EFFECT OF TEMPERATURES ON THE PARASITAEMIA OF *Plasmodium falciparum in vitro*

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ABSTRACT:

Background: *Plasmodium falciparum* is the most important human malaria parasite. To further improve the cultivation and related techniques of this parasite, effect of temperatures on the *P. falciparum* cultivation, *in vitro*, was studied. The total, ring and non-ring stages of synchronized culture, at different temperatures, were determined to evaluate their effects on the growth and development of the parasites.

Methods: Cultures of *P. falciparum* were exposed to different temperatures, 4, 34, 37 and 40 °C. The effect of temperature was determined as percent parasitaemia of ring and non-ring stage. K1CB1, T9/94RC17 and 3D7 clones were used in this experiment.

Results: The growth pattern of all 34 °C cultures were similar to control (37 °C) while the parasites were killed after 72 hours exposure at 4 and 40 °C. In 4 °C culture, the major population was mainly in ring stage. On the contrary, only the non-ring stage can be detected after 48 hours incubation at 40 °C.

Conclusion: At 4, 34 and 40 °C, the *P. falciparum* growth was lowered, compared with the control group at 37 °C. All parasites cannot be detected after 48 or 72 hours incubation at 4 and 40 °C. In 4 °C culture, most detected parasites were ring stage while in 40 °C, most detected parasites were non-ring stage. Stage preferences by different temperatures may applicable for synchronization technique improvement.

Keywords: *Plasmodium falciparum*; Temperature; Culture; Synchronization

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INTRODUCTION

Malaria is one of the most important parasitic diseases at the global scale. In 2015, it is estimated that the disease effects over 200 million people with over 400, 000 death [1]. The disease is caused by parasites in the Plasmodium genus in which *Plasmodium falciparum* is accounted for a major impact on human. Many research works have been focused on this parasite, in order to reveal its biological properties. The cultivation technique of *P. falciparum*, developed by Trager and Jensen [2], allows us to propagate the parasite *in vitro*. During cultivation, each parasites would alternatively develop through ring, trophozoite and schizont stages, similar to erythrocytic cycle development in human blood. Scientists can, now, clone and manipulate the parasite based on this cultivation technique. Knowledge from these studies helps us to understand many aspects of *P. falciparum*, e.g. drug susceptibility, population genetic, protein expression, cell physiology and response, etc. [3-8]. This knowledge also could be applied to drug, vaccine or diagnostic test development which may lead to improved drug treatment, control program design or the eradication of malaria disease [9, 10].

Before cultivation, *P. falciparum* is, sometimes, chemically or physically selected or synchronized,

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to get a specific stage. Sorbitol is commonly used chemical to treat *P. falciparum* culture with mixed stages. It is known to preferentially select for ring stage parasites. The survived rings can, then, be seeded a new culture in which they will developed at the same pace. The synchronization step can be repeated if required. Normally, the synchronized state would be maintained for 1 - 2 cycles which are long enough for the characterization of *P. falciparum*, *in vitro* [11]. In 2014, a simplified technique for synchronization had been published [12]. The technique is based on low temperature incubation for a short period, 8 – 24 hours, before cultivation. Low temperature was proposed to block schizont development or/and merozoite invasion process, as a result ring-stage parasites survived and further developed into later stage at the same time. Although, it had been proposed that temperature affects the growth of *P. falciparum*, *in vitro*, only few publications had demonstrated this effect. The objective of this work is to explore the effect of temperature on the growth and stages of different clones of *P. falciparum*, *in vitro*.

METHODS

Plasmodium falciparum clones

Three *P. falciparum* clones, K1CB1, T9/94RC17, and 3D7, were used in this study. All clones were established and characterized at Malaria Research Laboratory, Department of Biology, Faculty of Science, Chulalongkorn University. T9/94RC17 was cloned and re-cloned from the original clone T9 isolate collected from Tak province [13] while K1CB1 was cloned from the K1 isolate collected from Kanchanaburi province [14]. 3D7 was a cloned derived from the NF54 isolate originally collected from the Netherlands [15].

Parasite cultivation

Erythrocytic stages of the parasites were cultured *in vitro* at 37 °C in a candle jar, according to the modified protocols of Trager and Jensen [2]. The culture medium consisted of completed RPMI-1640 medium (RPMI-1640 medium, with glutamine (GIBCO™ Invitrogen Corporation), supplemented with 25 mM HEPES (SIGMA-ALDRICH) , 40 mg/l gentamycin (T.P. DRUG Laboratories), 24 mM sodium bicarbonate (MERCK), and 10% human serum). To maintain the parasite culture, culture medium and/or erythrocytes were added or replaced when the parasitaemia was higher than 5-10% [16].

Synchronization

Each culture was centrifuged at 1,500 rpm for 5 minutes prior to supernatant removal. The packed cells were treated with 5% D-sorbitol (FLUKA) for 10 minutes at room temperature [11]. The cells were washed 3 times with incompleted media (completed medium without human serum). These steps were repeated twice to ensure the ring stage selection. Finally, the parasites were cultured in RPMI-1640 medium as described above.

Effect of temperature changes

Synchronized cultured were counted and diluted by uninfected red blood cell to 0.3 – 0.5% parasitaemia. After dilution, their total parasitaemia, ring and non-ring stages were evaluated as time 0 hour. Ten microliters of parasite infected blood and cultured medium (100 microliter/well) were added into each well of 96-well plates. Three individual plates were set up with 9 wells of parasite culture in each plate. They were incubated at 4 °C for 72 hours. A separated plate with 9 wells of parasite culture was incubated at 37 °C as a control plate. At 24, 48 and 72 hours, medium and red blood cells from 3 wells, in each plate, were removed and determined their total parasitaemia, ring and non-ring stages. The experiment was repeated at 34 °C and 40 °C. Every day, the complete medium was removed and new completed medium (100 microliter/well) was added to each well. No uninfected red blood cell was added or replaced during the experiment.

Parasitaemia determination

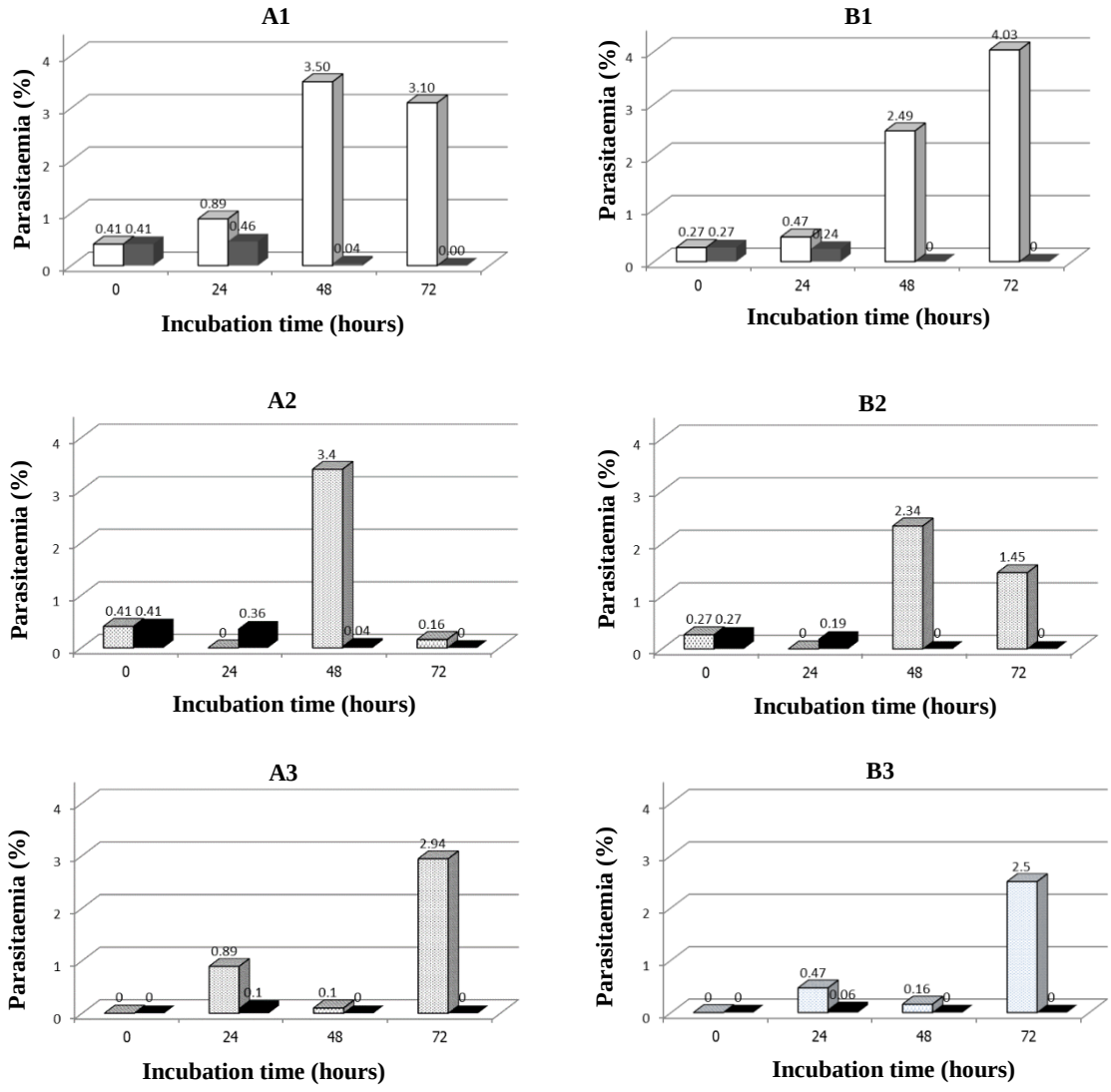
A thin smear, on a glass slide, was prepared from infected red blood cells from each well. Slide was fixed and stained with Giemsa. Stage (ring and non-ring) and the number of parasites were determined using a compound microscope at 1,000 times magnification. Percent parasitaemia were, then, calculated [17]. To differentiate parasite stages, parasites were determined as ring and non-ring stages.

RESULTS AND DISCUSSION

In all control group, at 72 hrs, K1CB1, T9/94RC17 and 3D7 gave different parasitaemia values, of which reflect different biological properties among these parasite clones. Both K1CB1 and 3D7 show lower parasitaemia than T9/94RC17 at 37 °C (Table. 1). K1CB1 and 3D7, in control group, exhibited synchronization growth throughout 72 hours while T9/94RC17 lost its

Table 1 Mean parasitaemia (%) of 3 clones in synchronized culture after 72 hours at 37 °C together with the parasitaemia of ring and non-ring stages in the culture

Clones	Mean parasitaemia (%)		
	at 72 hrs	ring	non-ring
K1CB1	2.79	0.12	2.67
T9/94RC17	4.59	1.98	2.61
3D7	2.39	0.08	2.31



A1 and B1 (total parasitaemia) : control group (37 °C), experiment group (4 °C)
A2 and B2 (ring), A3 and B3 (non-ring) : control group (37 °C), experiment group (4 °C)

Figure 1 Graphs show the effect of low temperature (4 °C) on the parasitaemia of synchronized *P. falciparum* at different incubation period; 0, 24, 48 and 72 hours. Results from two clones were shown here A) K1CB1 and B) T9/94RC17. The data include total parasitaemia (A1 and B1) together with ring (A2 and B2) and non-ring (A3 and B3) population at each time point

synchronized cycle at the end of incubation period. At 72 hour, the highest parasitaemia point, T9/94RC17 (mean value 4.59%) was mixed between ring (1.98%) and non-ring (2.61%) stage. It was unclear whether or not the high parasitaemia was responsible for the asynchronized status only after 48 hour cycle.

At 4 °C, although the non-ring stage increased after 24 hours, at low level (≤ 0.10%), as a result of ring development from 0 hour (Figure 1: A3 and

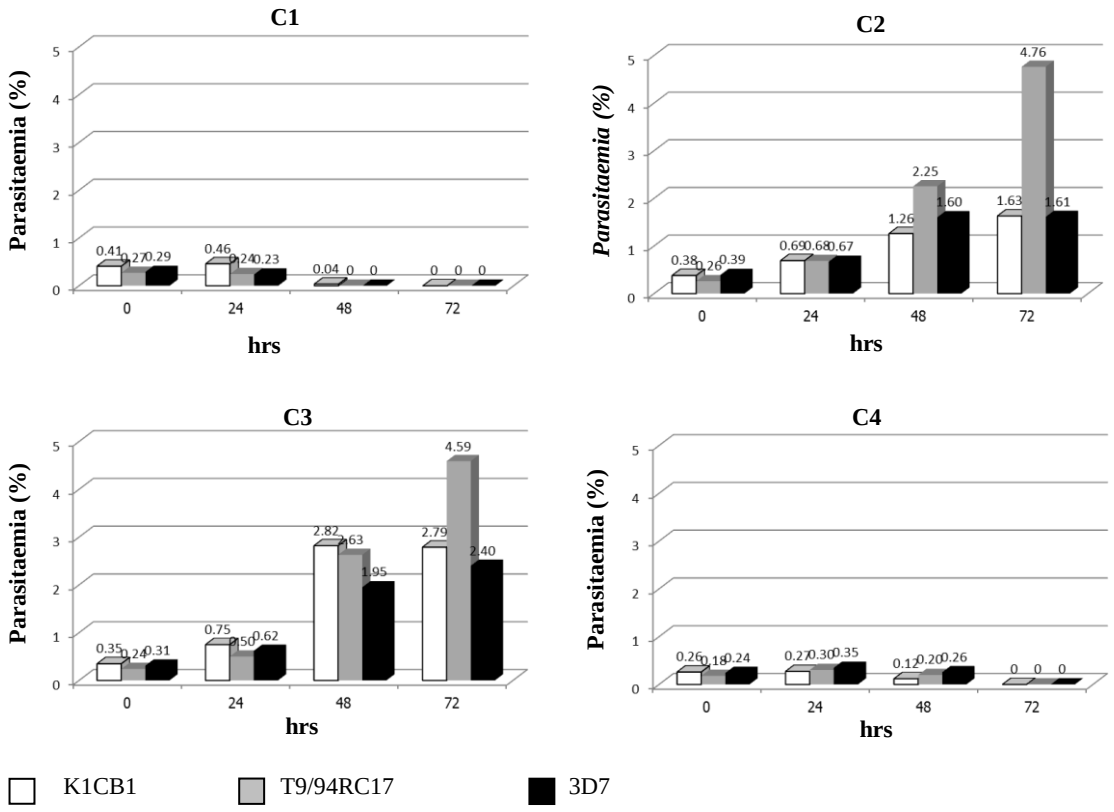


Figure 2 Graphs show the average parasitaemia of synchronized *P. falciparum* clones at different temperature: 4 °C (C1), 34 °C (C2), 37 °C (C3) and 40 °C (C4) during 72 hours.

B3), all parasite clones lowered their parasitaemia from 24 or 48 hours onward (Figure 1: A1 and B1). Most of the detected parasites were mainly ring stage (≥ 0.19%) after 24 hours exposure (Figure 1: A2 and B2). Non-ring stage cannot be detected at 48 hour onward in all clones (Figure 1: A3 and B3). The 3D7 data gave similar graph pattern to the K1CB1 clone and were not presented here. These suggested that prolong exposure, between 24 to 48 hours, to low temperature (4 °C) may eliminate nearly all non-ring parasites. It must be noted that the synchronization technique proposed by Yuan and his colleague use low temperature for stage selection. Ring stage was selected after short exposure (8-24 hours) at 4 °C, prior to routine culture [12]. Our results suggested that these procedure increased the proportion of ring/non-ring stage in all clones; K1CB1 (0.36/0.10), T9/94RC17 (0.19/0.06), 3D7 (0.20/0.03) after the parasites were incubated for 24 hours.

Three clones of *P. falciparum* similarly responded to different temperatures at 4, 34, 37 and 40 °C (Figure 2). The result showed that 37 °C was the best temperature for the growth of parasites compared to other temperatures (Figure 2: C3). Similar growth pattern was also observed at 34 °C with the tendency toward lower parasitaemia. At 40 °C (Figure 2: C4), similar to 4 °C, the parasite growth was largely affected compared to the parasite growth at 37 °C. A previous work had reported that temperature at 40 °C can inhibit the parasite development. It had also been postulated that high temperature, during malaria infection, may be a natural defense mechanism against these parasites in human [18]. Our results suggested that all parasite clones can develop into non-ring stage with small increase in their parasitaemia at 24 hour (Figure 2 and 3). After that, the total parasitaemia decreased and no parasite can be detected after 72 hour incubation at 40 °C. It is possible that high body temperature (≥ 40 °C) may partially inhibit the parasite growth in *vivo*.

It had been reported that 6 hours exposure to 40 °C has no effect to the parasite, but prolong exposure, for 12 hour, inhibited all parasite stages, *in vitro* [18]. Surprisingly, our work revealed that all parasites, at 48 hour, were non-ring stage (Figure 3) while the ring stage decreased abruptly at 24 hour and cannot be detected later on. The selective effect was opposite to the result observed at 4 °C. As proposed by Kwiatkowski, high temperature, at 40 °C, may be applicable for stage selection in

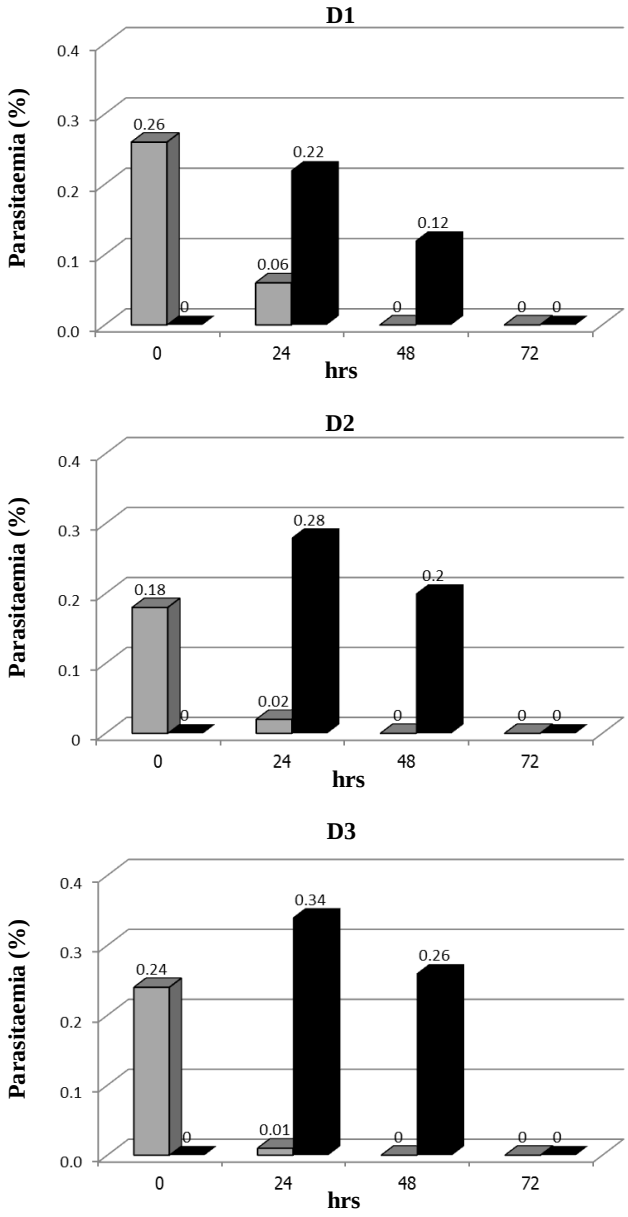


Figure 3 Graphs showed the parasitaemia of ring and non-ring stage in culture incubated at 40 °C for 72 hours: D1 (K1CB1), D2 (T9/94RC17), D3 (3D7)

P. falciparum to synchronize a parasite culture [19].

The changes of culture temperature have great influence to the growth and development of *P. falciparum* during *in vitro* culture. However, the mechanisms of these effects were still unclear. It is possible that, similar to other organisms, deviated temperature may affect their protein synthesis, transportation and/or stability and result in unusual growth and development. It must be noted that the *P. falciparum* also has heat shock protein system which will be synthesized during cell stress, e.g. elevated temperature or oxygen/glucose deprivation [20].

CONCLUSIONS

Different temperatures had different effects to the synchronized *P. falciaparum* culture. The growth pattern, at 34 °C, was similar to control (37 °C), with lower parasitaemia. Low (4 °C) and high temperature (40 °C), has effects on the parasite growth, the total parasitaemia was greatly decreased after a prolong exposure. No parasite was detected after 72 hours incubation at both temperatures. At low temperature, only ring stage was detected while only non-ring stage was detected after 48 hours incubation.

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