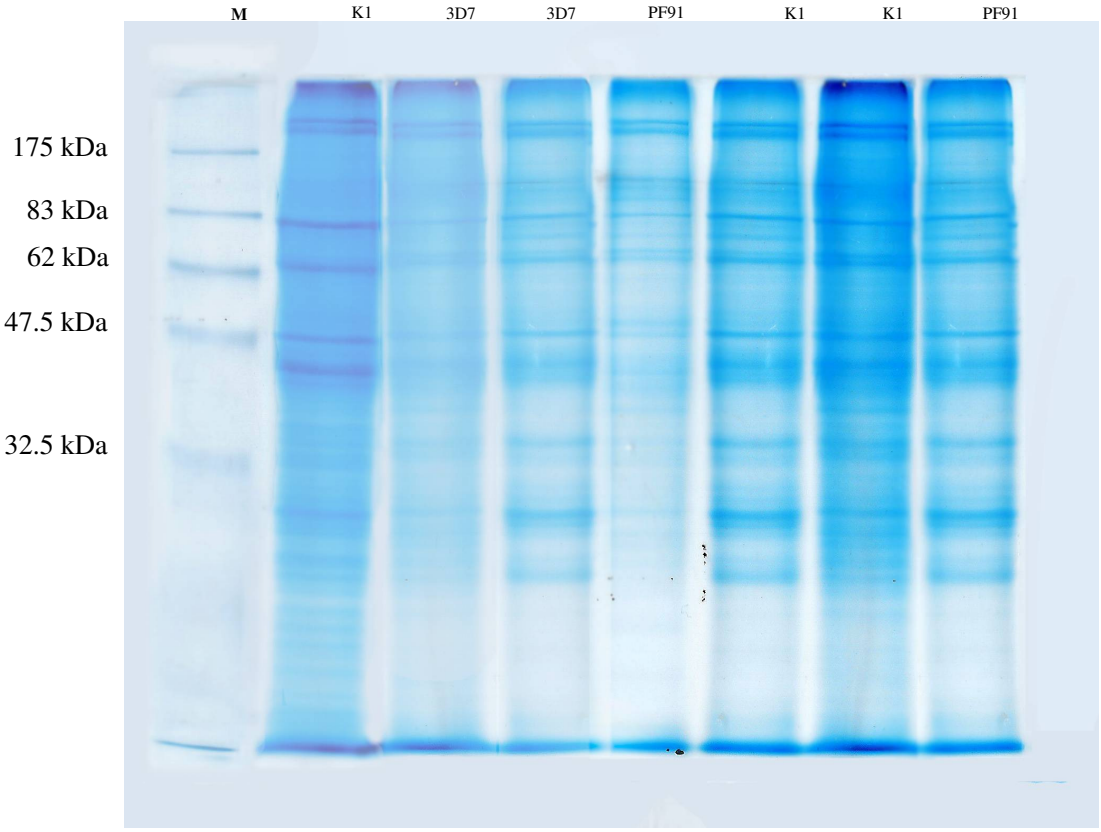


Figure 36 SDS PAGE showing the pattern of protein expression of *P. falciparum* strain K1, 3D7 and isolate PF91 grown under different conditions. M is protein maker.

4.2 Detection of *P. falciparum* heat shock protein 70 (*pf*HSP70s) by Immuno

Blotting

The total protein was transferred to nylon membrane and hybridize with antibody to *pf*HSP70. The results showed that *pf*HSP70 antibody could bind to protein with the molecular mass of 120 kDa in all conditions except K1 under HS with CQ. There were four hybridized bands from the protein of strain K1 grown under HS with CQ. The four bands had the molecular mass of 120 kDa, 83 kDa, 60 kDa and 40 kDa (**Figures 37-42**).

Figure 37 The immunoblotting membrane of *P. falciparum* strain K1, 3D7 and five isolates (PF235, PF112, TMPF225, TMPF224 and PF91) grown under non-heat shock (non-HS) conditions. Arrow indicates the band with the molecular mass of 120 kDa hybridized with *pf*HSP70 antibody. M is protein maker.

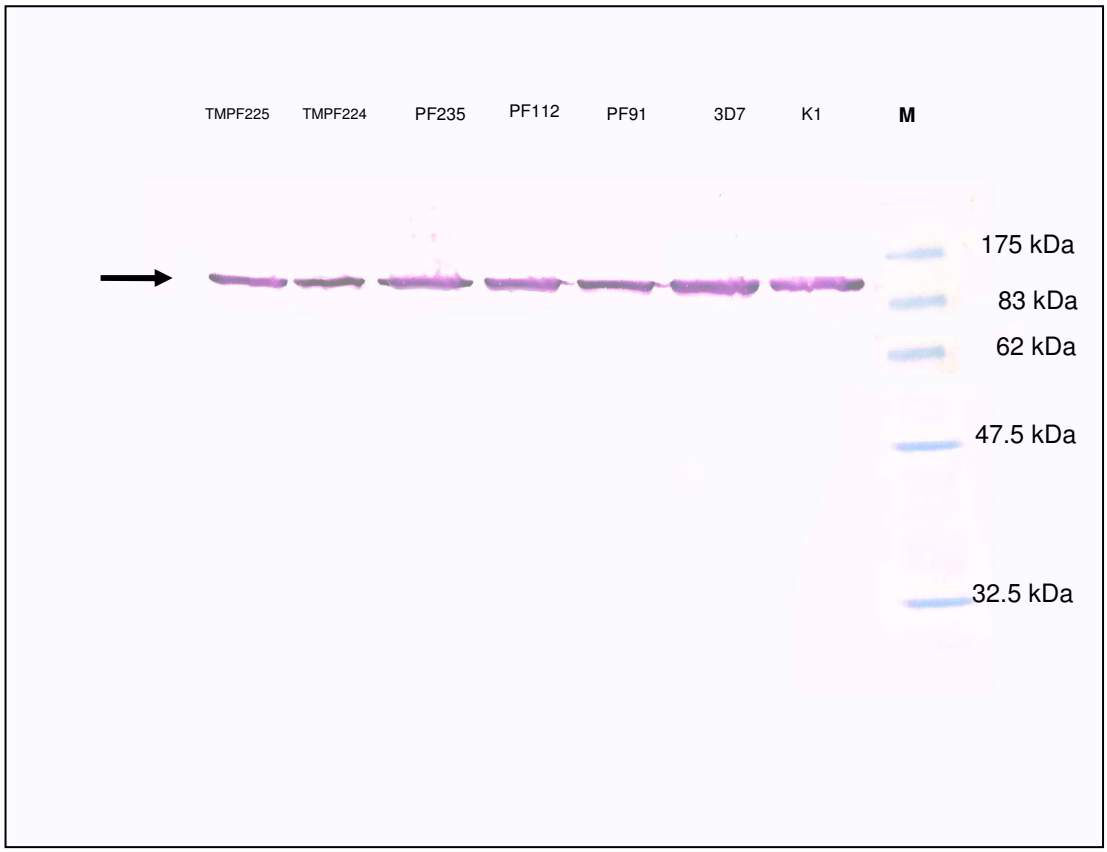


Figure 38 The immunoblotting membrane of *P. falciparum* strain K1, 3D7 and five isolates (PF235, PF112, TMPF225, TMPF224 and PF91) grown under heat shock (HS) conditions. Arrow indicates the band with the molecular mass of 120 kDa hybridized with *pf*HSP70 antibody. M is protein maker.

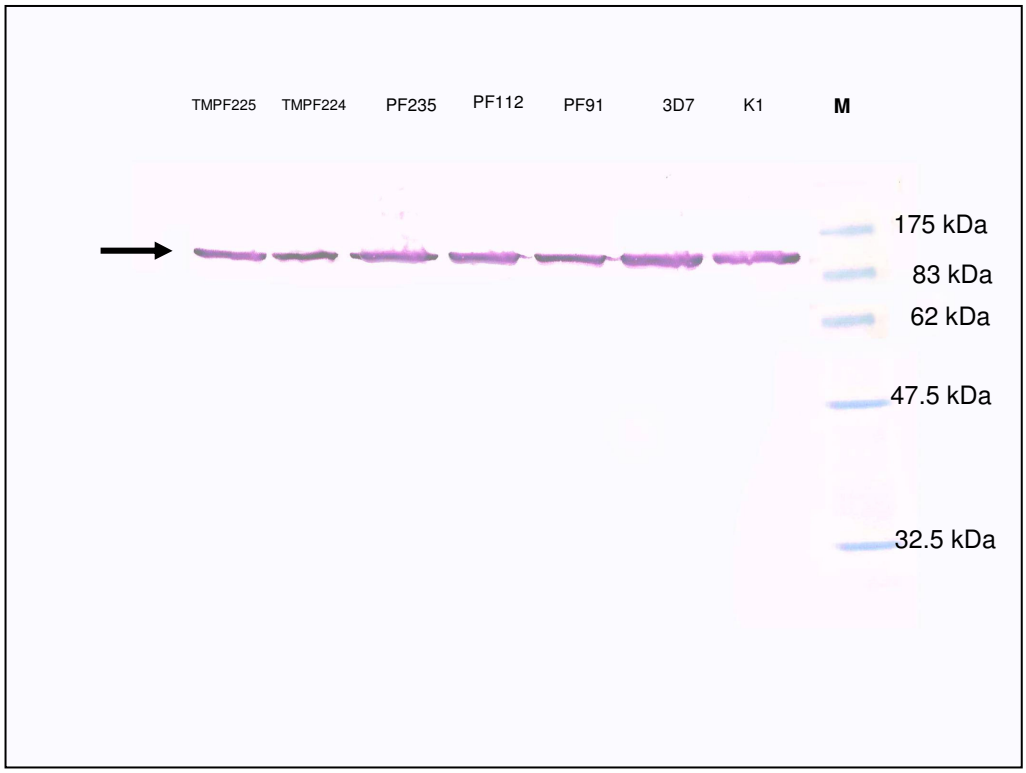


Figure 39 The immunoblotting membrane of *P. falciparum* strain K1, 3D7 and five isolates (PF235, PF112, TMPF225, TMPF224 and PF91) grown under non-heat shock with Chloroquine (non-HS CQ) conditions. Arrow indicates the band with the molecular mass of 120 kDa hybridized with *pf*HSP70 antibody. M is protein maker.

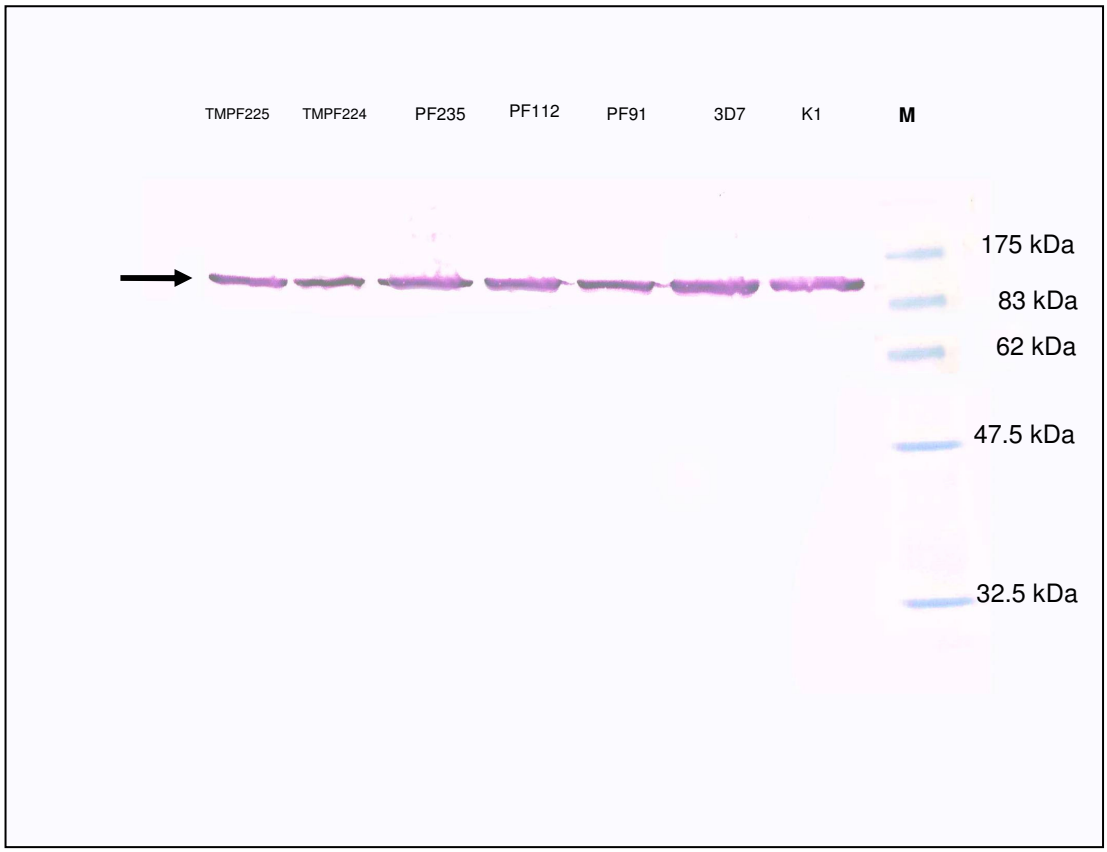


Figure 40 The immunoblotting membrane of *P. falciparum* strain K1, 3D7 and five isolates (PF235, PF112, TMPF225, TMPF224 and PF91) grown under non-heat shock with Artesunate (non-HS ARS) conditions. Arrow indicates the band with the molecular mass of 120 kDa hybridized with *pf*HSP70 antibody. M is protein maker.

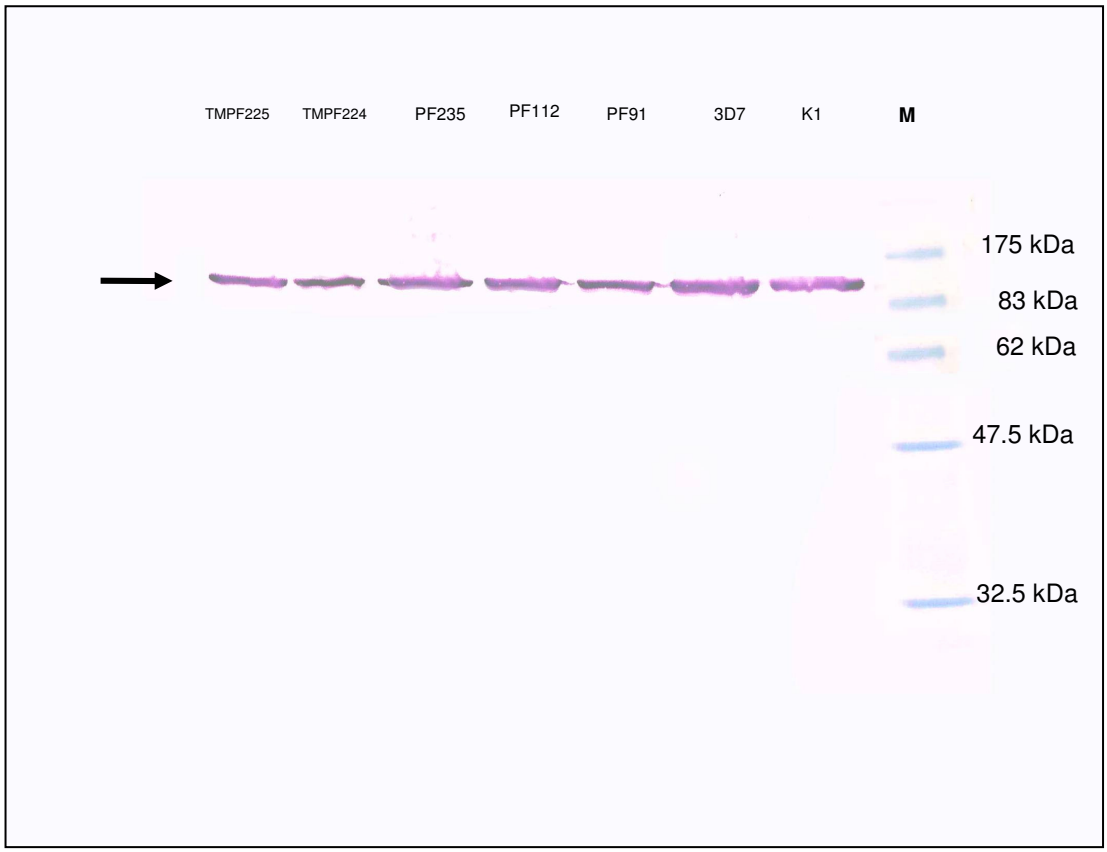


Figure 41 The immunoblotting membrane of *P. falciparum* strain K1, 3D7 and five isolates (PF235, PF112, TMPF225, TMPF224 and PF91) grown under heat shock with Artesunate (HS ARS) conditions. Arrow indicates the band with the molecular mass of 120 kDa hybridized with *pf*HSP70 antibody. M is protein maker.

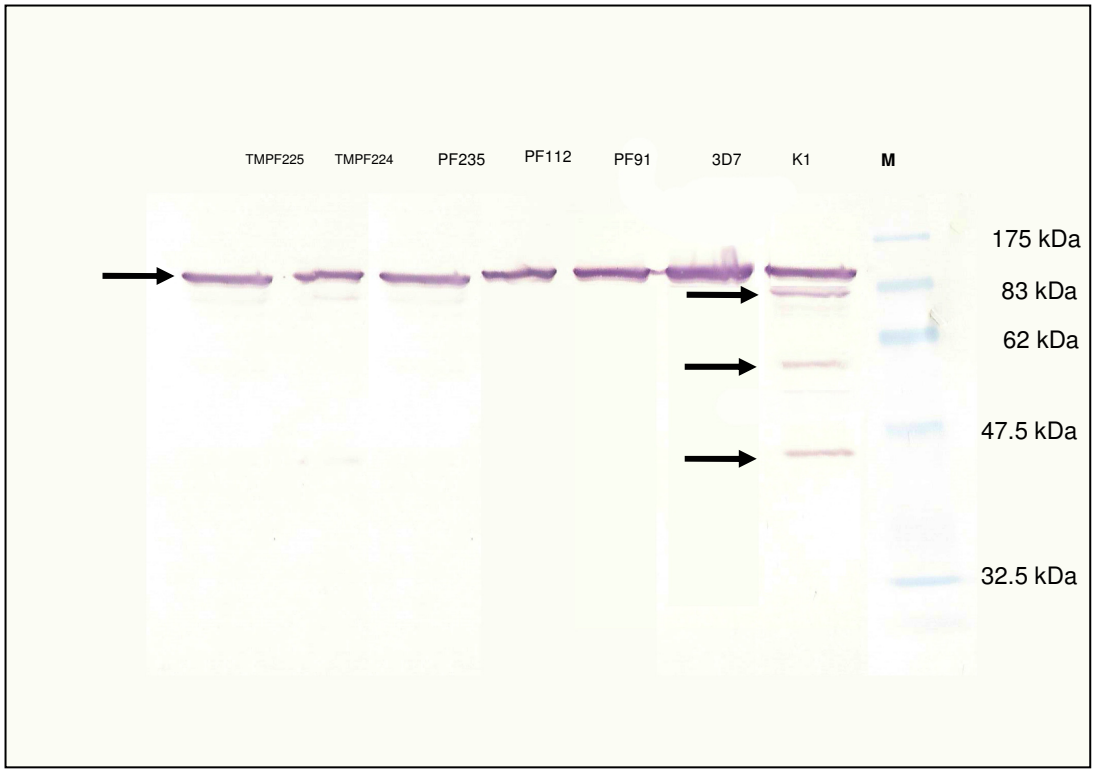


Figure 42 The immunoblotting membrane of *P. falciparum* strain K1, 3D7 and five isolates (PF235, PF112, TMPF225, TMPF224 and PF91) grown under heat shock with chloroquine (HS CQ) conditions. Arrows indicate four bands with the molecular mass of 120, 83 kDa, 60 kDa and 40 kDa hybridized with *pf*HSP70 antibody. M is protein maker.

4.3 Protein identification by Mass spectrometry

In order to identify the proteins hybridized with *pf*HSP70 antibody, the mass spectrometry was used. The proteins on SDS-PAGE gel with the same molecular mass with the hybridized bands on immunoblotting membrane were excised and separately digested with trypsin and identified by MALDI-TOF peptide mass fingerprint (Bioservice Service Unit, NSTDA). After searching through the database, the band with molecular mass of 120 kDa was identified as *Plasmodium* heat shock protein (score 110) and *pf*HSP 70 (score 87) as shown in **Figure 43**. Three bands hybridized with *pf*HSP70 antibody with molecular mass of 83 kDa, 60 kDa and 40 kDa were identified as elongation factor 1 alpha of *P. falciparum* (score 220), *pf*HSP 86 (score118) and phosphoethanolamine N-methyltransferase (score 110), respectively (**Figures 44-46**).

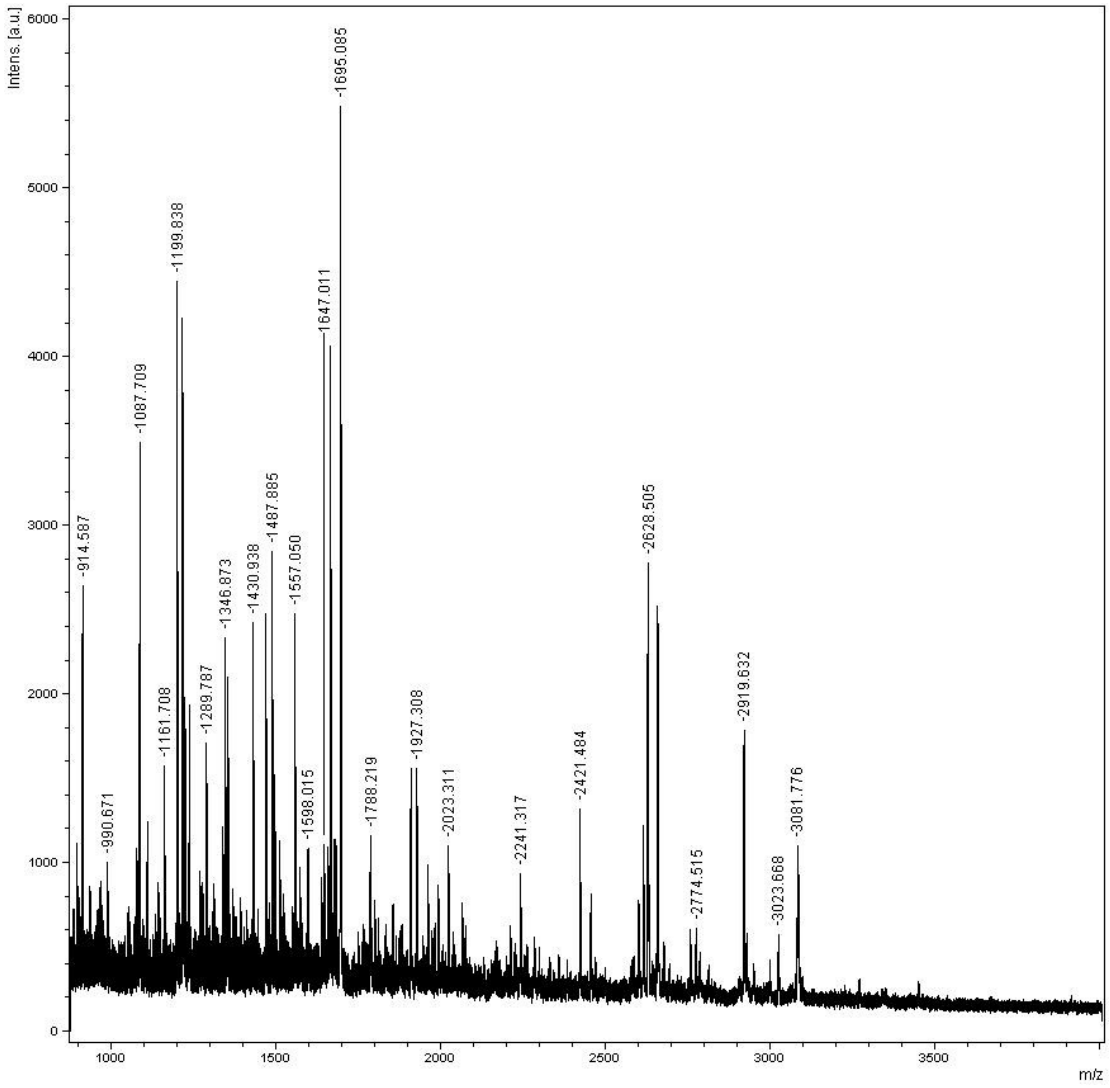


Figure 43 The MALDI-TOF peptide mass spectro of the hybridized 120 kDa protein band identified as *Plasmodium* heat shock protein (score 110) and *pf*HSP 70 (score 87).

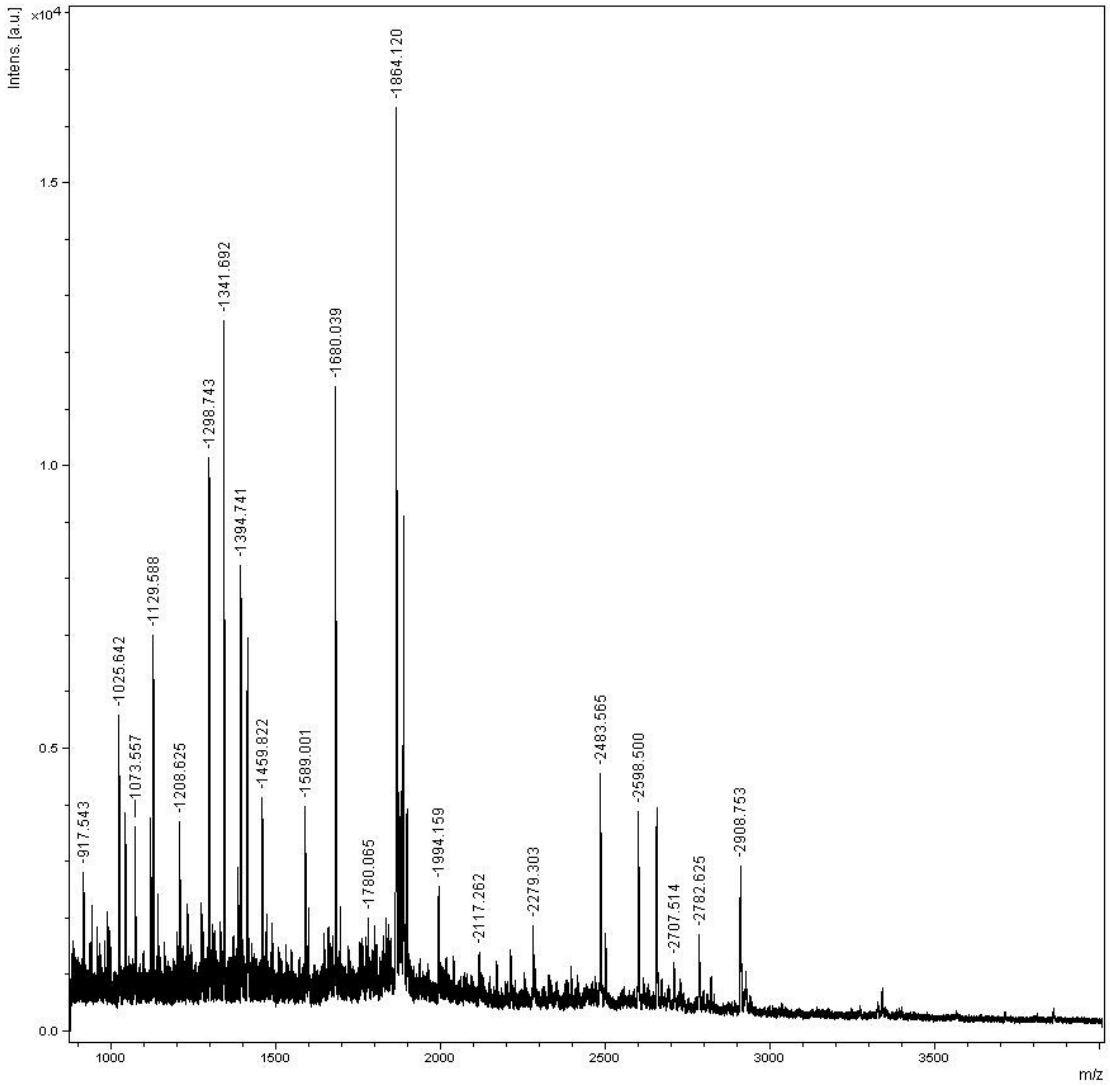


Figure 44 The MALDI-TOF peptide mass spectro of the hybridized 83 kDa protein band identified as elongation factor 1 alpha of *P. falciparum* (score220).

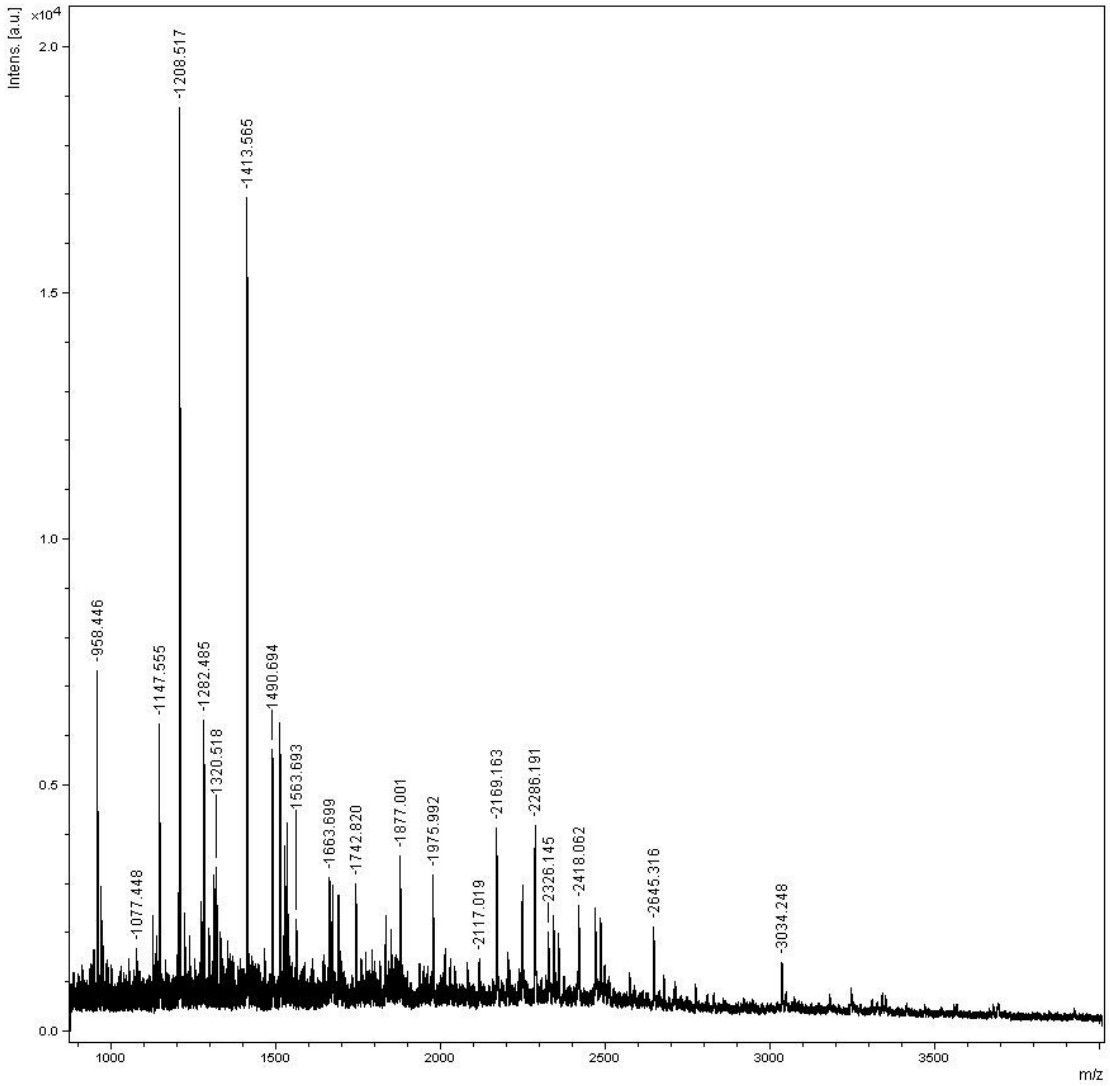


Figure 45 The MALDI-TOF peptide mass spectro of the hybridized 60 kDa protein band identified as *pf*HSP 86 (score 118).

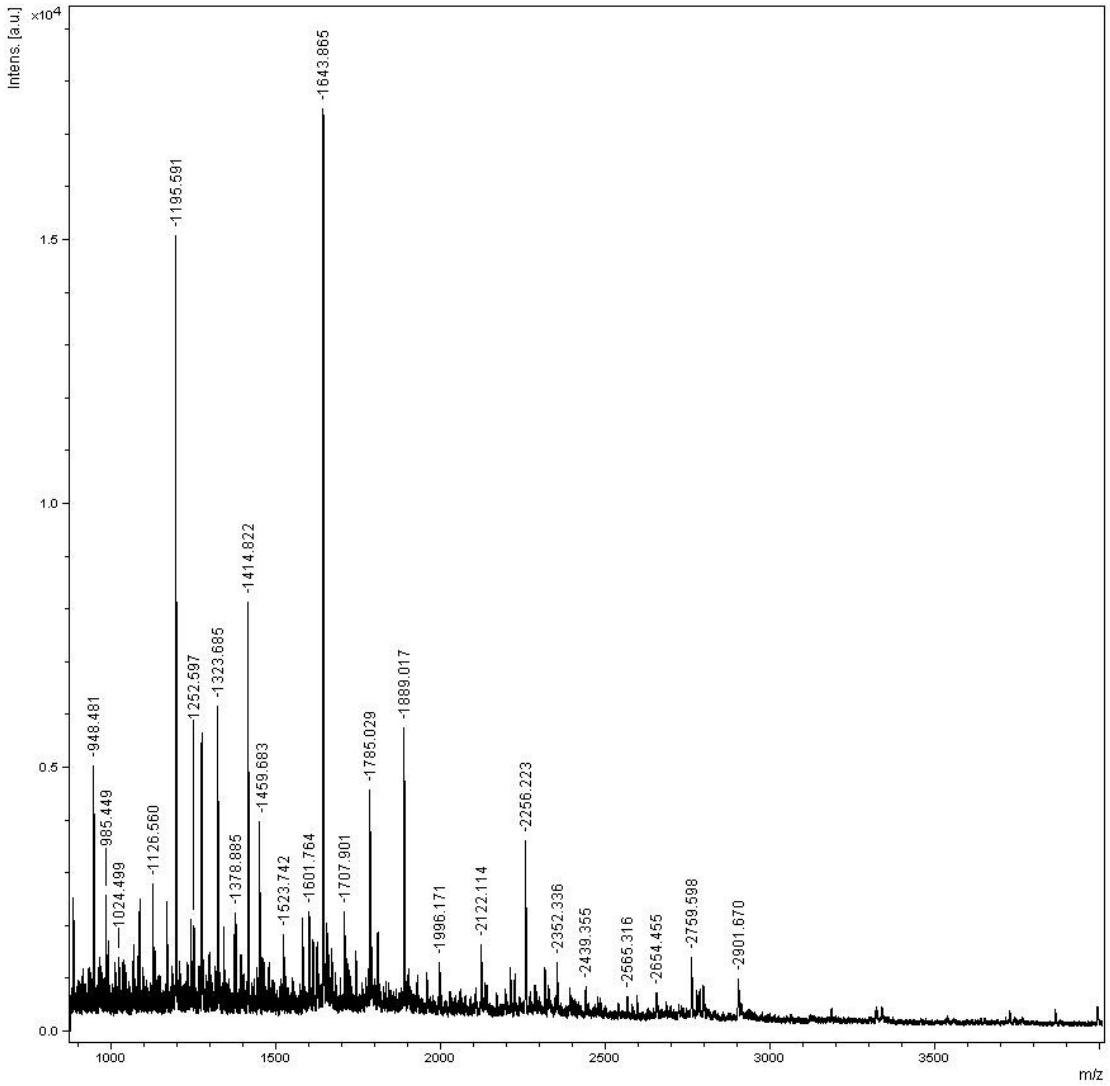


Figure 46 The MALDI-TOF peptide mass spectro of the hybridized 40 kDa protein band identified as phosphoethanolamine N-methyltransferase (score 110).