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Long-Term *In vitro* Cultivation of *Plasmodium falciparum* in a Novel Cell Culture Device

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Abstract. The standard *in vitro* cultivation procedure for *Plasmodium falciparum* requires gas exchange and a microaerophilic atmosphere. A novel system using a commercially available cell culture device (Petaka G3™; Celartia Ltd., Powell, OH) was assessed for long-term cultivation of a *P. falciparum* reference laboratory clone in normal air. Parasite growth during 30 days was similar, or better, in Petaka G3 than that in the standard cultivation method with gas exchange in a CO₂ incubator. The successful cultivation of *P. falciparum* in the Petaka G3 device suggests that low O₂ content available in hemoglobin and dissolved gas in the blood is sufficient for long-term cultivation. This finding may open the way to novel methods to cultivate and adapt *P. falciparum* field isolates to *in vitro* conditions with more ease.

INTRODUCTION

Long-term cultivation of *Plasmodium falciparum* malaria parasites is one of the research tools that have played a crucial role in drug development and in the studies on parasite biology and immunology. The understanding of *in vitro* requirements and development of long-term cultivation techniques took more than 60 years of research since the first attempt by Bass to maintain a successful short-term cultivation during one or two parasite life cycles.^{1,2} In 1976, two independent American research teams had finally discovered the set of requirements for successful continuous *in vitro* cultivation.^{3,4} These include culture media supplemented with buffers and serum, culture vessels that allow shallow static culture favoring gas exchange (flask, microtiter plate, Petri dish, or flow vial), and microaerophilic atmosphere. Other basic requirements include a constant temperature (37–38°C), humidity, and human (or simian) erythrocytes. The original methods used either medium 199 with Earle's modified salts (enriched with 2 mg/mL glucose, 2 mM glutamine, 3 × 10⁻⁵ M 2-mercaptoethanol, and 30 µg/mL α-tocopherol) or RPMI 1640 (Dominique Dutscher SAS, Brumath, France) buffered with either 10 mM TES (for medium 199) or 25 mM HEPES-25 mM NaHCO₃ (for RPMI 1640) and supplemented with 10–15% fetal bovine serum or human serum. Subsequent experiments have shown that several standard media for eukaryotic cell culture, animal sera, and serum substitutes (e.g., lipid-enriched bovine albumin [Albumax®; Gibco, Grand Island, NY]) also support *P. falciparum* growth *in vitro* for short-term and long-term cultivation.^{5–8} Static culture, as opposed to suspension culture, was also found to be unnecessary.^{9–11} The method developed by Trager and Jensen,⁴ rather than the similar technique of Hayes et al.,³ has become a standard laboratory procedure and is currently used with or without minor modifications in malaria research laboratories around the world.

Plasmodium falciparum cultivation has been performed under microaerophilic conditions since 1976. In the original methods developed by Haynes et al.³ and Trager and Jensen,⁴

cultures were placed in a controlled gas mixture of 3–7% CO₂ and 1–6.6% O₂ in CO₂ incubators with variable oxygen control. Subsequent experiments have shown that sophisticated incubators with oxygen control are not necessary. A premixed CO₂–O₂–N₂ gas can be flushed into culture flasks and their caps closed airtight before placing them in a room-air incubator. A suitable atmosphere can also be obtained by various alternative ways, including 5% CO₂ incubators, which lower the O₂ content to about 17%; candle jar (approximately 5% CO₂ and 17% O₂ when the candle burns out); or generator sachet placed in an airtight box or plastic bag.^{12–16}

The requirement for a microaerophilic atmosphere has been one of the limiting factors that have hampered the more widespread application of *P. falciparum* cultivation in field laboratories. A novel system designed to perform eukaryotic cell cultivation independently of CO₂ and humidity has been developed in recent years. Moreover, the contents of the culture vessel are isolated from the external environment, reducing the risk of microbial contamination. The aim of the present study was to assess the performance of long-term *P. falciparum* cultivation using the Petaka G3 device (Celartia Ltd.).

MATERIALS AND METHODS

Cell culture. The drug-sensitive *P. falciparum* reference clone 3D7 (of unknown origin derived from NF54 isolate from a case of airport malaria in Amsterdam, the Netherlands) was cultivated for 30 days under three different conditions using culture flasks or the Petaka G3 device. This latter device, a rectangular chamber (size 85.5 × 127.5 × 5.0 mm and 25 mL of internal volume), was specifically designed for cell cultivation on a large surface area without any internal air space to avoid contamination and allows minimal gas exchange with normal air (internal oxygen tension: 15–45 mmHg) through a 0.2-µm filter for an automatic gas diffusion control system. Its injection port and 0.2-µm air exhaust filter allow introduction of fresh culture medium and removal of spent medium, with or without cell retrieval, in sterile conditions. Many of the cultivation steps can be performed on a laboratory bench without a laminar flow system.

The culture medium–blood mixture was slightly modified from the original methods developed by Haynes et al.³ and

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Trager and Jensen.⁴ For each group, it consisted of type A⁺ erythrocytes at 5% hematocrit suspended in RPMI 1640 medium supplemented with 25 mM HEPES, 25 mM NaHCO₃, 1 mg/L hypoxanthine, 2 mM L-glutamine (GE Healthcare Europe, Vélizy-Villacoublay, France), and 0.5% Albumax II[®]. The initial parasitemia was set at 0.5%.

Group 1 was the control culture performed in 25-cm² culture flasks containing 5 mL of culture medium–blood mixture closed with a vented cap containing a hydrophobic filter for gas exchange (total volume capacity, 50 mL; Falcon[®], Corning, NY) and placed in a CO₂ incubator (5% CO₂ controlled atmosphere with 95% humidity; INCO 108, Memmert GmbH, Schwabach, Germany). Negative control group 2 was identical to group 1 but was placed in regular room-air microbiological incubator type B15 (Thermo-Scientific, Langensfeld, Germany). Group 3 (experimental group) consisted of a 75-cm² Petaka G3 nonadherent cell device containing 25 mL of culture medium–blood mixture to fill the internal chamber completely without leaving any air space inside, as recommended by the manufacturer, which was placed in the room-air microbiological incubator, as in group 2. All culture flasks and devices were incubated at 37°C. Cultures were maintained in 12 flasks or Petaka G3 devices for each group, that is, a total of 36 flasks or culture devices, for 30 days. The culture medium was changed on a daily basis in groups 1 and 2 and every 4 days in group 3 using sterile syringes and needles to remove the spent supernatant, except during holidays and weekends. In all groups, fresh uninfected type A⁺ erythrocytes were added to reduce the parasitemia by half when parasitemia attained 10%. The red cell pellet (2 mL) was divided into two equal fractions. One fraction was cryopreserved, whereas the other fraction was returned to its original flask or device, to which 1 mL of uninfected erythrocytes was added. The dilution factor was taken into account to calculate parasitemia and multiplication rate.

Parasite growth. Parasitemia was evaluated daily in one of the randomly selected replicates from each group by microscopic examination of Giemsa-stained thin blood films. The number of infected erythrocytes was counted against 2,000 erythrocytes and expressed as the percentage of infected erythrocytes.

For cytometric parasite count, parasite growth was measured weekly, that is, on days 8, 15, 22, and 29. The culture medium–blood mixture (25 µL) was transferred from 36 flasks or culture devices to a 96-well plate and fixed with 200 µL of paraformaldehyde and stained with 20 µL of Hoechst 33342 solution (44 µM) (H3570; Life Technologies Corporation, Eugene, OR) in each well.¹⁷ The plate was incubated for 45 minutes at 4°C in the dark, and 50,000 occurrences were analyzed for each sample or measured for 3 minutes when there was a high degree of hemolysis (especially in the negative control group 2), using a CytoFLEX flow cytometer (Beckman Coulter, Villepinte, France).

Statistical analysis. Weekly parasite growth determined individually from 12 replicates in each group using flow cytometry was expressed as the median percentage of parasite growth, quartiles, and range. The Kruskal–Wallis test was used to compare the median of the 3 groups, followed by a 2 × 2 Mann–Whitney test. The significant level was fixed at $P < 0.05$. All analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

The daily parasite growth evaluated using microscopy from day 1 (the first day of cultivation is denoted Day 0) is illustrated in Figure 1A and B. Flow cytometry clearly distinguished between red blood cells infected with different stages of *P. falciparum* and uninfected red blood cells (Figure 2). However, the results obtained with cytometry overestimated the actual parasitemia since all nucleated cells, including multiple trophozoites in a single erythrocyte, or cellular elements, such as merozoites in schizonts, were counted as individual parasites (whereas in microscopy, multiple parasites or merozoites in schizonts in an erythrocyte are counted as a single infected erythrocyte).

Parasites grown in the standard 5% CO₂ atmosphere (positive control group 1) showed a regular increase in parasitemia, requiring a regular dilution with fresh uninfected erythrocytes during the first 14 days. By contrast, parasites in the negative control group 2, cultivated in normal air, failed to grow after 14–15 days, as expected. Before day 7, pyknotic forms characteristic of dying parasites were observed in group 2, and at least some of the asexual parasites included in parasite counting between day 7 and day 15 were probably nonviable. The growth curve of parasites cultivated in the Petaka G3 device (group 3) generally followed a similar tendency of parasite growth as that of group 1. The growth curves of groups 1 and 3 indicated parasite multiplication after the addition of fresh uninfected erythrocytes. At the end of the first week (day 8), groups 1 and 3 resulted in comparable percentage of parasite growth ($P > 0.05$) (Figure 3). Likewise, on day 15 (before dilution with fresh erythrocytes), the growth curves of groups 1 and 3 exhibited similar increasing tendencies. Although parasite growth was not optimal between days 15 and 29 in both groups 1 and 3, probably because of inadequate dilution with fresh uninfected erythrocytes, on day 29, parasites exhibited better growth in group 3 than in group 1 ($P < 0.05$).

DISCUSSION

Several attempts have been made to improve the yield of *P. falciparum* cultivation based on methods modified from the original technical procedures developed by Haynes et al.³ and Trager and Jensen.⁴ For this purpose, modified culture vessels have been developed and applied to *P. falciparum* cultivation, mostly for large-scale production of parasites. In the first attempt, deep stirred cultivation was developed to produce high parasite yields in a single, large vessel (15 L) with semi-automatic medium replacement.¹⁹ In this technique, one-third of the deep culture vessel volume is filled with a gas mixture (5% O₂, 5% CO₂, and 90% N₂) for optimal parasite growth. This deep cultivation system proved to be suitable for optimal parasite growth and yield. More recent experiments have used bioreactors. Cultivation of malaria parasites in a hollow-fiber capillary bioreactor has been shown to increase parasite yield and can be performed even at 100% hematocrit.²⁰ The entire system consisting of the bioreactor (cartridge), pump, and bottle with culture medium is kept either inside an airtight box filled with 5% CO₂, 5% O₂, and 90% N₂ in a room-air incubator or in a 5% CO₂ incubator.^{20,21} A wave bioreactor, in which a controlled wave motion is produced

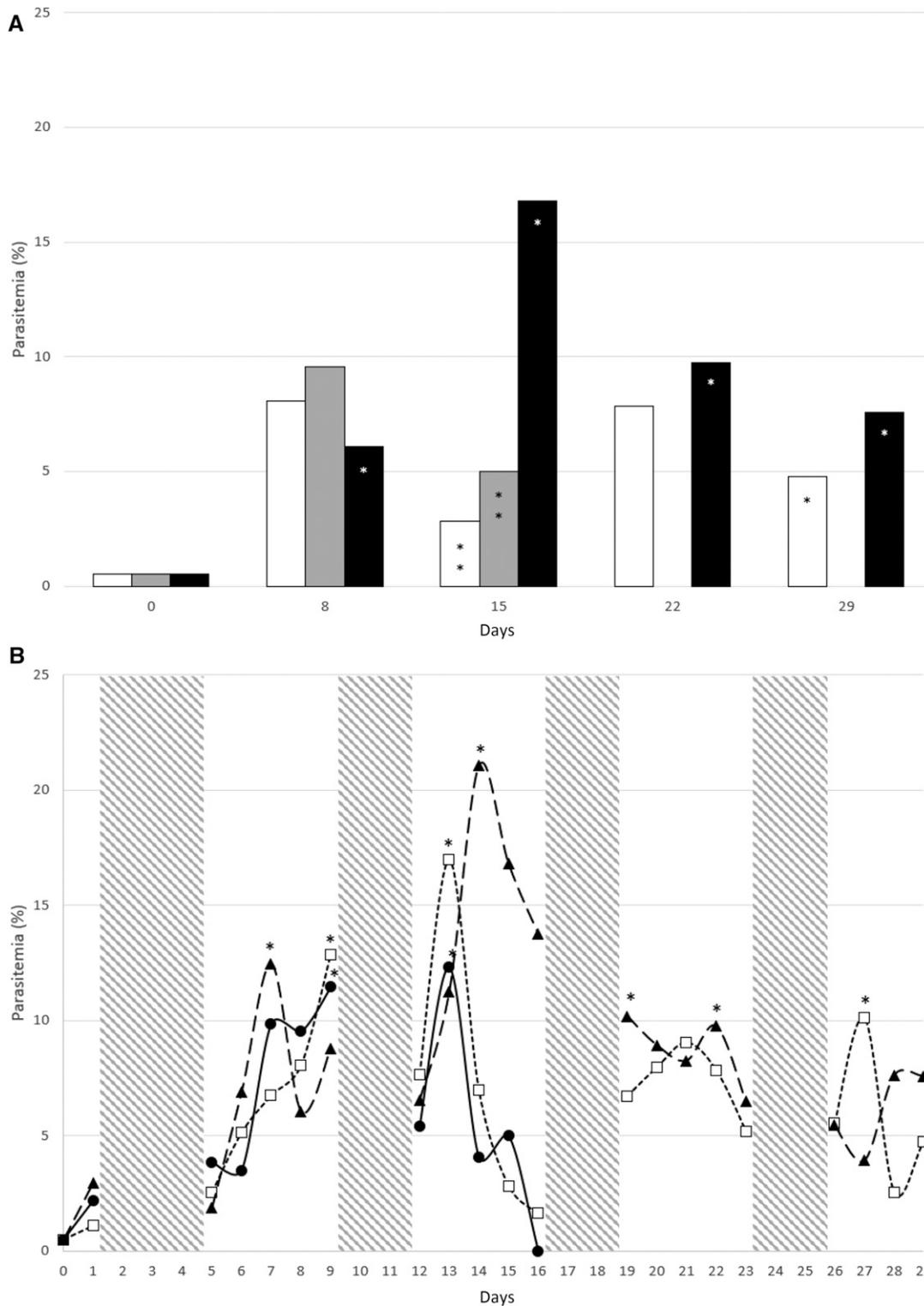


FIGURE 1. (A) Weekly *in vitro* parasitemia under different atmospheric conditions determined by microscopy. Group 1 (white bars), culture flasks in 5% CO₂ (positive control); group 2 (gray bars), culture flasks in normal air; group 3 (black bars), Petaka G3 device under the same conditions as for group 2. After day 15, viable asexual parasites were not observed in group 2. *Number of dilutions made before the evaluation of parasitemia. (B) Daily parasite growth of 3D7 clone in three different atmospheric conditions (group 1, white squares; group 2, black circles; and group 3, black triangles). The hatched vertical bars denote days when cultures were diluted but parasitemia was not determined. Culture media were not changed during weekends and national holidays. Growth was optimal until day 15 in all three groups. Both groups 1 and 3 showed suboptimal growth between days 16 and 29 because of inadequate dilution with fresh uninfected erythrocytes. Group 2 failed to grow beyond day 15. * Dilution of parasitemia with fresh uninfected erythrocytes.

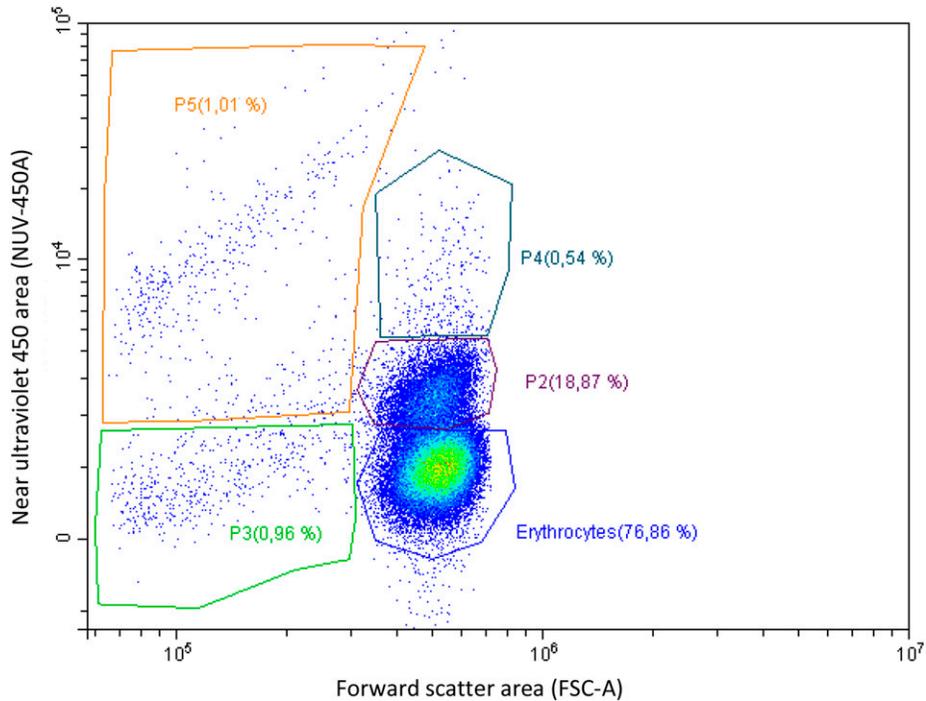


FIGURE 2. Illustration of the cytometric count of various cell components in the blood–culture medium suspension by CytoFLEX flow cytometer (Beckman Coulter) with CytExpert 2.0 software (Beckman Coulter). Here, the term “erythrocytes” denotes uninfected red blood cells; P2, red blood cells with young trophozoites (rings); P3, cell debris; P4, red blood cells with mature trophozoites and schizonts; and P5, free merozoites.¹⁸ Forward scatter area (FSC–A) denotes particle size. Near ultraviolet 450 area (NUV–450–A) corresponds to the Hoescht fluorescence emission. Both X and Y axes are expressed as logarithmic values of fluorescence intensity. This figure appears in color at www.ajtmh.org.

within the device to create and enhance hydrodynamic conditions favorable for cell cultivation and gas exchange (1–2 hours of 3% O₂, 5% CO₂, and 92% N₂ gas flow into the Cellbag), has also been used successfully to maintain the same rate of *P. falciparum* growth as in the static control flask.²² However, cultivation in deep culture vessel or

bioreactors still requires humidity and gas exchange to obtain microaerophilic conditions.

The development of asexual intraerythrocytic *P. falciparum* parasites is characterized by a 48-hour cycle. During the first 24 hours, young trophozoites maintain a low metabolic rate and do not display any major morphological change other than

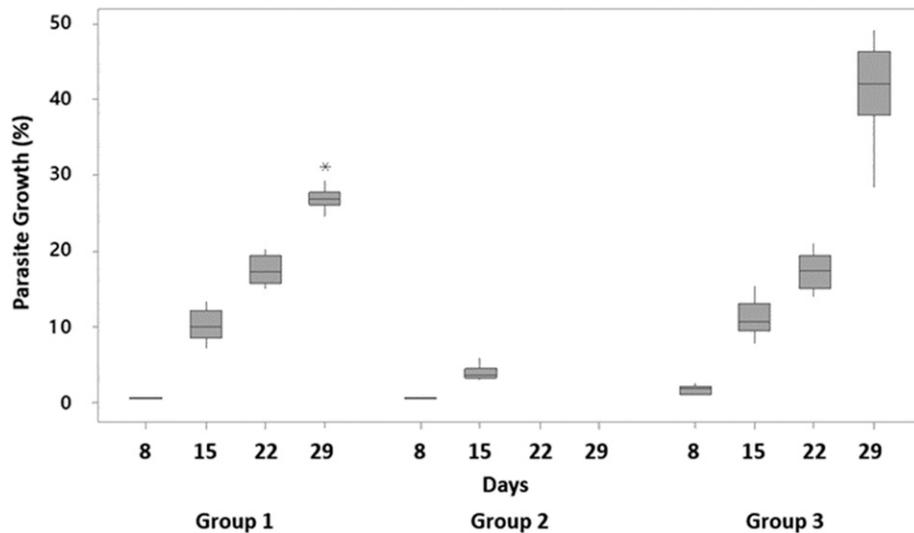


FIGURE 3. Weekly *in vitro* parasite growth under different atmospheric conditions (group 1, culture flasks in 5% CO₂; group 2, culture flasks in normal air; and group 3, Petaka G3 device in normal air) determined by cytometry, with a starting parasitemia of 0.5% on day 0. Dilution factors were included to adjust parasite growth. The box and whisker plots show the median percentage of parasite growth (horizontal bars), their quartiles (box), and range (whiskers). Measurements were obtained from 12 replicates of each group. *One outlier value was observed in group 1. After day 15, asexual parasites with normal morphology and schizonts were not observed in group 2 by microscopic examination of thin smears.

thickening of the cytoplasm. During the second 24-hour period, intense metabolism, accompanied by a large consumption of glucose, increased production of lactic acid, increased cytoplasmic volume occupying most of the available space in the erythrocyte, DNA replication, production of hemozoin, and formation of merozoites, occurs in mature trophozoites and schizonts.²³ Oxygen is the terminal electron acceptor of mitochondrial respiration in humans and malaria parasites. *Plasmodium falciparum* possesses a single mitochondrion with few cristae,^{24,25} which may partly explain why the parasite requires O₂ at a much lower extent, as compared with the human host cells. *In vivo*, only young trophozoites come in contact with relatively high O₂ levels (15–21% O₂ in expired and inhaled air, respectively) during their short transit in the pulmonary alveolar capillaries. Most O₂ molecules (97%) are reversibly bound to the heme iron of the hemoglobin for transport to tissues and cells in humans. A small proportion of O₂ (3%) is dissolved in the plasma. *In vivo*, mature trophozoites and schizonts obtain most of the required O₂ from gas dissolved in the blood in small vessels of deep organs to which they adhere, where the O₂ content at tissue and cellular levels ranges from 0.6 to 5.3%.^{26,27} Mature intraerythrocytic stage parasites may also possibly obtain O₂ molecules carried by hemoglobin during hemoglobin digestion. O₂ as seen in the microaerophilic environment has been shown to be essential to the parasite's growth.^{28,29}

The respiratory physiology of the malaria parasites suggests that *P. falciparum* does not require much gas exchange between the atmosphere and liquid medium during cultivation. The successful cultivation of *P. falciparum* in the Petaka G3 device suggests that low O₂ content available in hemoglobin and dissolved gas is sufficient for long-term cultivation without additional CO₂. Further experiments will be required to extend our observation to a panel of reference laboratory strains of *P. falciparum* and freshly collected isolates. If *P. falciparum* from various sources can be adapted to this cultivation system, malaria parasites can be collected and cultivated in moderately well-equipped field laboratories with more ease in normal air microbiological incubators and less risk of external contamination. Although the unit price of the Petaka G3 device is about 5–6 times more expensive than classical culture flasks (10.00 versus 1.82 US dollars), it is largely offset by the lower cost of a microbiology incubator (with the ambient air), the cost of which is about one-fifth of the 5% CO₂ incubator (including the cost of CO₂ gas supply). The lighter weight and greater mechanical simplicity of the microbiology incubator are also more amenable to use in field laboratories.

This study showed that it is possible to use the Petaka G3 device to cultivate *P. falciparum* under simple working conditions, without high CO₂ concentration to create a low O₂ atmosphere. Before testing the Petaka G3 device in the field, it is important to further develop the standard operating conditions that are suitable for different *P. falciparum* strains.

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