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## Improving *in vitro* continuous cultivation of *Plasmodium cynomolgi*, a model for *P. vivax*

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Mis en forme : Surlignage

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### Abstract

The absence of a routine continuous *in vitro* cultivation method for *Plasmodium vivax*, an important globally distributed parasite species causing malaria in humans, has restricted investigations to field and clinical sampling. Such a method has recently been developed for the Berok strain of *P. cynomolgi*, a parasite of macaques that has long been used as a model for *P. vivax*, as these two parasites are nearly indistinguishable biologically and are genetically closely related. The availability of the *P. cynomolgi* Berok in routine continuous culture provides for the first time an opportunity to conduct a plethora of functional studies. However, the initial cultivation protocol proved unsuited for investigations requiring extended cultivation times, such as reverse genetics and drug resistance. Here we have addressed some of the critical obstacles to this, and we propose a set of modifications that help overcome them.



## 1. Introduction

Continuous *in vitro* culture of *Plasmodium* parasites has only been achieved for a few species, of which only one, *P. falciparum* specifically infects humans. This has greatly advanced knowledge on this parasite's biology, biochemistry, genetics, etc. To date, *in vitro* cultivation of *P. vivax*, a parasite responsible for increasing proportions of global malaria infection (1) has yet to be achieved (2). Laboratory-based investigations on this important species have thus been restricted to brief *ex-vivo* manipulations of samples collected from patients, often harbouring genetically diverse strains, making comparative studies challenging. Traditionally, *P. cynomolgi*, a parasite species that naturally infects macaques in southeast Asia, has served as the animal model for *P. vivax*, because it recapitulates the morphological and biological characteristics of *P. vivax*, and it has now been confirmed to be phylogenetically highly close to *P. vivax* (3). Previous demonstration that some strains of *P. cynomolgi* can be maintained in culture have recently spurred the development of a robust routine cultivation protocol for the *P. cynomolgi* Berok strain, the usefulness of which has been validated through extended length drug assays, isolation of a cloned line, etc. (4). Although this surrogate model has made it possible to address long-standing issues on *P. vivax*, researchers have faced challenges specific to the *in vitro* maintenance of *P. cynomolgi*.

It was early realised that many of the standard conditions used for *P. falciparum* cultivation were not suitable for *P. cynomolgi*. For instance, *P. cynomolgi* unlike *P. falciparum*, does not grow under standard 5% CO<sub>2</sub>, and serum replacements such as Albumax result in abnormal growth. Furthermore, the standard use of the antibiotic gentamycin to minimise risk of bacterial contamination, a particularly important consideration when cultures need to be maintained for extended periods, is not compatible with *P. cynomolgi* cultivation because this species is susceptible to gentamycin. Finally, the need for non-human primate blood and serum can impose substantial costs both monetary and bureaucratic if these are to be imported.

Here we present the results of our investigations aimed at optimizing *P. cynomolgi* culture conditions, which included testing serum substitutes, diverse antibiotic combinations, and synchronization methodologies, and we provide thereby optimised cultivation conditions that facilitate the maintenance and use of this parasite.

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## 2. Methods

### 2.1. Media

2.1.1.1. Red blood cells: Heparinised *Macaca fascicularis* (Mf) whole blood was obtained from the Monash Animal Research Platform (Monash University, Gippsland, Australia), centrifuged at 600 g for 10 min. The plasma was then removed and replaced with sterile PBS (pH 7.2) to 50% hematocrit (Hct). The cell suspension was gravity fed through a non-woven filter (Antoshin Pte. Ltd.) to remove white blood cells. Red blood cells (RBCs) were stored thereafter at 4°C and 50% Hct in Base media.

2.1.1.2. Base media: RPMI 1640 containing 25 mM HEPES (Gibco™ cat. no. 22400089) supplemented with 2 g/L D-Glucose (Sigma-Aldrich cat. no. G7021) and 400 μM hypoxanthine (Alfa Aesar cat. no. A11481.14).

- 2.1.3. Serum assay: Complete medias were made by addition of one of the following sera and/or serum substitutes (v/v) to base media: Mf serum (MFS) to 20%, Normal Horse serum (NHS) (Gibco™ cat. no. 16050122) to 20%, foetal bovine serum (FBS) ([Pan Biotech Lot#P160608#](#)) to 20%, Mf serum + FBS each to 10%, or Albumax II™ (Gibco™ cat. no. 11021045) to 5 g/L + horse serum to 10%.
- 2.1.4. Antibiotics assays: Various antibiotic concentrations and combinations were tested (12): 3 concentrations of Penicillin G (25 µg, 250 µg, 500 µg), of Cefquinome (0.5 µg/mL, 12.5 µg/mL, 50 µg/mL), or of Gentamycin (0.5 µg/mL, 12.5 µg/mL, 50 µg/mL), as well as 3 low dose combinations of each (25 µg/mL penicillin + 0.5 µg/mL cefquinome, 0.5 µg/mL cefquinome + 0.5 µg/mL gentamycin, 0.5 µg/mL gentamycin + 25 µg/mL penicillin). In all cases these were used in base media supplemented with 5 g/L Albumax and 10% horse serum.
- 2.1.5. Asexual RBC stage synchronization: Sterile stocks of 5% (w/v) D-sorbitol (sorbitol) and 1.4% (w/v) guanidine hydrochloride (GuHCl) were prepared. Media used in synchronization assays was Base Media supplemented with 5g/L Albumax II, 10% horse serum, 25 µg/mL penicillin and 0.5 µg/mL cefquinome.
- 2.2. Continuous culture: *P. cynomolgi* Berok K4A7 parasites were expanded in base media supplemented with 20% FBS at 2-5% Hct using Mf RBCs. Parasitaemia was maintained at 1 – 5% with daily media changes and when adequate by addition of fresh RBCs and incubation at 37°C in a [mixed gassed](#) chamber (5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub>). [Standard 5% CO<sub>2</sub> in air does not support robust continuous growth of \*P. cynomolgi\* Berok.](#)
- 2.3. Flow Cytometry: Parasite growth in all assays was measured by flow cytometry using a FACSCanto II (BD biosciences, San Jose, USA). Cells were stained by adding 10 µL of culture to 80 µL of PBS containing 0.1 µL/mL of SYBR Green I (Invitrogen cat. no. S7563) and 100 nM of Mitotracker Deep Red (Invitrogen cat. no. M22426) followed by incubation at 37°C for 15 min. Samples were diluted in PBS before acquisition and differentiation of parasitized cells by positive fluorescence in both SYBR (488 nm laser, 530/30 nm detector) and Mitotraker (640 nm laser, 670/14 nm detector) channels for 20,000 events.
- 2.4. Media selection assay: Cultured K4A7 parasites were diluted to 1% parasitaemia and 2% Hct with one of the 5 media types and placed into duplicate 1 mL flat bottom culture wells. Media was changed and parasitaemia determined every 2 days by flow cytometry. Parasitaemia was adjusted to 1% on day 4 and 8 and measured again to obtain an accurate measure of parasitaemia increase.
- 2.5. Antibiotics assay: K4A7 parasites cultured in 5 g/L Albumax II™ and 10% horse serum were diluted to 1% parasitaemia and 2% haematocrit. This culture was then grown in the presence of one of 12 antibiotic concentrations and combinations for 15 days (see media 2.1.4). Every 2 days media containing fresh antibiotics was replaced and parasitaemia was determined. On day 4 and 8 the infected PRBCs were diluted 1:10 with fresh uninfected RBCs, and parasitaemia measured before and after dilution.
- 2.6. Bacterial challenge: The ability of media containing antibiotics to protect against a common lab contaminant was set up similarly to the antibiotics assay above, except the RBCs used to initiate culture contained a small number of gram-negative rods (undetectable by microscopy) taken from a previously contaminated culture. Each 1 mL culture had its media changed every 2 days and then split 1:10 on days 5, 9, 13 and 17. Parasitaemia was measured by flow cytometry on

days 0, 6, 11, 15 and 21. Bacterial contamination was monitored by microscopic observation of stained thin smears at each parasitaemia measurement.

2.7. Synchronization: The effectiveness of guanidine hydrochloride and sorbitol to eliminate older *P. cynomolgi* asexual erythrocytic stages but not young rings was tested. Synchronous ring stage K4A7 were produced by 5% sorbitol treatment of cultures initiated three hours earlier using MACs column-purified schizonts and fresh RBCs. The resulting culture of 0-3 hour old ring stage parasites was divided into 1 mL cultures in base media supplemented with 5 g/L Albumax II™ and 10% horse serum at 1.5% haematocrit. Starting at hour 6 and at 6 hour intervals, each pair was treated either with 140 mM guanidine HCl (1.4 % w/v) or with 275 nM sorbitol (5 % w/v) for 15 minutes, then washed twice with fresh medium and returned to a new culture plate that was incubated in mixed gas at 37°C. Parasitaemia was then measured in all wells by flow cytometry at hour 72. Compounds yielding adequate synchronization should have little effect on ring stages compared to untreated control and should eliminate all late-stage parasites.

2.7.2.8. Statistics: As most of the data collected in this study violates normality, non-parametric analysis such as Kruskal-Wallis (with or without Multiple comparisons) or in the case of paired or repeated measures a Wilcoxon or Friedman's Test were used respectively.

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### 3. Results

#### 3.1. Components from *Macaca*

The *P. cynomolgi* Berok K4A7 cloned line was obtained from an uncloned culture of parasites that had adapted to *in vitro* cultivation (4). The process was conducted using red blood cells and serum obtained from naïve *M. fascicularis*. Access to such material can be restrictive and expensive as its shipment requires CITES permits. To date, adaptation of the K4A7 parasite to growth in human red blood cells has not been achieved. The availability of serum is particularly challenging as it requires repeated bleeding due to the low volume able to be obtained from *M. fascicularis* at any one time (25 mL on average per animal once every two weeks).

We tested different combinations of serum supplements for their capacity to sustain the K4A7 cultures (Fig. 1). Media supplemented with Mf serum (MFS) produced the best parasite growth, with an average fold increase in parasitaemia of 5.61 per cycle when 20% Mf serum was used. This was only marginally reduced to 5.32-fold when a combination of 10% Mf serum + Albumax II was used. We then tested foetal bovine serum (FBS) and normal horse serum (NHS), two readily available materials. The average fold increases in parasitaemia were similar for media supplemented with 20% FBS or NHS, 2.7 and 2.6, respectively, with a higher value of 3.2-fold for the 10% NHS + Albumax II.

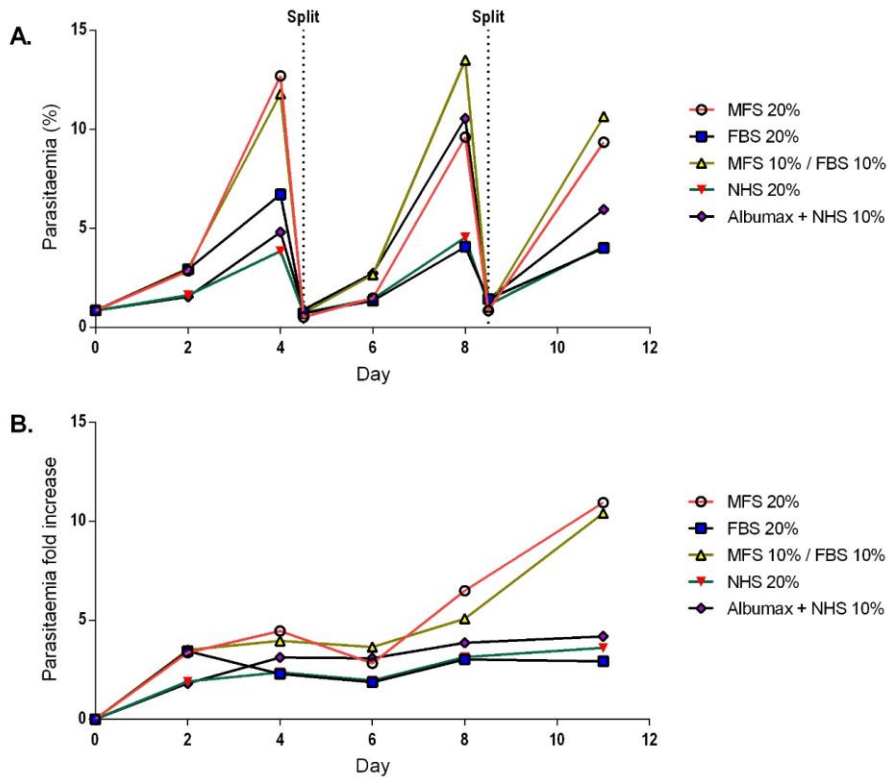


Figure 1. *P. cynomolgi* K4A7 growth at 2% Hct in paired 1 mL cultures media supplemented with 20% MFS, 20% FBS, 20% NHS, 10% MFS + 10% FBS, or 10% NHS + Albumax II (5 g/L). In all 10 wells parasitaemia was measured every 2 days and the cultures split to 1% parasitaemia on day 4 and day 8. **A.** Average parasitaemia of paired assay wells over time. **B.** average parasitaemia fold increase compared to the previous measurement over time. Culture supplements had a significant effect on the parasitemia of in vitro over the 12-day period ( $P=0.012$ ; Friedman Statistic = 12.80)

### 3.2. Antibiotics assay

The repeated manipulation of *in vitro* cultures introduces risk of bacterial contamination that increases as the period of cultivation extends. Such contaminations can be particularly unwelcome when selecting for genetically modified parasites, a generally lengthy process. Three antibiotics were first tested, Penicillin G, Cefquinome, and Gentamycin. At the concentrations tested these antibiotics negatively affected parasite multiplication in a dose dependent manner, as compared to that measured in the absence of antibiotics (Fig. 2). By day 15 after initiation of the cultures, only those exposed to 25 µg/ml Penicillin G, or 0.5 µg/mL Cefquinome maintained a



fold increase exceeding 50% that of control cultures. These observations confirmed the potent inhibitory effect of Gentamycin, and the relative resistance of K4A7 to Cefquinome. When the lower doses of each antibiotic were tested in pairs, the Penicillin G + Cefquinome combination proved the least detrimental to parasite multiplication.

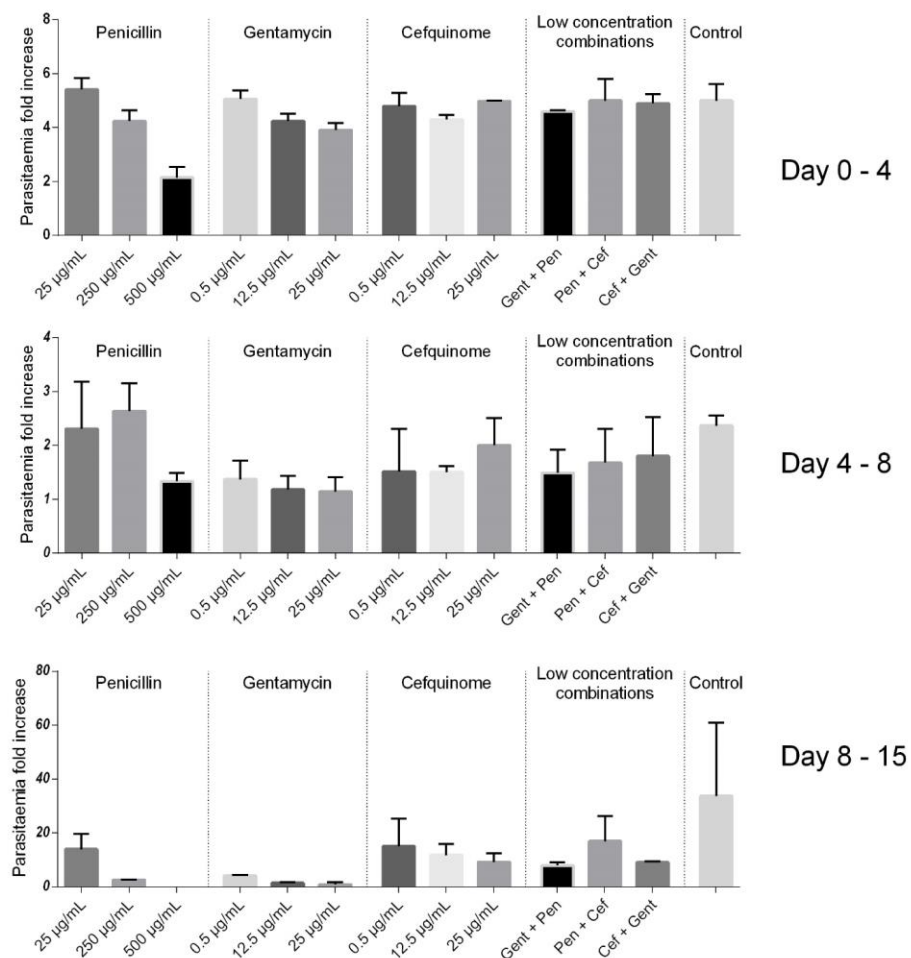


Figure 2. *P. cynomolgi* K4A7 growth in 1 mL of base media supplemented with Albumax (5g/L) + 10% horse serum, 2% Mf RBCs and either; no antibiotics, 3 concentrations of either Penicillin G, Gentamycin or Cefquinome and low dose paired combinations of each (26 individual cultures). Parasitaemia was measured by flow cytometry on days 0, 4, 8 and 15 before and after a 1 in 10 split of parasitized RBCs with fresh Mf RBCs. Parasitaemia fold change from day 0-4, 4-8 and 8-15 is plotted. Friedman's test analysis showed a significant effect of antibiotics on parasite

growth only by Day 8-15, with only low does of Penicillin, Cefquinome and Pen & Cef (combination) allowing significant parasite growth (P=0.0453; Friedman's statistic=21.36)

### 3.3. Bacterial challenge

Given that contamination might occur at any point during cultivation, we wished to test the effectiveness of various antibiotic concentrations and combinations to prevent bacterial growth while maintaining continued parasite multiplication. To this end, cultures were deliberately contaminated with a low dose of Gram-negative bacteria obtained from a previously contaminated culture, thus approximating a contamination event (Fig. 3). In all cases of treatment with a single antibiotic, the cultures either became contaminated by the 6<sup>th</sup> day, as for the untreated control culture, or became parasite free by Day 21. The only exception being for the culture treated with Penicillin G at 25 µg/mL. Similar outcomes were obtained for the antibiotic paired combinations except for the Penicillin G + Gentamycin and the Penicillin G + Cefquinome combinations, with this latter combination showing the greater multiplication rate by Day 21.

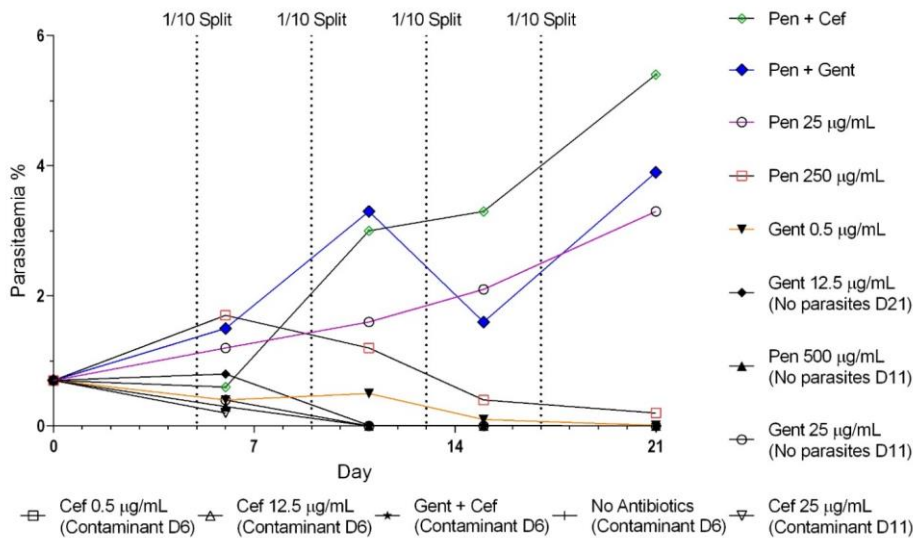


Figure 3. Efficacy of anti-bacterial treatment on 26 paired *P. cynomolgi* K4A7 cultures. Dotted line indicates the days when cultures were split 1:10 with fresh Mf RBCs. Pen = Penicillin, Gent = Gentamycin, Pen = Penicillin G. The day of culture failure (D) and cause is noted in the legend. Significant effects of the antibacterial treatment were found at days 11, 15 and 21 (P<0.01; Kruskal-Wallis Statistic 27.05)

### 3.4 Parasite synchronization.

Various investigations, such as drug assays or transcriptomic analyses, require synchronous parasites. This is usually achieved by treating parasite cultures with chemical agents that

exclusively damage older stages. Sorbitol and GuHCl, which have been used to synchronise other species of *Plasmodium* parasites, were assessed for their ability to do so with *P. cynomolgi*. Aliquots of cultures containing only K4A7 young rings, which were obtained from MACs column-purified mature schizonts that were then allowed to invade fresh red blood cells, were briefly exposed to either sorbitol or GuHCl starting from six hours after initiation of the cultures, and then at six hourly intervals (12 to 48 hours), before being replaced in normal medium and allowed to develop until hour 72. Thus, the reduction in the parasitaemia at hour 72 for each treatment time point as compared to that of the control provides a measure of life stage susceptibility to each agent (Fig. 4).

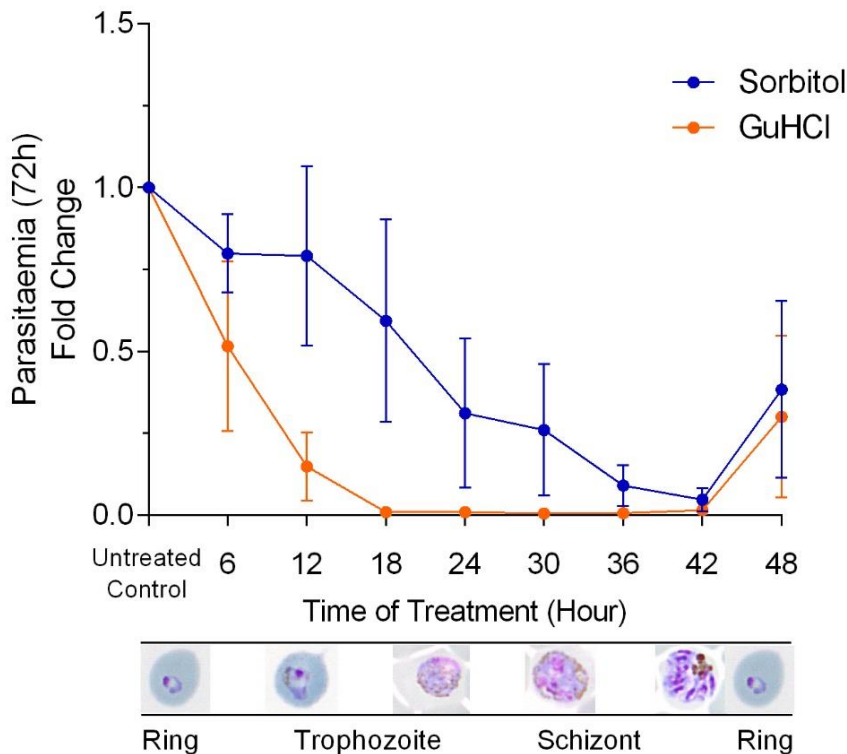


Figure 4. Average fold change of parasitaemia at 72 hours after assay initiation with ring stage (0-3 hours old) *P. cynomolgi* cultures that were either left untreated or exposed for 15 min- to either 5% sorbitol or 1.4% GHCl, at hours 6, 12, 18, 24, 30, 36, 42 or 48. Results from two experiments are shown, one of which was in duplicate [\[54 individual 1 mL cultures\]](#). Photos of Giemsa-stained *P. cynomolgi* during their maturation (ring, young trophozoite, late trophozoite, early schizont, late schizont, and ring) are provided below. [Wilcoxon matched-pairs signed rank](#)

test showed a significant difference in the median fold parasitemia between the Sorbitol and GUHCl treatments (P=0.0078)

Treatment with GuHCl eliminated most of the parasites from all the cultures treated from 12 hr on, and substantially reduced the parasitaemia in the cultures treated at hour 6. By contrast, sorbitol was effective at eliminating parasites that were 36 hours old or more, while leaving relatively unaffected those that were 12 hours or less old. The parasitaemia increase observed in cultures treated at hour 48 was due to those merozoites that had egressed and invaded fresh red blood cells by the time of treatment.

## 1. Discussion

The path to successful routine cultivation and multiplication of malaria parasites can only be determined through empirical testing, a process that might extend for many years as was the case for *P. falciparum*, the first *Plasmodium* species for which a continuous culture was achieved (5). However, the methodology and culture components successfully used for one parasite species are not necessarily suitable for the cultivation of another. To date continuous cultivation has been obtained for a relatively small number of *Plasmodium* species. Moreover, it was also noted that some but not all distinct lines of a particular species can be adapted to cultivation. This was indeed the case for *P. cynomolgi*, where the conditions suitable for cultivation of the Berok strain did not sustain that of the M or B strains of this species (4).

A major hurdle in the cultivation of *Plasmodium* species of non-human primates is the provision of blood and serum from the host animal, in this case *M. fascicularis* or *M. mulatta*. In addition to being particularly onerous, this is exacerbated by oft protracted procedures to obtain CITES permissions when shipment is to be made between countries. A potential solution is to adapt the parasites to growth in human blood, though this might require many months of adaptation with no guarantee of success. The provision of *Macaca* blood is less problematic than that of serum because the RBCs that can be obtained from a single collection can be cryopreserved and thus sustain cultures over extended periods. However, even in laboratories with ready access to the animals, the provision of serum when large cultures volumes are needed remains restrictive because the collection of blood from these relatively small animals is restricted in volume and in time. This makes it necessary to reserve those animals with suitable serum for extended periods, which is often difficult. Moreover, in our experience sera from some naïve animals failed to sustain the Berok line and its cloned line cultures. Therefore, our demonstration that readily available components (foetal bovine serum and normal horse serum) can adequately substitute for *Macaca* serum provides a significant step towards making the cultivation of *P. cynomolgi* accessible to a wider number of researchers.

The susceptibility of the *P. cynomolgi* Berok line to gentamicin, the standard anti-bacterial supplement for the cultivation of other *Plasmodium* species, made it risky to maintain cultures over extended periods, as the likelihood of bacterial contamination increases both with time and culture manipulation. Indeed, the nutrient rich culture medium provides an optimal environment for bacterial growth, and is unforgiving of the slightest mistake. Our results provide a solution to this problem with a combination of two antibiotics (penicillin G and cefquinome) at doses that are tolerated by the parasites. Nonetheless, their addition to

the cultures does reduce the multiplication rate, and their use should therefore be restricted to short intermittent periods, or when a potential contamination is suspected. Their use should also be avoided when the parasites are “stressed” (for e.g. after transfection), and when drug assays are to be conducted.

The availability of highly synchronised parasites is necessary for diverse of biological and functional assays, where a particular stage of the parasite’s developmental erythrocytic cycle is being investigated. This is especially important when conducting the ring stage survival assay (6), when defining the stage specificity of novel compounds (7), or to optimise transfection. Sorbitol, which is generally used to synchronise *P. falciparum* to the ring stage (8), was not found to be as effective for *P. knowlesi*, another parasite of macaques, for which GuHCl was found to be more efficient (9). For the *P. cynomolgi* line cultivated here, although the early ring stages resisted GuHCl nearly all the older stages were adversely affected. GuHCl should therefore be used cautiously, as it is likely to substantially reduce the parasitaemia in treated asynchronous cultures. The inhibitory spectrum of sorbitol is less extensive, with only 24 hour and older parasites significantly eliminated by treatment. We also noted that the morphology of parasites following GuHCl treatment was adversely affected, which was not the case following sorbitol treatment. This should council against using GuCL repeatedly, especially as the Berok lines have a lower replication rate *in vitro* than that of *P. falciparum* or *P. knowlesi*. We recommend favouring repeated sorbitol treatment to maintain synchronicity, while reserving GuHCl for occasions where highly synchronous parasites are needed urgently and when the starting culture has a high parasitaemia. At present, we have not tested whether the disruption caused by GuHCl has a detrimental downstream effect on multiplication rates or indeed on the duration of the synchronicity. Synchronisation can also be initiated via the more onerous magnetic sorting of hemozoin-rich late-stage parasites (10) that can then be allowed to invade fresh red blood cells prior to a sorbitol treatment (7).

The usefulness of *P. cynomolgi* as an experimental model, in addition to its fundamental interest, resides in its status as an ideal model for *P. vivax*, an important parasite of humans that is not amenable to a broad swathe of laboratory investigations. It is hoped that the improvements to the cultivation protocols described here for the *P. cynomolgi* Berok line will promote its wider use by the community, making it also possible to exploit it to unravel fundamental biological processes as has been elegantly demonstrated using the recently cloned *P. knowlesi* H line (11, 12).

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