



Original Article

Continuous Cultures of *Plasmodium Falciparum* Established in Tanzania from Patients with Acute Malaria

Florence Urio*^{1,2}, Matilda Mkombachepa^{1,3}, Gratian Rwegasira¹, Twilumba Makene⁴, Billy Ngasala⁴, Teddy Mselle², Julie Makani^{1,5} and Lucio Luzzatto⁵.

¹ Muhimbili Sickle Cell Programme, Muhimbili University of Health and Allied Sciences, Tanzania.

² Department of Biochemistry, Muhimbili University of Health and Allied Sciences, Tanzania.

³ Department of Parasitology, Muhimbili National Hospital.

⁴ Department of Parasitology and Medical Entomology, Muhimbili University of Health and Allied Sciences, Tanzania.

⁵ Department of Hematology and Blood Transfusion, Muhimbili University of Health and Allied Sciences, Tanzania.

Competing interests: The authors declare no conflict of Interest.

Abstract. Background: Malaria morbidity and mortality, almost entirely from *Plasmodium falciparum*, are still rampant in Africa: therefore, it is important to study the biology of the parasite and the parasite-host cell interactions. *In vitro* cultivation of *Plasmodium falciparum* is most useful for this purpose, as well as for investigating drug resistance and possible new therapies. Here we report that the Trager & Jensen continuous culture of *P. falciparum* can be established in a laboratory in Tanzania with minimal facilities and with modest expenditure.

Methodology: This was an *in-vitro* set up of continuous culture of *Plasmodium falciparum* study, carried out in 2016-2020 at Muhimbili university of health and allied sciences, Dar-es salaam. Parasite samples were obtained from patients with acute malaria, frozen parasites, and live cultures. Data was collected and analyzed using GraphPad Prism version 8.

Results: We have successfully achieved exponential growth of existing strains that are used worldwide, as well as of parasites in clinical samples from patients with acute malaria. In the aim to optimize growth we have compared human serum and bovine serum albumin as components of the culture media. Additionally, culture synchronization has been achieved using sorbitol.

Conclusion: This experimental system is now available to our institution and to researchers aiming at investigating drug sensitivity and mechanisms of protection against *Plasmodium falciparum* that accrue from various genes expressed in red cells.

Keywords: *Plasmodium falciparum*; *in vitro* cultures; Albumax II and Human sera.

Citation: Urio F., Mkombachepa M., Rwegasira G., Makene T., Ngasala B., Mselle T., Makani J., Luzzatto L. Continuous cultures of *Plasmodium falciparum* established in tanzania from patients with acute malaria. *Mediterr J Hematol Infect Dis* 2021, 13(1): e2021036, DOI: <http://dx.doi.org/10.4084/MJHID.2021.036>

Published: May 1, 2021

Received: February 11, 2021

Accepted: April 16, 2021

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by-nc/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Correspondence to: Florence Urio. Department of Biochemistry and Muhimbili Sickle Cell Programme, Muhimbili University of Health and Allied Sciences, P. O. Box 65001, Dar-es-Salaam, Tanzania. Tel: +255716894860. E-mail: flosu28@gmail.com

Introduction. All countries in tropical Africa are severely affected by malaria, where *Plasmodium falciparum* accounts for most of malaria morbidity and mortality,¹ with an estimated 400,000 deaths per year.² In Tanzania, we are far from elimination of malaria: this is one good reason why we need to understand better the

biology of the parasite and of parasite-host cell interactions.

The introduction of continuous culture of *P. falciparum* by William Trager over 40 years ago has been of tremendous value in malaria research since viable parasites can be studied at each stage of the intra-erythrocytic cycle.² Compared to long-established laboratory strains, clinical parasite isolates tend to have low rates of multiplication *in vitro*, at least initially. The precise mechanisms underlying adaptation to *in vitro* culture are still incompletely known but adaptation may be associated with loss or gain of large chromosomal regions, as well as with specific mutations^{3,4,5} meaning that established strains are almost certainly genetically different from fresh isolates.

RPMI 1640 has been the reference medium for *P. falciparum* cultures, and it has been used to investigate parasite behaviour, drug action, and potential targets for future therapies.^{6,7} Human serum is known to enhance parasite growth; however, it may contain inhibitory antibodies especially if obtained from donors in malaria-endemic countries:⁸ in view of this, various alternatives have been tested.^{9,10,11,12,13,14,15} Among these, the most popular is commercially available lipid-enriched bovine serum albumin, Albumax I.^{2,16,17,18}

The main aim of our study was to define what are the minimum facilities required to obtain continuous culture of *P. falciparum* in a laboratory in a low resource setting. We report here that both established strains and parasites from patients reporting to hospital with acute malaria can be grown successfully.

Methods.

Study area. This work has been carried out in the Molecular Biology Research Laboratory in the MPL Building at Muhimbili University of Health and Allied Sciences (MUHAS) in Dar-es-Salaam, Tanzania

Study design. *In-vitro* set up of continuous culture of *Plasmodium falciparum* in 2016-2020.

Source of Samples. (a) Samples from consenting patients with acute malaria (*P. falciparum*) were obtained from Emergency Department at Muhimbili National Hospital (MNH).

(b) Frozen parasites: These were obtained from (Kenya Medical Research Institute-KEMRI), Kilifi, Kenya; (Ifakara Health Institute-IHI) Bagamoyo Tanzania and (National Institute for Medical Research-NIMR) Korogwe, Tanzania.

(b) Live cultures: these were obtained from (University of Witwatersrand) Johannesburg, South Africa; (University of Ghana) Accra, Ghana; (University of Milan) Milano, Italy; (National Institute of Health) Rome, Italy and (NIMR) Korogwe, Tanga

Continuous Culture of *Plasmodium falciparum*. For (a) and (c) we have followed the original Trager & Jensen methodology;² for (b) we have used in addition the thawing techniques detailed in Protocols.¹⁹

Clinical isolates. Fresh clinical isolates were obtained from patients with acute malaria residing in Dar es salaam before initiation of anti-malarial drugs. For our attempts to establish continuous cultures from such isolates we selected patients who had at least 30000 parasites/microlitre (**Figure 1**). This was estimated in the Parasitology diagnostic laboratory from the white blood cell count of each patient, and it was then confirmed in the malaria culture lab before starting cultures.

Culture technique. All Venous blood samples (from malaria patients or from normal donors) were collected in EDTA tubes. After initial centrifugation the buffy coat was removed, and the red cells were washed three times in RPMI 1640. To prepare a 25% hematocrit of uninfected red cell suspension, 1-2mls of RPMI 1640 was added to the red cell suspension. The hematocrit was then confirmed by using an automated hematology analyzer (Sysmex XT 2000i Kobe, Japan). The prepared red cell suspension was used for up to 8 days. The culture medium contained NaHCO₃ (25 mmol/liter) and was supplemented with HEPES (25mmol/liter), Gentamicin (80 mg/2ml) and L-glutamine (200mM). Infected red cells were diluted with medium and fresh non-infected red cells to a hematocrit of 2 to 4% (0.08 to 0.16 ml of packed red cells were added to 4ml of 'complete malaria culture media' cMCM) and to an initial parasitemia of 0.1% to 1%. Cultures were grown

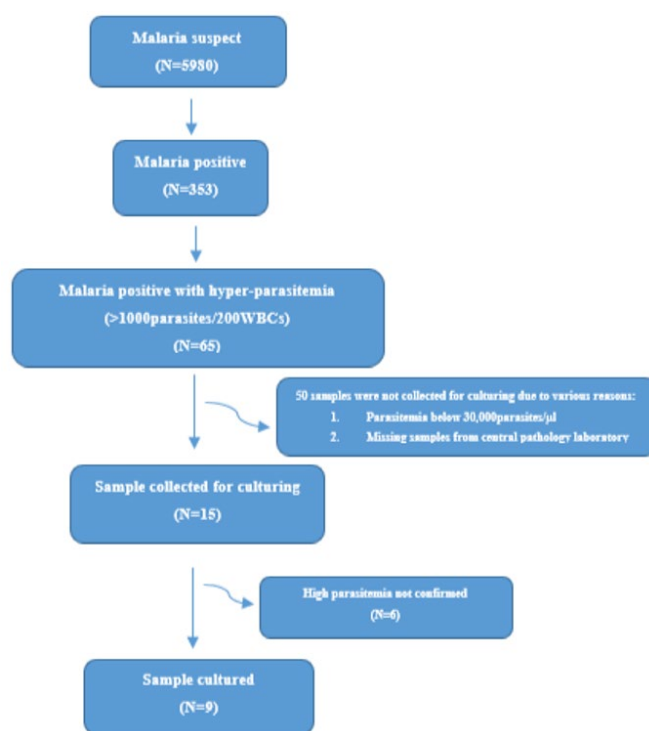


Figure 1. Selection of Clinical malaria samples for *in vitro* cultures.

in 25 cm² flasks, or in small petri dishes, or in 6 well microtiter plates. The cMCM included, in addition to the above, either 10% (vol/vol) group A human serum, or 10% Albumax II solution, or a combination of both in equal parts. Human serum was obtained from donors who had not had malaria for at least the past one year. Flask screw caps were loosened before transfer to the candle jar. The cMCM was replaced on an alternate day and if the culture had parasitemia of 3% and above, group O+ve red cells were added to lower the parasitemia.

The development and growth of parasites was assessed using the light microscope. Percentage parasite count was calculated by counting 300-1000 red cells.

Ethics approval and consent to participate. The study was granted ethical approval by Muhimbili University of Health and Allied Science (MUHAS) Institutional Review Board (Reference number: 2016-7-21/AEC/Vol.x/04).

Results.

Laboratory set-up. For petri dishes or flasks containing red cells in a nutrient medium a major threat is contamination by bacteria (despite gentamycin in the medium) or by fungi: therefore, a Biosafety cabinet (Class II) is the main piece of equipment required (**Figure 2A, B**). The cabinet is equipped with a HEPA (High Efficiency Particulate Air) filter, capable of retaining 0.3-micron particles with 99.99% efficiency. We made sure that the airflow met specifications and that the cabinet was regularly serviced. We installed a UV lamp which was turned on at least 30 minutes before use. Then, with the UV lamp turned off, we exposed open blood-agar and nutrient agar microbiology plates for 12 hours and confirmed there was no bacterial growth. The cabinet was always kept free of any unnecessary items. All manipulations involving cultures or reagents needed for cultures were carried out in this cabinet with sterile precautions. We always wear gloves and sleeveless

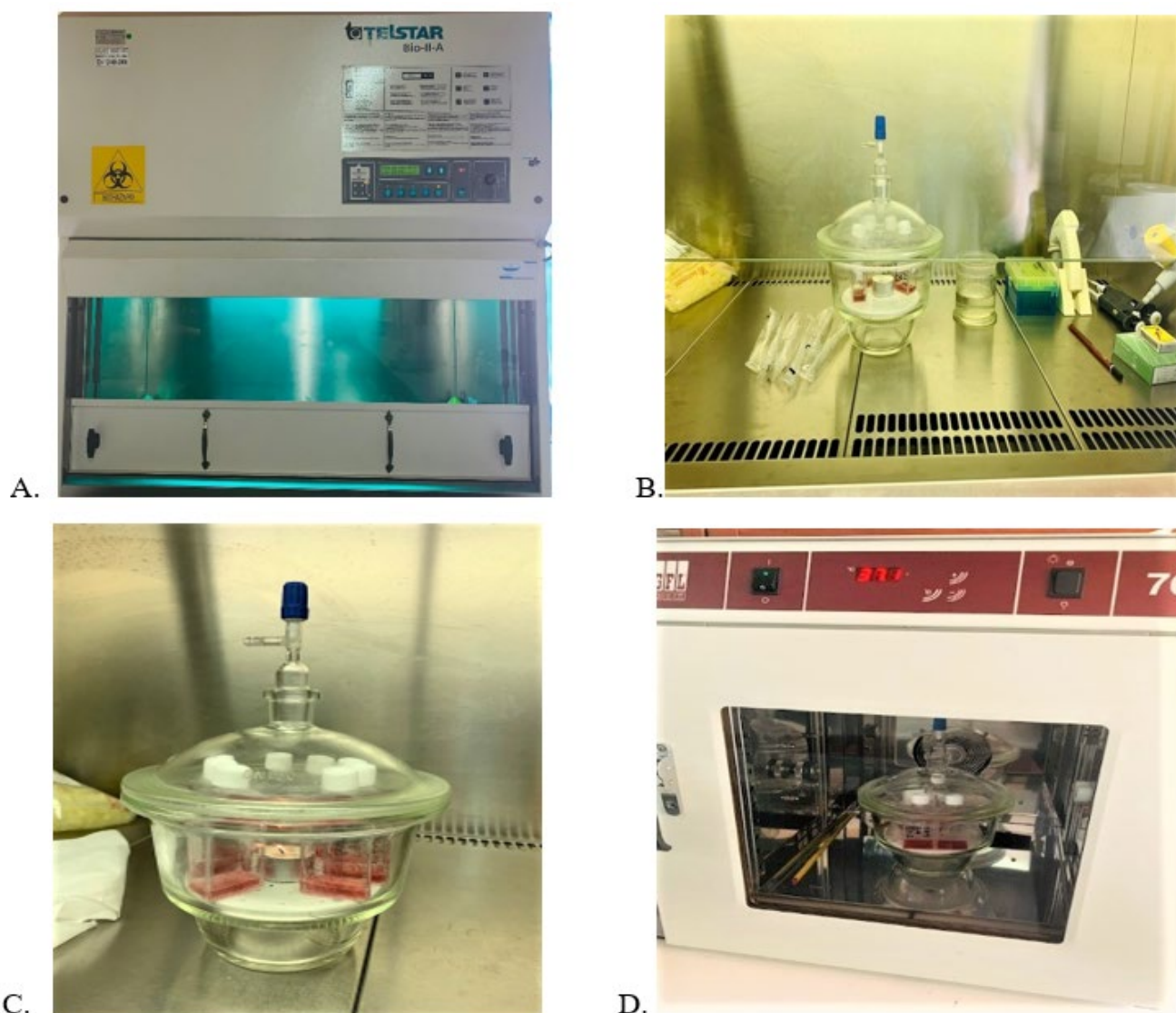


Figure 2. Laboratory set-up for *Plasmodium falciparum* culture. A: Telstar Biosafety Cabinet Class II A with UV light on when not in use. B: Same cabinet when in use. C: Close-up of candle jar with lighted candle. D: Candle jar (flame off) in 37 °C incubator.

Table 1. List of sources for *Plasmodium falciparum* cultures.

<i>Strain</i>	<i>Source</i>	<i>Live/frozen</i>	<i>Date received</i>
3D7	KEMRI (Kilifi, Kenya)	Frozen	27/07/2016
	University of Witwatersrand (Johannesburg, South Africa)	Live	06/08/2016
	University of Ghana (Accra, Ghana)	Live	20/07/2018
	NIMR (Korogwe, Tanzania)	Live	02/04/2019
NF54	University of Witwatersrand (Johannesburg, South Africa)	Live	06/08/2016
	University of Milan (Milano, Italy)	Live	14/04/2018
	National Institute of Health (Rome, Italy)	Live	18/02/2018
	NIMR (Korogwe, Tanzania)	Live	02/04/2019
	IHI (Bagamoyo, Tanzania)	Live	24/09/2020
FCR 3	NIMR (Korogwe, Tanzania)	Live	02/04/2019
DD2	University of Ghana (Accra, Ghana)	Live	20/07/2018
W2Mef	University of Ghana (Accra, Ghana)	Live	20/07/2018
BFM 4782	NIMR (Korogwe, Tanzania)	Frozen	12/02/2019

Trial attempts of malaria cultures from different strains. We are currently culturing the NF54 and FCR3 strains.

gowns on sleeveless arms. Reduced oxygen is known to be essential for optimal growth of *P. falciparum*.² Rather than continuous flow of a gas mixture from an *ad hoc* cylinder, we chose the so-called ‘candle jar’ approach for several reasons. (i) It is free of charge. (ii) Supply of the appropriate gas mixture cylinders may be erratic. (iii) In a sealed candle jar, if it is sterile to begin with, the cultures are completely protected from contamination (the same is not necessarily true in CO₂ incubators with continuous gas flow). By the candle jar method O₂ is 17% and CO₂ is 3%.²⁰ The jar we used was a vacuum desiccator made of heavy glass (**Figure 2C**) with a 2 cm ground glass edge, and the lid has a similar edge (we found vacuum desiccators made of plastic not equally reliable). In order to obtain a perfect seal, we apply a thin but generous layer of high vacuum grease (Dow Corning Corporation, USA) to both edges and to the ground glass

device incorporating the tap. The jar, when open, is handled only under the biosafety cabinet. We lay the flasks or dishes inside the jar, light a white candle, and put in place the lid with the tap open; when the flame goes out, we immediately close the tap.

The sealed jar is then carefully transferred to the incubator, that must have a good temperature control, and must be checked to be never outside the range of 36.8-37.1°C (**Figure 2D**).

Culture of established P. falciparum strains. Thanks to the courtesy of many colleagues (**Table 1**) we have obtained several culture samples, some live and some frozen. The data in **Table 1** indicate that, despite our precautions, infection was a significant problem especially at the beginning. In some cases, cultures may have failed because frozen parasites were no longer

Table 2. Data on individual culture attempts from clinical samples in year 2020.

	Original Parasitemia <i>in vivo</i> (parasites/μl)	Date culture started	Initial parasite count %	Maximum parasite count %	End Date (<i>In vitro</i>)	Comment
PAT-1	175,352	13 May	0.03	0.03	22 May	Bacterial contamination
PAT-2	159,417	18 May	2.7	15	To-date	Successful continuous culture
PAT-3	44,560	18 May	1	1.7	26 May	After 2 cycles culture died
PAT-4	140,250	8 June	0.03	0.03	12 June	After 1 cycle culture died
PAT-5	56,588	12 June	0.03	0.03	17 June	After 1 cycle culture died
PAT-6	115,000	16 June	3	10	To-date	Successful continuous culture
PAT-7	29,593	05 August	1.3	4.0	31 August	After 13 cycles there was bacterial contamination
PAT-8	52,668	13 August	0.67	2.67	31 August	After 13 cycles there was bacterial contamination
PAT-9	46,260	13 September	0.03	2.64	23 September	After 5 cycles culture died

In 2 out of 9 cases continuous culture was established, Re-growth was obtained after thawing samples that had been frozen and stored at -80°C for up to 60 days. Of the 4 samples with *in vivo* parasitemia >100,000 one was unfortunately contaminated; of the remaining 3, 2 yielded successful cultures. This suggests that the level of *in vivo* parasitemia is a major determinant of whether you can establish a long-term (continuous) culture.

viable as a result of prolonged storage or problems associated with transportation.

Cultures of *P. falciparum* from clinical isolates. In our attempts to culture parasites from patients we have selected, for obvious reasons, those who had high parasitemia (**Table 2**). In 9 attempts (leaving aside one in which the culture suffered early bacterial contamination), we initially observed gametocytes in all the cultured clinical isolates for up to 30 days. We also saw the production of new rings (**Figure 3A**) in 8 cases: however, in 4 of these parasite growths stopped after one to five cycles. In the remaining 4 cases we obtained continuous cultures, but two of these were later lost (again because of bacterial contamination). With PAT-2 and PAT-6 we were able to document protracted exponential growth (**Figure 4**) with high parasite counts (**supplementary table 1**). The multiplication factor per cycle (48hrs) of clinical isolates ranged from 1.6 to 5.5, whereas it was 8.0-11.1 for the NF54 strain.

Composition of culture media. Since the original notion of Trager & Jensen that a strong buffer (HEPES) was required, and that 10-20% human serum would help to optimize growth, attempts to improve culture media have not gone far: except that human serum has been often replaced by bovine albumin (Albumax). For a start we preferred human serum because it is easily available and

free of charge from generous donors; however, we were aware that human serum in a malaria-endemic setting is likely to contain antibodies that may inhibit *P. falciparum* growth. In several experiments we observed that a 1:1 mixture of human serum with Albumax was either equivalent or superior to Albumax alone (**Figure 5**).

Synchronization. We have used the sorbitol technique¹⁹ and the refrigeration technique.²¹ Starting from a culture with a parasite count of 8.6%, of which 65% rings, 25% trophozoites and 20% schizonts, we obtained a culture that had 77% rings after one round of sorbitol treatment, and 92% rings after two rounds of sorbitol.

Recovery of frozen parasites. Ideally parasitized red cells should be stored frozen in liquid nitrogen (i.e. at -195°C). However, since this was not available, we have stored parasitized red cells in a -80°C deep-freezer and recovered them successfully after up to 120 days. The freezing solution consisted of 28% Glycerol; 3% Sorbitol; 0.65% NaCl in distilled water; the thawing solution was 3.5% NaCl.

Discussion. *In vitro* cultivation of continuous *Plasmodium falciparum* cultures was established more than 40 years ago, and it has been a tremendous booster for research.² Formerly *P. falciparum* malaria could be investigated only in endemic countries [or

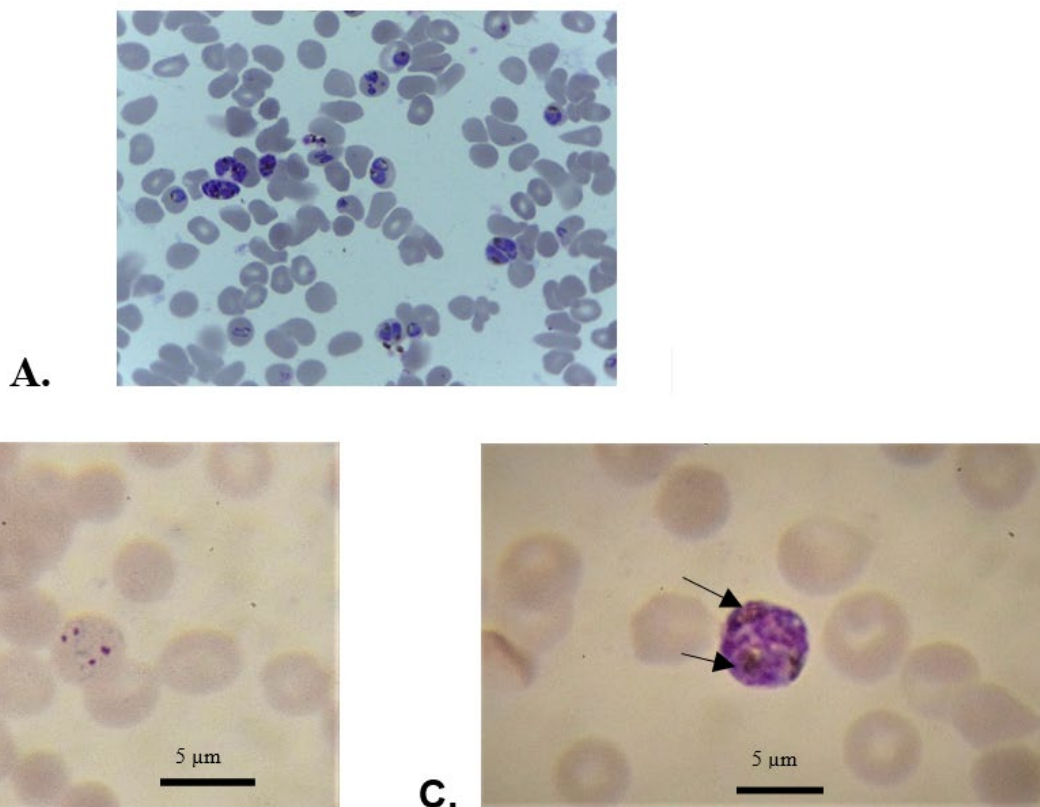


Figure 3. Microscopic images of *Plasmodium falciparum* from *in vitro* cultures A: Low power view of culture smear: all stages of parasite development are seen. B: High power view: red cell with multiple rings C: Schizont with hemozoin (malarial pigment).

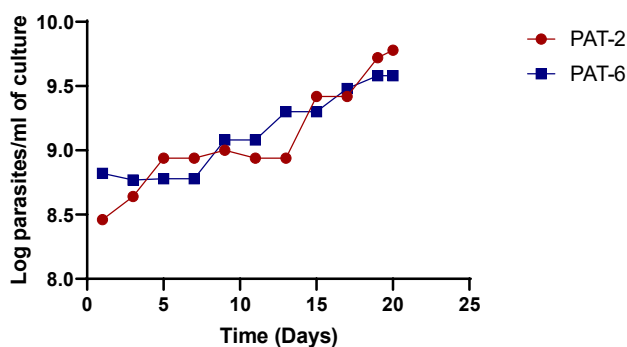


Figure 4. Exponential growth of *Plasmodium falciparum* in cultures from clinical isolates. A mixture of serum/albumax 50:50 was used for culturing. Values on the ordinate axis were calculated from parasite counts on smears, taking into account the dilution of parasitized red cells by non-parasitized red cells each time fresh red cells were added every 2 days.

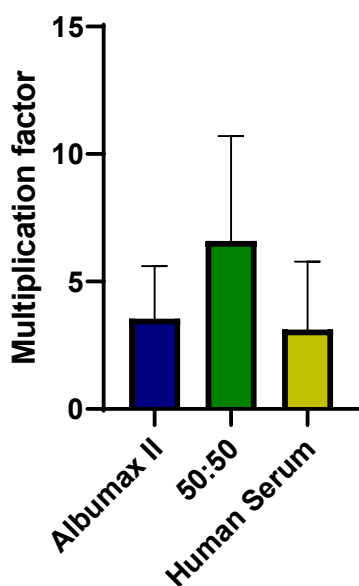


Figure 5. Supplementation of media with a mixture of human serum and bovine serum optimizes parasite growth. Parasites were cultured in complete culture medium containing i) Albumax II ii) 50:50 Albumax II and human serum A+ and iii) Human Serum A+. The experiment was performed in two replicates. Paired t-test was used to compare the mean differences. Serum vs Albumax ($p=0.0567$); 50:50 vs serum ($p=0.0123$) and 50:50 vs Albumax (0.0636).

experimentally in *Aotus trivigatus*, the owl monkey].²²

With *in vitro* cultures available, there has been a reversal: research on *P falciparum* has become easy in non-endemic areas; whereas it may be lagging where cultures are not carried out. For this reason, set up of culture facilities in endemic areas has become very important, and it has been done in several countries in Africa, in order to conduct studies in immunology, molecular biology, genetics, pharmacology and biochemistry.^{23,24,25,26} In this paper, we have reported in detail how this can be done successfully with minimal resources.

Since exponential growth is probably the best proof

that the culture is doing well, we find that for small scale experiments the candle jar method is entirely satisfactory: it does not require customized gas mixtures, nor a dedicated CO₂ incubator. Since maintaining cultures all the time is demanding in terms of labor, media, and supply of fresh red cells, it is convenient to resort to freezing whenever live parasites are not needed. From this point of view, it is of practical importance that storage at -80°C is satisfactory for 2-4 months.

In our initial experiments we have supplemented media with human serum, in keeping with the original formula of Trager & Jensen.² However, these authors worked in a malaria-free setting in New York City. We have observed, not surprisingly, that sera from different donors give different results: it stands to reason that if donors who have been exposed to malaria – the rule rather than the exception in Tanzania – their serum may contain inhibitory antibodies.²⁷ Dohutia et al found that the combination of fresh human serum and Albumax might be superior, which is similar to our findings with strain NF54.²⁸ Therefore, it may be expedient, though more expensive, to use Albumax instead of human serum, or a 1:1 mixture of both: the latter worked well in our hands.

P. falciparum strains that are used worldwide, such as 3D7, FCR-3 are a great asset, because they make it possible to compare results, no matter where an experiment is carried out. On the other hand, it is abundantly clear that laboratory-adapted strains are different from ‘wild’ parasites. In this respect, a unique advantage of malaria cultures being carried out in a malaria-endemic area is that one may obtain parasites that are indeed wild, as previously shown by.^{29,30,31,32} In our small series this was successfully achieved in 4 out of 9 cases. In all these cases we have observed low rates of multiplication in the first 10 days followed by exponential growth (Figure 4), even though the multiplication factor was still low compared to that of well-established laboratory strains. This is similar to what was observed in previous studies.^{5,33} It will be clearly interesting to determine why in some cases adaptation to laboratory conditions is so prompt, whereas in other cases it fails.

The main limitation of our study has been the significant incidence of bacterial or fungal contamination, that on several occasions has forced us to discard cultures. We have learnt that one can never be too careful in this respect: for instance, to keep the laminar flow cabinet free from clutter is imperative.

A different kind of limitation is that of resources. We have already enumerated the equipment needed. As for running costs, if we add up maintenance of the laminar flow cabinet, media, plastic, and other consumable materials, with a mean of 4-8 culture flasks in use our best cost estimate is of approximately 490 US\$ per month.

Despite the limitations, this study has highlighted some of the technical difficulties and solutions for setting up continuous *in vitro* cultures of malaria in an endemic region. Similar studies were conducted previously in Mali and Nigeria.^{29, 30}

Conclusions. Our first and foremost aim in establishing continuous *in vitro* cultures of *P. falciparum* was to make these available to our scientific community. In the meantime, we have been also recently asked by the Tanzania Medicines and Medical Devices Authority (TMDA) to provide cultures for quality testing of malaria rapid diagnostic test kits. In addition, we plan to investigate in greater depth the mechanisms whereby red cells with different genotypes play host to *P. falciparum*: this endeavour is currently in progress. Most importantly, we believe our malaria culture lab will enable malaria research into real life clinical isolates and drug resistance.

Acknowledgements. We are grateful to the members of the Department of Biochemistry, particularly the late Mr. Idrisa Mshanga for his tireless support in setting up the

laboratory; we also thank Dr. Francis Dida for kindly making laboratory space available. We thank Mr. Ally Athuman Sule for his great support in data collection at Muhimbili National Hospital. We appreciate greatly the support from Dr. Daniel Minja and Mr. Gerson Maro during data collection at NIMR-Korogwe and Dr. Lucas Matemba from NIMR-Morogoro. We are grateful to the following, who provided frozen parasites and live cultures of *Plasmodium falciparum*: Mr. Mgeni Tambwe and Mr. Lorenz Hofer from Ifakara Health Institute, Bagamoyo; Professor Jeffrey Dorfman from University of Capetown, South Africa; Professor Maureen Coetzer University of Witwatersrand, Johannesburg, South Africa; Dr. Gordon Awandare University of Ghana; Dr. Donatella Taramelli, University of Milano, Italy; Dr. Pietro Alano Istituto Superiore di Sanità, Rome, Italy. We acknowledge the financial support from MAGI and MMV, Italy and MUHAS-SIDA small grant, Tanzania. Lastly, we thank all staff of Muhimbili Sickle Cell Programme, Muhimbili University of Health and Allied Sciences and Muhimbili National Hospital.

References:

1. <https://www.who.int/news-room/feature-stories/detail/world-malaria-report-2019>
2. Trager W, Jensen J. Human malaria parasites in continuous culture. *Science* (80-) [Internet]. 1976 Aug 20;193(4254):673-5. <https://doi.org/10.1126/science.781840> PMID:781840
3. Bhasin VK, Clayton C, Trager W, Cross GAM. Variations in the organization of repetitive DNA sequences in the genomes of *Plasmodium falciparum* clones. *Mol Biochem Parasitol* [Internet]. 1985 May;15(2):149-58. [https://doi.org/10.1016/0166-6851\(85\)90116-1](https://doi.org/10.1016/0166-6851(85)90116-1)
4. Corcoran LM, Forsyth KP, Bianco AE, Brown G V., Kemp DJ. Chromosome size polymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite populations. *Cell* [Internet]. 1986 Jan;44(1):87-95. [https://doi.org/10.1016/0092-8674\(86\)90487-3](https://doi.org/10.1016/0092-8674(86)90487-3)
5. Summary of discussions on *in vitro* cultivation of malaria parasites. *Bull World Health Organ*. 1977;55(2-3):411-9.
6. Desai SA. Insights gained from *P. falciparum* cultivation in modified media. *Sci World J*. 2013;2013. <https://doi.org/10.1155/2013/363505> PMID:23956690 PMCID:PMC3727134
7. Ringwald P, Meche FS, Bickii J, Basco LK. *In vitro* culture and drug sensitivity assay of *Plasmodium falciparum* with nonserum substitute and acute-phase sera. *J Clin Microbiol*. 1999;37(3):700-5. <https://doi.org/10.1128/JCM.37.3.700-705.1999> PMID:9986835 PMCID:PMC84528
8. Asahi H, Kanazawa T. Continuous cultivation of intraerythrocytic *Plasmodium falciparum* in a serum-free medium with the use of a growth-promoting factor. *Parasitology* [Internet]. 1994 Nov 6;109(4):397-401. Available from: <https://doi.org/10.1017/S0031182000080641> PMID:7800407
9. Divo AA, Jensen JB. Studies on serum requirements for the cultivation of *Plasmodium falciparum*. I. Animal sera. *Bull World Health Organ*. 1982;60(4):565-9.
10. Ifediba T, Vanderberg JP. Peptones and Calf Serum as a Replacement for Human Serum in the Cultivation of *Plasmodium falciparum*. *J Parasitol* [Internet]. 1980 Apr;66(2):236 <https://doi.org/10.2307/3280810>
11. Lingnau A, Margos G, Maier WA, Seitz HM. Serum-free cultivation of several *Plasmodium falciparum* strains. *Parasitol Res* [Internet]. 1994;80(1):84-6. <https://doi.org/10.1007/BF00932631> PMID:8153133
12. Desjardins RE, Alexander BM, Weatherly NF, Bowdre JH, Oduola AMJ. Use of Non-Human Plasma for *In Vitro* Cultivation and Antimalarial Drug Susceptibility Testing of *Plasmodium falciparum*. *Am J Trop Med Hyg* [Internet]. 1985 Mar 1;34(2):209-15. <https://doi.org/10.4269/ajtmh.1985.34.209> PMID:3885768
13. Sax LJ, Rieckmann KH. Use of Rabbit Serum in the Cultivation of *Plasmodium falciparum*. *J Parasitol* [Internet]. 1980 Aug;66(4):621. <https://doi.org/10.2307/3280518>
14. Willet GP, Canfield CJ. *Plasmodium falciparum*: Continuous cultivation of erythrocyte stages in plasma-free culture medium. *Exp Parasitol* [Internet]. 1984 Feb;57(1):76-80. [https://doi.org/10.1016/0014-4894\(84\)90065-1](https://doi.org/10.1016/0014-4894(84)90065-1)
15. Binh VQ, Luty AJF, Kremsner PG. Differential Effects of Human Serum and Cells on the Growth of *Plasmodium falciparum* Adapted to Serum-Free *In Vitro* Culture Conditions. *Am J Trop Med Hyg* [Internet]. 1997 Nov 1;57(5):594-600. <https://doi.org/10.4269/ajtmh.1997.57.594> PMID:9392601
16. Cranmer SL, Magowan C, Liang J, Coppel RL, Cooke BM. An alternative to serum for cultivation of *Plasmodium falciparum* *in vitro*. *Trans R Soc Trop Med Hyg* [Internet]. 1997 May;91(3):363-5. [https://academic.oup.com/trstmh/article-lookup/doi/10.1016/S0035-9203\(97\)90110-3](https://academic.oup.com/trstmh/article-lookup/doi/10.1016/S0035-9203(97)90110-3) [https://doi.org/10.1016/S0035-9203\(97\)90110-3](https://doi.org/10.1016/S0035-9203(97)90110-3)
17. Flores MVC, Berger-Eiszele SM, Stewart TS. Long-term cultivation of *Plasmodium falciparum* in media with commercial non-serum supplements. *Parasitol Res* [Internet]. 1997 Aug 1;83(7):734-6. <https://doi.org/10.1007/s004360050330> PMID:9272569
18. Wang P, Read M, Sims PFG, Hyde JE. Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. *Mol Microbiol* [Internet]. 1997 Mar 31;23(5):979-86. <https://doi.org/10.1046/j.1365-2958.1997.2821646.x> PMID:9076734
19. http://ki.se/sites/default/files/methods_in_malaria_research
20. Trager W, Jensen J.B. Cultivation of erythrocytic stages. *Bulletin of the World Health Organisation*. 1977; 55:363-365

21. Yuan L, Hao M, Wu L, Zhao Z, Rosenthal BM, Li X, et al. Refrigeration provides a simple means to synchronize in vitro cultures of *Plasmodium falciparum*. *Exp Parasitol* [Internet]. 2014 May;140:18-23. <https://doi.org/10.1016/j.exppara.2014.03.010> PMID:24632190 PMCID:PMC4018460
22. TRAGER W. A New Method for Intraerythrocytic Cultivation of Malaria Parasites (*Plasmodium coatneyi* and *P. falciparum*). *J Protozool*. 1971;18(2):239-42. <https://doi.org/10.1111/j.1550-7408.1971.tb03314.x> PMID:4997037
23. Awandare GA, Nyarko PB, Aniweh Y, Ayivor-Djanie R, Stoute JA. *Plasmodium falciparum* strains spontaneously switch invasion phenotype in suspension culture. *Sci Rep*. 2018;8(1):1-10. <https://doi.org/10.1038/s41598-018-24218-0> PMID:29636510 PMCID:PMC5893586
24. Amoah LE, Kakaney C, Kwansa-Bentum B, Kusi KA. Activity of Herbal Medicines on *Plasmodium falciparum* Gametocytes: Implications for Malaria Transmission in Ghana. Lanz-Mendoza H, editor. *PLoS One* [Internet]. 2015 Nov 12;10(11):e0142587. <https://doi.org/10.1371/journal.pone.0142587> PMID:26562778 PMCID:PMC4642932
25. Lusakibanza M, Mesia G, Tona G, Karemere S, Lukuka A, Tits M, et al. In vitro and in vivo antimalarial and cytotoxic activity of five plants used in congolese traditional medicine. *J Ethnopharmacol* [Internet]. 2010 Jun;129(3):398-402. <https://doi.org/10.1016/j.jep.2010.04.007> PMID:20430094
26. Engelbrecht D, Coetzer TL. Sunlight inhibits growth and induces markers of programmed cell death in *Plasmodium falciparum* in vitro. *Malar J* [Internet]. 2015 Dec 29;14(1):378. <https://doi.org/10.1186/s12936-015-0867-0> PMID:26419629 PMCID:PMC4588498
27. Khandros E, Huang P, Peslak SA, Sharma M, Abdulmalik O, Giardine BM, et al. Rapid emergence of clonal interference during malaria parasite cultivation. *Blood* [Internet]. 2011;10(1):271. <http://www.malariajournal.com/content/10/1/271>
28. Dohutia C, Mohapatra PK, Bhattacharyya DR, Gogoi K, Bora K, Goswami BK. In vitro adaptability of *Plasmodium falciparum* to different fresh serum alternatives. *J Parasit Dis* [Internet]. 2017 Jun 30;41(2):371-4. <https://doi.org/10.1007/s12639-016-0808-z> PMID:28615843 PMCID:PMC5447585
29. Sodeinde O, Williams CK. Continuous in-vitro cultivation of *Plasmodium falciparum* in Ibadan: solutions to scientific and logistical problems. *Afr J Med Med Sci* [Internet]. 1990 Jun;19(2):71-6. <http://www.ncbi.nlm.nih.gov/pubmed/2115731>
30. Djimde AA, Kirkman L, Kassambara L, Diallo M, Plowe C V, Welles TE, et al. [In vitro cultivation of field isolates of *Plasmodium falciparum* in Mali]. *Bull Soc Pathol Exot* [Internet]. 2007 Feb;100(1):3-5. <https://doi.org/10.3185/pathexo2883> PMID:17402683
31. Held J, Zanger P, Issifou S, Kreamsner PG, Mordmüller B. In vitro activity of tigeicycline in *Plasmodium falciparum* culture-adapted strains and clinical isolates from Gabon. *Int J Antimicrob Agents*. 2010;35(6). <https://doi.org/10.1016/j.ijantimicag.2010.02.003> PMID:20227854
32. Deans AM, Nery S, Conway DJ, Kai O, Marsh K, Rowe JA. Invasion pathways and malaria severity in Kenyan *Plasmodium falciparum* clinical isolates. *Infect Immun*. 2007;75(6):3014-20. <https://doi.org/10.1128/IAI.00249-07> PMID:17438038 PMCID:PMC1932858
33. Murray L, Stewart LB, Tarr SJ, Ahouidi AD, Diakite M, Amambua-ngwa A, et al. Multiplication rate variation in the human malaria parasite *Plasmodium falciparum*. *Sci Rep* [Internet]. 2017;(July):1-8. <https://doi.org/10.1038/s41598-017-06295-9> PMID:28743888 PMCID:PMC5527095

Supplementary Data:

Table 1. Growth of *Plasmodium falciparum* in cultures from clinical isolates. The actual parasitemia is presented with days in which the culture was diluted with uninfected red blood cells (presented in bold and italics).

Day	PAT-2	PAT-6
1	1.3	3
2	2	2.7
3	5	3.3
4	6	3.3
5	7.7	6
6	4	3
7	5	4.7
8	15	3.3
9	4	5
10	11	6
11	12	3