GROWTH AND DRUG SUSCEPTIBILITY OF ARTIFICIALLY MIXED-CLONES OF *PLASMODIUM FALCIPARUM* DURING *IN VITRO* CULTIVATION

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ABSTRACT:

To establish an *in vitro* culture of the human malaria parasite *Plasmodium falciparum*, an infected blood sample must be collected and transferred to culture medium in which parasites are adapted to propagate in controlled environment. Genetically mixed parasite populations are highly prevalent in a malarial infection in an endemic area and individual clones are diverse in adaptability to the *in vitro* culture. How proportional variation of parasite clones could affect growth patterns and susceptibility to an anti-malarial drugs during *in vitro* culture remains largely unknown. To address this question, single- and mixed clone cultures of *P. falciparum* consisting of a pyrimethamine sensitive clone (T9/94RC17) and/or a pyrimethamine resistant clone (K1CB1) were established at various proportions and followed for 72 hours (short-term) and 90 days (long-term) to monitor for asexual growth patterns and susceptibility to pyrimethamine, respectively. In the single clone cultures, T9/94RC17 produced higher parasitaemia than K1CB1, while in the mixed clone cultures the growth patterns and stage-specific parasitaemia were varied according to the initial proportions of the parasite clones. In the 100:1 and 10:1 T9/94RC17:K1CB1 mixed clone cultures, the growth patterns were similar to that of T9/94RC17, while in the 1:100 T9/94RC17:K1CB1 mixed clone cultures, the growth pattern was identical to that of K1CB1. In contrast, the intermediate growth patterns between the two clones were observed in the mixtures containing 1:1 and 1:10 T9/94RC17:K1CB1. Drug susceptibility tests revealed also the gradual changes in minimum inhibitory concentration (MIC) and half maximal inhibitory concentration (IC_{50}) values of pyrimethamine in the parasite mixtures following 90-day cultivation, while in the single clone cultures these values remained entirely stable, indicating the contribution of the proportion variation of clones in the cultures to anti-malaria drug responses. In summary, our study demonstrated that asexual growth and susceptibility to pyrimethamine were invariable in short-term and long-term *P. falciparum* cultivations of single parasite clones, but not the mixed clone populations.

Keywords: *Plasmodium falciparum*, *In vitro* culture, Pyrimethamine susceptibility, Minimum inhibitory concentration, Half maximal inhibitory concentration

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INTRODUCTION

Malaria is a serious infectious disease that causes severe clinical symptoms and deaths in humans. Causative agents of human malaria are parasitic protozoa that belong to the genus *Plasmodium*, comprising five species: *Plasmodium falciparum*, *P. vivax*, *P. ovalae*, *P. malariae* and *P. knowlesi* [1-2]. Because a natural vector of human malaria diseases, a female mosquito in the genus *Anopheles*, requires warm climate and suitable habitats for breeding [3], the transmission of human malaria parasites, *P. falciparum* and *P. vivax*, is widely distributed in the tropics and subtropics [4]. In endemic regions including East Africa and Southeast Asia, human infections are often caused by mixed parasite species and mixed clones.

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In addition, in most *P. falciparum* cases, the multi-clonal infections are common [5-12]. For example, our previous study showed that the prevalence of multiple clone infections of *P. falciparum* in six provinces of Thailand in 2009 was as high as 32% [13]. These parasite clones could exhibit variation in phenotypic traits such as asexual multiplication rate, virulence, transmissibility and responses to anti-malaria drugs.

To characterize phenotypes of the malaria parasites of natural isolates, blood samples must be collected and transported to laboratory for *in vitro* propagation and maintenance. The parasite must be transferred to culture medium and maintained in the controlled environment in the laboratory. For over 40 years, the candle jar method has been successfully used for *P. falciparum* culture [14], facilitating biological and molecular characterizations of the parasites. Thaithong and colleagues followed this approach and applied it for cultivation of *P. falciparum* Thai isolates originated from finger prick blood samples [15]. Briefly, a small volume of human blood (20-50 µl) was collected from a subject in an endemic area and transported to a laboratory within 24 hours to reduce the loss of parasite viability. Upon arrival, the parasites were transferred to culture medium and, thereafter, they were maintained for up to several months, depending on the adaptability of parasites and the initial parasitaemia in the blood samples. After cultured, some parasite may be adapted and expand to microscopically detectable levels. Because natural populations in malaria parasites often comprise parasites of various genetic backgrounds and phenotypes, the variation of the clonal composition may result in differential growth patterns and responses to an anti-malaria drug [16-18].

In the present study, we established short and long-term *in vitro* cultures of single and mixed - clones of *P. falciparum* containing T9/94RC17 (pyrimethamine sensitive) and K1CB1 (pyrimethamine resistant) clones at different proportions to monitor for growth patterns and drug susceptibility.

MATERIALS AND METHODS

Parasite clones and maintenance

Two cloned lines of *P. falciparum* used in this study were T9/94RC17and K1CB1. They were originally cloned from the T9/94 clone (pyrimethamine sensitive) [5] and the K1 isolate (pyrimethamine resistant) [19] by micromanipulation, respectively [20]. The minimum inhibitory concentration (MIC) value of pyrimethamine against T9/94RC17 was 0.05 µM [21] while the MIC value of pyrimethamine against K1CB1 was 100 µM [unpublished data]. These two clones were grown continuously in the laboratory according to the simple method of Trager and Jensen [13] and routinely use as a control for phenotype and genotype investigations. In brief, parasites were cultured in Petri dish and maintained in RPMI-1640 medium (GIBCO™ Invitrogen Corporation, USA) containing human O+ red blood cells (RBCs) at 2% haematocrit. The medium was supplemented with 25 mmol/L HEPES (SIGMA-ALDRICH, USA), 24 mmol/L sodium bicarbonate (MERCK, Germany) and 40 mg/L Miramycin (40 mg/ml Gentamycin base: Atlantic Laboratories Corp. Ltd., Bangkok, Thailand) with 10% (v/v) pooled heat-inactivated human serum. The cultures were placed in a desiccator and incubated at 37ºC in an atmosphere produced by candle lighting with approximately 5% CO_2, 5% O_2 and 90% N_2.

Analysis of asexual growth of T9/94RC17 and K1CB1 parasites

T9/94RC17 and K1CB1 clones were synchronized with sorbitol treatment to final ring-stage parasites according to standard protocols [22]. Briefly, asynchronous parasites of each clone from culture were centrifuged and then the medium was discarded. The pack cells were resuspended in 5 times volume of 5% (v/v) D-Sorbitol and leaved for 10 minutes. To remove sorbitol, the cell suspension was centrifuged and the pellet was washed three times with an excess RPMI medium without serum. The synchronized parasites were diluted immediately with 50% haematocrit of fresh uninfected RBCs to the desired parasitaemia (0.5 to 1%). A 200 µl of the diluted parasite was added to the Petri dish containing 5 ml of culture medium to achieve the final of 2% haematocrit. The assays were performed in triplicate. The culture medium was changed daily without the addition of fresh uninfected RBCs. The thin blood smears were prepared at 24, 48 and 72 hours of cultivation. After staining with Giemsa, the numbers of rings and schizonts per 1,000 RBCs were counted under the light microscope at 1,000x magnifications and expressed as percent total parasitaemia (ring + schizont), ring-stage parasitaemia and schizont-stage parasitaemia. The mean parasitaemia and standard deviation were calculated using Microsoft Excel software.

Preparations of mixed-clone cultures

T9/94RC17 and K1CB1 clones were grown to an approximately 30% parasitaemia with at least 20% ring forms. The cultivated parasite clones were synchronized and adjusted to 20% parasitaemia. Both clones were diluted to 2%, 1%, 0.2% and

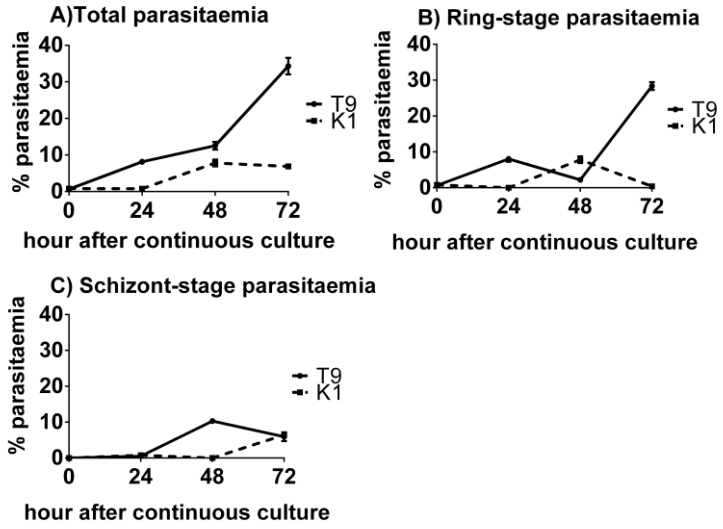

Figure 1 - Total parasitaemia and stage-specific parasitaemia of *Plasmodium falciparum* clones T9/94RC17 (T9) and K1CB1 (K1) during 72 hours after *in vitro* continuous culture. At 0 hour, T9 and K1 cultures comprised of 0.66 and 0.83% parasitaemia respectively. Each group consisted of three independent parasite cultures (n=3). Error bars indicate standard deviation (SD). A, % total parasitaemia; B, % ring stage parasitaemia; C, % schizont stage parasitaemia

0.02% with the fresh uninfected RBCs and mixed to prepare mixed-clone cultures at final concentrations of (i) 0.5% + 0.5%, (ii) 0.1% + 1%, (iii) 1% + 0.1%, (iv) 1% + 0.01% and (v) 0.01% + 1% of T9/94RC17 and K1CB1, respectively. The ratios of mixtures were 1:1, 1:10, 10:1, 100:1 and 1:100% T9/94RC17 and K1CB1, respectively. All the suspensions were adjusted to 2% haematocrit in 5 ml of cultured medium/dish. The single and mixed-clone cultures were set up in triplicate. The medium was changed once or twice a day, depending on the parasite growth and parasitaemia. The cultures were maintained at 2% haematocrit and approximately 2-5% parasitaemia throughout 90 days period of cultivation. The parasite growth was determined on day 0 at 24, 48 and 72 hours. The parasites were harvested for the analyses of drug susceptibility on days 0, 20, 44, 60 and 90 post-cultivation (see below).

Parasite susceptibility to pyrimethamine

On days 0, 20, 44, 60 and 90 post-cultivation, the MIC and half maximal inhibitory concentrations (IC_{50}) were measured, according to standard procedures [23]. Briefly, two single clones and five mixed-clones parasites were harvested and adjusted to 0.5 % parasitaemia. A 10^{-2} M pyrimethamine (SIGMA-ALDRICH, USA) stock solution in 1:1 dimethyl sulphoxide (SIGMA-ALDRICH, USA) and absolute ethanol (MERCK, Germany) was added to the culture medium to obtain final concentrations of 10^{-9} M, $5x10^{-9}$ M, 10^{-8} M, $5x10^{-8}$ M, 10^{-7} M, 10^{-6} M, $5x10^{-6}$ M, 10^{-5} M, $5x10^{-5}$ M, 10^{-4} M, and $2x10^{-4}$ M. A 95 µl of pyrimethamine solutions or culture medium (as a control) were dispensed into a 96-well, flat bottom plate (Thermo SCIENTIFIC NUNC™, Denmark) in triplicate. Five microlitres of infected blood were added in each well to obtain 2% haematocrit. The cultures were further incubated at 37ºC for 72 hours and the culture medium was changed daily. After the incubation, thin blood smears were prepared and stained with Giemsa's stain prior to microscopic examination. The parasitaemia was also recorded and used to calculate the MIC and IC_{50} values using GraphPad Prism 6 software.

RESULTS

Growth patterns of T9/94RC17 and K1CB1 in single clone culture

To investigate growth patterns of the *P. falciparum* clones, T9/94RC17 and K1CB1 cultures were synchronized by sorbitol treatment and diluted to an initial parasitaemia of 0.66% and 0.83%, respectively. The synchronized T9/94RC17 parasite consisted of mature rings, young and late trophozoites (>95%) and early schizonts while K1CB1 parasite was mostly in early ring form (>99%). The parasites were subsequently cultured in separated dishes and monitored for levels of parasitaemia at 24, 48 and 72 hours. T9/94RC17 developed into mostly ring stage within 24 hours due to the effect of sorbitol treatment on the wide age range parasites in stock cultures. The parasite developed into schizont stage at 48 hours and ring

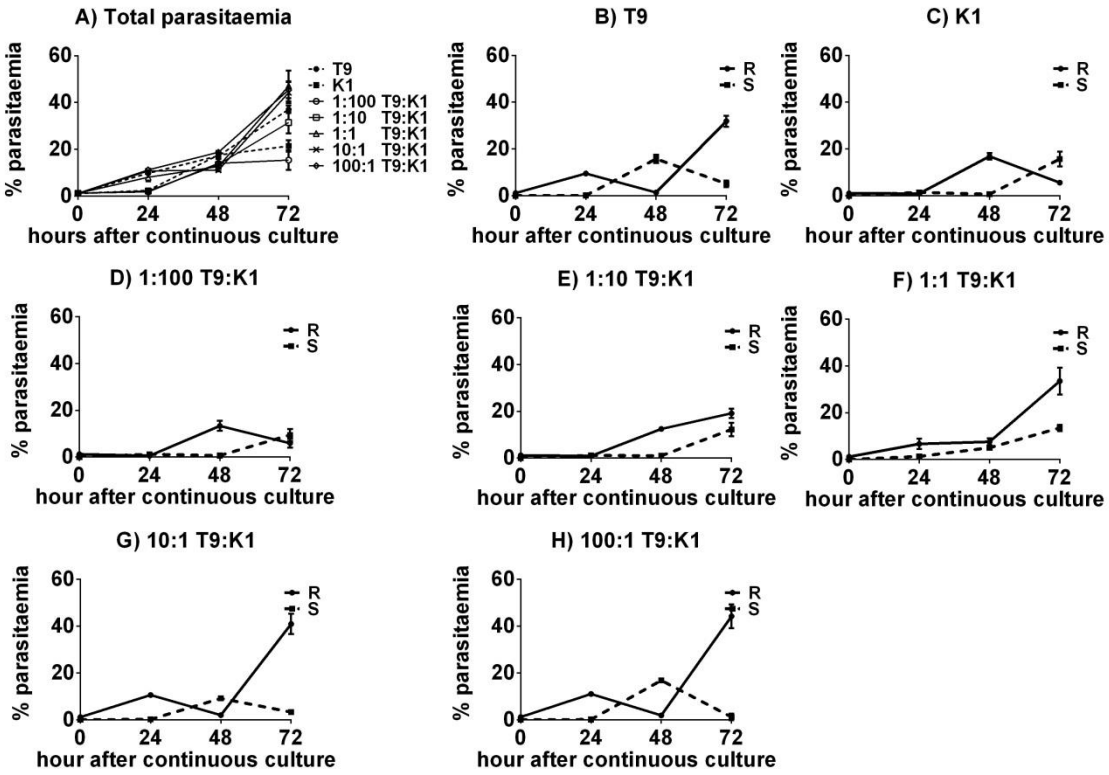

Figure 2 Total parasitaemia and stage-specific parasitaemia of the human malaria parasite *Plasmodium falciparum* clones T9/94RC17 (T9), K1CB1 (K1) and mixed clones during 72 hours after initiation of *in vitro* culture. Each group consisted of three independent parasite cultures (n=3). Error bars indicate standard deviation (SD). A, % total parasitaemia of T9 and K1 clones and mixed clones; B, % ring and schizont of T9; C, % ring and schizont of K1; D, E, F, G and H, % ring and schizont in the mixtures with proportions of 1:100, 1:10, 1:1, 10:1 and 100:1 T9 and K1, respectively. (R, % ring form; S, % schizont)

stage again at 72 hours. Growth of T9/94RC17 reached a peak parasitaemia of ~30% at 72 hours (Figure 1A). At this point, parasite growth was asynchronous (Figure 1B-C). In contrast, K1CB1 showed more synchronous growth in the culture. The ring-stage parasites developed to the schizont-stage parasite after 24-hour incubation (Figure 1C). Then, the parasite developed into ring stage at 48hours and reached its peak parasitaemia of ~10% (Figure 1B). Subsequently, the parasite changed into schizont stages at 72 hours, without affecting the overall parasitaemia (Figure 1A and 1C). These results demonstrated that T9/94RC17 and K1CB1 had different growth patterns. T9/94RC17 showed much faster growth rate *in vitro* and produced higher parasitaemia than K1CB1 in the single clone cultures.

Growth pattern of mixed clones

T9/94RC17 and K1CB1 clones were mixed at different proportions of 100:1, 10:1, 1:1, 1:10 and 1:100, respectively, as described in *Materials and Methods* As shown in Figure 2, growth of these parasite cultures was expressed as the average total parasitaemia and the ring- and schizont-stage parasitaemia. In the mixed parasite cultures with ratios of 100:1 and 10:1 T9/94RC17:K1CB1 (Figure 2G-H), the parasites developed into rings at 24 and 72 hours and schizonts at 48 hours. These growth patterns of the mixtures were similar to those of the T9/94RC17 clone (Figure 2B). In contrast, the mixtures with proportions of 1:1, 1:10 and 1:100 T9/94RC17:K1CB1 grew more slowly and developed mostly into ring forms at 48 hours (Figure 2D-F) At 72 hours, the mixtures containing 1:1 and 1:10 T9/94RC17:K1CB1 grew to higher parasitaemia of 30% and 20% respectively, and most parasites developed into ring forms. This was likely due to the increase in the proportions of parasites in the mixed clone cultures could affect the growth pattern and development cycles of blood stage malaria parasite in the *in vitro* cultures.

Measurement of drug responses to pyrimethamine during *in vitro* culture

The MIC of pyrimethamine against T9/94RC17

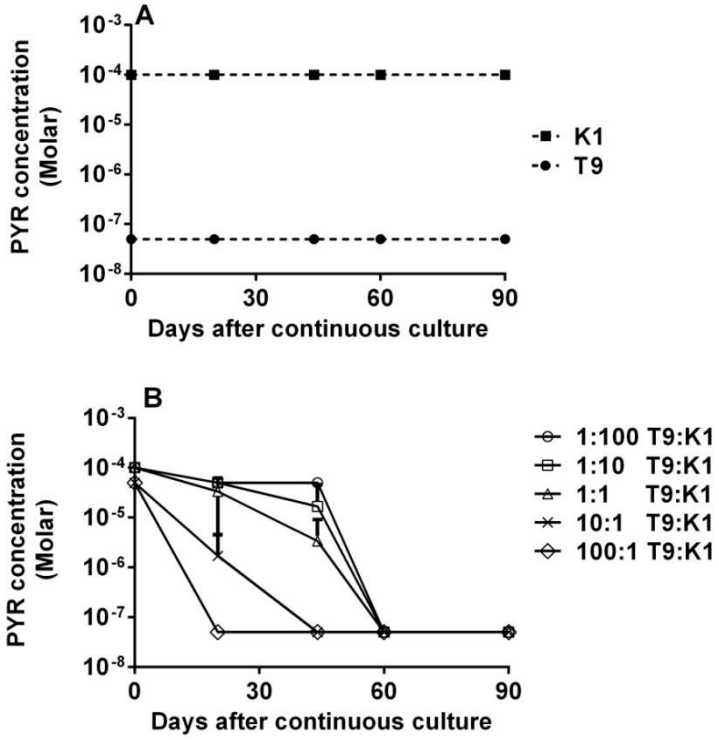

Figure 3 - Minimum inhibitory concentration (MIC) of pyrimethamine for the single and mixed-cloned blood stage cultures of *Plasmodium falciparum* clones T9/94RC17 (T9) and K1CB1 (K1). MIC values were measured at days 0, 20, 44, 60 and 90 after initiation of culture. Each value was derived from three independent assays. Concentration of pyrimethamine (PYR) is expressed in micromolar (µM) unit. A, MIC of single-clone culture T9 (black circle) and K1 (black square); B, MIC of mixed-cloned cultures between T9 and K1 in ratios of 1:100 (white circle), 1:10 (white square), 1:1 (white triangle), 10:1 (cross, x) and 100:1 (white diamond).

and K1CB1 and five mixtures of T9/94RC17 and K1CB1 in proportions of 100:1, 10:1, 1:1, 1:10 and 1:100 were measured at day 0, 20, 44, 60 and 90. The results showed that T9/94RC17 had the MIC value of 0.05 µM, while K1CB1 had the MIC value of 100 µM (Figure 3A). MIC value of both clones was stable thoroughly 90 days of *in vitro* culture**.** On the other hand, MIC values of all mixed cultures of T9/94RC17 and K1CB1 were changed toward 0.05 µM, the MIC value of clone T9/94RC17, during 20 - 60 days (Figure 3B). The rate of changing in MIC value depended on the starting proportions of T9/94RC17 clone. The 1:100 mixed clones was the slowest culture to change its MIC values to 0.05 µM at day 60, compared to the 100:1 mixed clone which changed its MIC to the same level at day 20.

Percent parasitaemia in all cultures were also used to calculate the percent growth inhibition and the IC_{50} against pyrimethamine at day 0, 20, 44, 60 and 90. Percent growth inhibition and IC_{50} of single clone T9/94RC17 and K1CB1 cultures were quite stable with small fluctuation from day 0 to day 90, due to the difference of an initial parasitaemia of each test and their growth patterns (Figure 4A-B, 4H-I). In 100:1, 10:1, 1:1 and 1:10 mixed cultures, their percent growth inhibitions and IC_{50} were similar to the value of T9/94RC17 (Figure 4C-F, 4J-M). Only at higher K1CB1 proportion of 1:100, the percent growth inhibition and IC_{50} resembled to the MIC value of K1CB1 until day 20 but changed to the T9/94RC17 MIC value after day 44 (Figure 4G and 4N).

DISCUSSION

Mixed-clones normally exist in *P. falciparum* isolates collected from any endemic areas [7, 11, 16, 24-26]. *In vitro* culture allowed each clones to grow but the survival of a clone depended on its ability to adapt under *in vitro* condition. Parasites that well adapted to the culture condition may multiply and became a major population while some clones may co-exist as minor populations [17]. However, these minor populations can affect biological characteristics of the isolate, such as the IC_{50} value [18].

In this experiment, two synchronized clones were used, T9/94RC17 (pyrimethamine-sensitive) and K1CB1 (pyrimethamine-resistance). Normally,

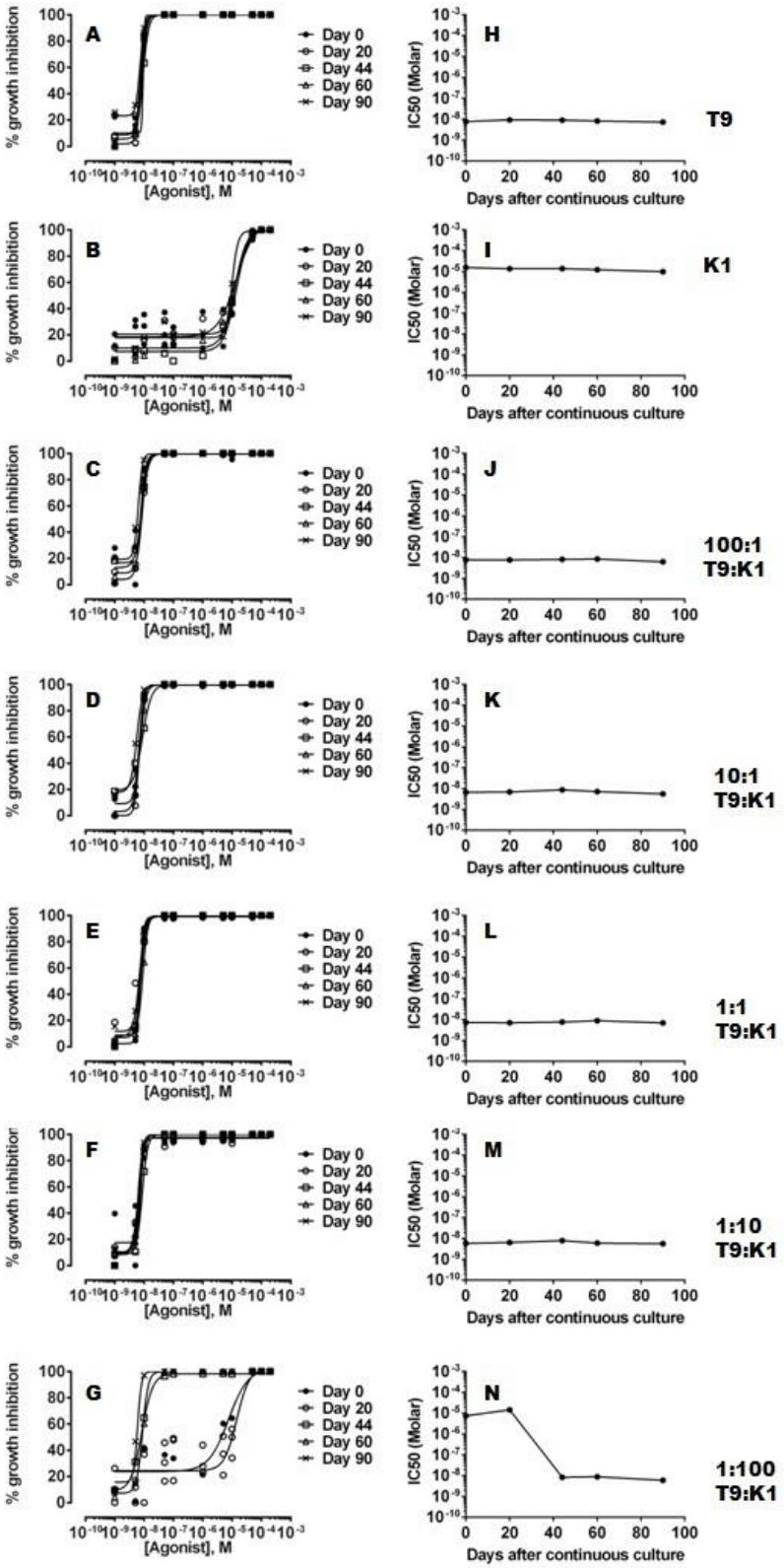

Figure 4 - Percent growth inhibition and half maximal inhibitory concentration (IC_{50}) of pyrimethamine in the single and mixed-cloned blood stage cultures of *Plasmodium falciparum* clones T9/94RC17 (T9) and K1CB1 (K1). Percent growth inhibition of pyrimethamine (agonist) at concentrations 1 nM to 0.2 mM in the single clone cultures of T9 (A) and K1(B) and in the mixed cloned cultures between T9 and K1 in proportion of 100:1 (C), 10:1(D), 1:1 (E), 1:10 (F) and 1:100 (G) during a 90-day period of parasite culture. A to G shows MIC of pyrimethamine for day 0 (dark circle); day 20 (white circle); day 44 (white square); day 60 (white triangle) and day 90 (cross, x). H to N shows IC_{50} of pyrimethamine in the single and mixed cloned cultures during a period of study

in the erythrocytic phase of *P. falciparum*, the merozoites invade new RBCs to form ring-stage and develop into schizont-stage parasites within 24 hours. The development will progress further into ring-stage parasites again in another 24 hours [27]. In this experiment, the slightly increasing in number of ring-stage of T9/94RC17 clone was found at 24 hours after cultivation, it was not implied that T9/94RC17 clone possessed the erythrocytic cycle shorter than 48 hours. But it was due to the fact that the synchronized T9/94RC17 at initiation of cultivation contained late trophozoites and few young schizonts which could develop into ring-stage parasites within 24-hour incubation. However, after 72 hour incubation, T9/94RC17 clone developed into ring stage again and produced higher parasitaemia than K1CB1 clone.

To imitate the adaptation of the natural parasite isolates, the synchronized T9/94RC17 and K1CB1 were mixed in different proportions to evaluate the growth patterns over the short-term *in vitro* cultivation. Among these artificial mixtures, two mixed clones with proportions of 100:1 and 10:1 T9/94RC17:K1CB1 produced the similar growth pattern to the T9/94RC17 culture. In contrast, another culture with proportion of 1:100 T9/94RC17:K1CB1 had similar growth pattern to K1CB1 clone. In the other mixtures with ratios of 1:1 and 1:10 T9/94RC17:K1CB1, the parasites showed the intermediate growth patterns of T9/94RC17 and K1CB1 when the parasitaemia of rings and schizonts were determined. This finding suggested that the growth pattern of an isolate may depend on the major population at the initiation point.

After 90-day cultivation of all mixed clones, the MIC values were gradually decreased toward the value of T9/94RC17 clone (pyrimethamine sensitive). The higher proportion of T9/94RC17 was present, the faster the MIC was changed. In case of the growth inhibition and the IC_{50} value nearly all mixed clones gave similar values or patterns to T9/94RC17. On the contrary, the mixed clone 1:100 showed similar pattern or value to K1CB1 (pyrimethamine resistant) and changed to the pyrimethamine sensitive value. These results suggested that the T9/94RC17 may be better than the K1CB1 in adaptation to the *in vitro* culture condition and outgrowth to become the major population in all mixed-clone cultures after a long period of cultivation (90 days). It must also be pointed out that the MIC evaluation method can be more effective than the growth inhibition and IC_{50} evaluation and can represent the characteristics of minor population in *in vitro* culture at some degrees.

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REFERENCES

1. Garnham PCC. Malaria parasites of man: life cycles and morphology (excluding ultrastructure). In: Wernsdorfer WH, Mcgregor Sir. I., editors. Malaria: principles and practice of parasitology (Volume 1). London: Churchill Livingstone; 1998. p. 61-96
2. Lee KS, Divis PC, Zakaria SK, Matusop A, Julin RA, Conway DJ, et al. *Plasmodium knowlesi*: reservoir hosts and tracking the emergence in humans and macaques. PLoS Pathog. 2011; 7(4): e1002015. DOI: 10.1371/journal.ppat.1002015
3. Service MW. The *Anopheles* vector. In: Gilles HM, Warrell DA, editors. Bruce-Chwatt's essential malariology (3rd ed.). London: Arnold; 1993. p. 97-117.
4. Gilles HM. Epidemiology of malaria. In: Gilles HM and Warrell DA, editors. Bruce-Chwatt's essential malariology (3rd ed.). London: Arnold; 1993. p.124.
5. Rosario V. Cloning of naturally occurring mixed infections of malaria parasites. Science. 1981; 212(4498): 1037-8.
6. Thaithong S, Beale GH, Fenton B, McBride J, Rosario V, Walker A, et al. Clonal diversity in a single isolate of the malaria parasite *Plasmodium falciparum*. Trans R Soc Trop Med Hyg. 1984; 78(2): 242-5.
7. Zakeri S, Bereczky S, Naimi P, Pedro Gil J, Djadid ND, Farnert A, et al. Multiple genotypes of the *merozoite* surface proteins 1 and 2 in *Plasmodium falciparum* infections in a hypoendemic area in Iran. Trop Med Int Health. 2005; 10(10): 1060-4.
8. Babiker HA, Ranford-Cartwright LC, Walliker D. Genetic structure and dynamics of *Plasmodium falciparum* infections in the Kilombero region of Tanzania. Trans R Soc Trop Med Hyg. 1999; 93 Suppl 1: 11-4.
9. Vafa M, Troye-Blomberg M, Anchang J, Garcia A, Migot-Nabias F. Multiplicity of *Plasmodium falciparum* infection in asymptomatic children in Senegal: relation to transmission, age and erythrocyte variants. Malar J. 2008; 7: 17. DOI: 10.1186/1475-2875-7-17
10. Soulama I, Nebie I, Ouedraogo A, Gansane A, Diarra A, Tiono AB, et al. *Plasmodium falciparum* genotypes

diversity in symptomatic malaria of children living in an urban and a rural setting in Burkina Faso. Malar J. 2009; 8: 135. DOI: 10.1186/1475-2875-8-135

11. Kobbe R, Neuhoff R, Marks F, Adjei S, Langefeld I, von Reden C, et al. Seasonal variation and high multiplicity of first *Plasmodium falciparum* infections in children from a holoendemic area in Ghana, West Africa. Trop Med Int Health. 2006; 11(5): 613-9.
12. Druilhe P, Daubersies P, Patarapotikul J, Gentil C, Chene L, Chongsuphajaisiddhi T, et al. A primary malarial infection is composed of a very wide range of genetically diverse but related parasites. J Clin Invest. 1998; 101(9): 2008-16.
13. Pumpaibool T, Arnathau C, Durand P, Kanchanakhan N, Siripoon N, Suegorn A, et al. Genetic diversity and population structure of *Plasmodium falciparum* in Thailand, a low transmission country. Malar J. 2009; 8: 155. DOI: 10.1186/1475-2875-8-155
14. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science. 1976; 193(4254): 673-5.
15. Thaithong S, Seugorn A, Beale GH. Culturing *Plasmodium falciparum* from finger-prick samples of infected blood. Trans R Soc Trop Med Hyg. 1994; 88(4): 490.
16. Viriyakosol S, Siripoon N, Zhu XP, Jarra W, Seugorn A, Brown KN, et al. *Plasmodium falciparum*: selective growth of subpopulations from field samples following *in vitro* culture, as detected by the polymerase chain reaction. Exp Parasitol. 1994; 79(4): 517-25.
17. Chen K, Sun L, Lin Y, Fan Q, Zhao Z, Hao M, et al. Competition between *Plasmodium falciparum* strains in clinical infections during *in vitro* culture adaptation. Infect Genet Evol. 2014; 24: 105-10.
18. Liu S, Mu J, Jiang H, Su XZ. Effects of *Plasmodium falciparum* mixed infections on *in vitro* antimalarial drug tests and genotyping. Am J Trop Med Hyg. 2008; 79(2): 178-84.
19. Thaithong S, Beale GH. Resistance of ten Thai isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine by *in vitro* tests. Trans R Soc Trop Med Hyg. 1981; 75(2): 271-3.
20. Beale GH, Thaithong S, Siripool N. Isolation of clones of *Plasmodium falciparum* by micromanipulation. Trans R Soc Trop Med Hyg. 1991; 85(1): 37.
21. Thaithong S, Ranford-Cartwright LC, Siripoon N, Harnyuttanakorn P, Kanchanakhan NS, Seugorn A, et al. *Plasmodium falciparum*: gene mutations and amplification of dihydrofolate reductase genes in parasites grown *in vitro* in presence of pyrimethamine. Exp Parasitol. 2001; 98(2): 59-70.
22. Lambros C, Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol. 1979; 65(3): 418-20.
23. Thaithong S, Beale GH, Chutmongkonkul M. Susceptibility of *Plasmodium falciparum* to five drugs: an *in vitro* study of isolates mainly from Thailand. Trans R Soc Trop Med Hyg. 1983; 77(2): 228-31.
24. Yuan L, Zhao H, Wu L, Li X, Parker D, Xu S, et al. *Plasmodium falciparum* populations from northeastern Myanmar display high levels of genetic diversity at multiple antigenic loci. Acta Trop. 2013; 125(1): 53-9.
25. Ochong E, Tumwebaze PK, Byaruhanga O, Greenhouse B, Rosenthal PJ. Fitness consequences of *Plasmodium falciparum* pfmdr1 polymorphisms inferred from ex vivo culture of Ugandan parasites. Antimicrob Agents Chemother. 2013. 57(9): 4245-51.
26. Thaithong S, Beale GH, Chutmongkonkul M. Variability in drug susceptibility amongst clones and isolates of *Plasmodium falciparum.* Trans R Soc Trop Med Hyg. 1988; 82(1): 33-6.
27. Trager W, Jensen JB. Continuous culture of *Plasmodium falciparum:* its impact on malaria research. Int J Parasitol. 1997; 27(9): 989-1006.