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Original Article

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

Florence Urio*[1,2], Matilda Mkombachepa[1,3], Gration Rwegasira[1], Twilumba Makene[4], Billy Ngasala[4], Teddy Mselle[2], Julie Makani[1,5] and Lucio Luzzatto[5].

[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.

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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.

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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. A*ll 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

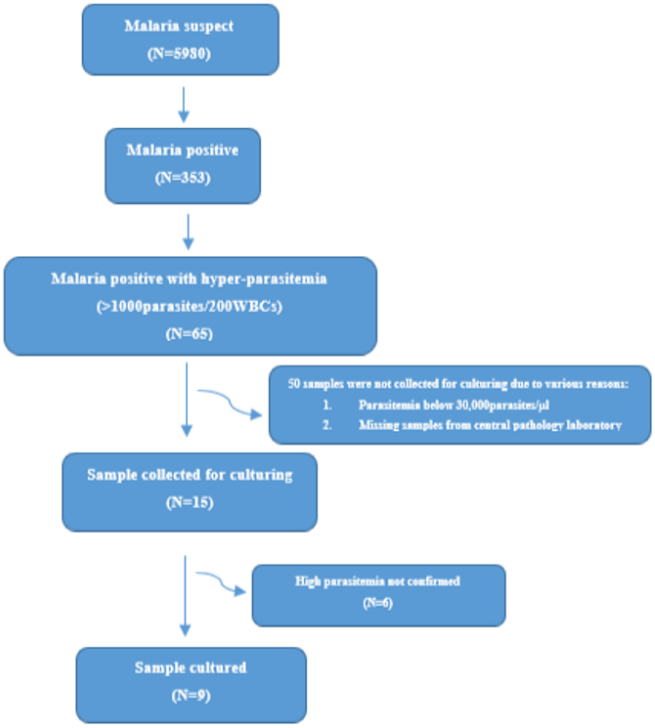

Figure 1. Selection of Clinical malaria samples for *in vitro* cultures.

in 25 cm^2 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

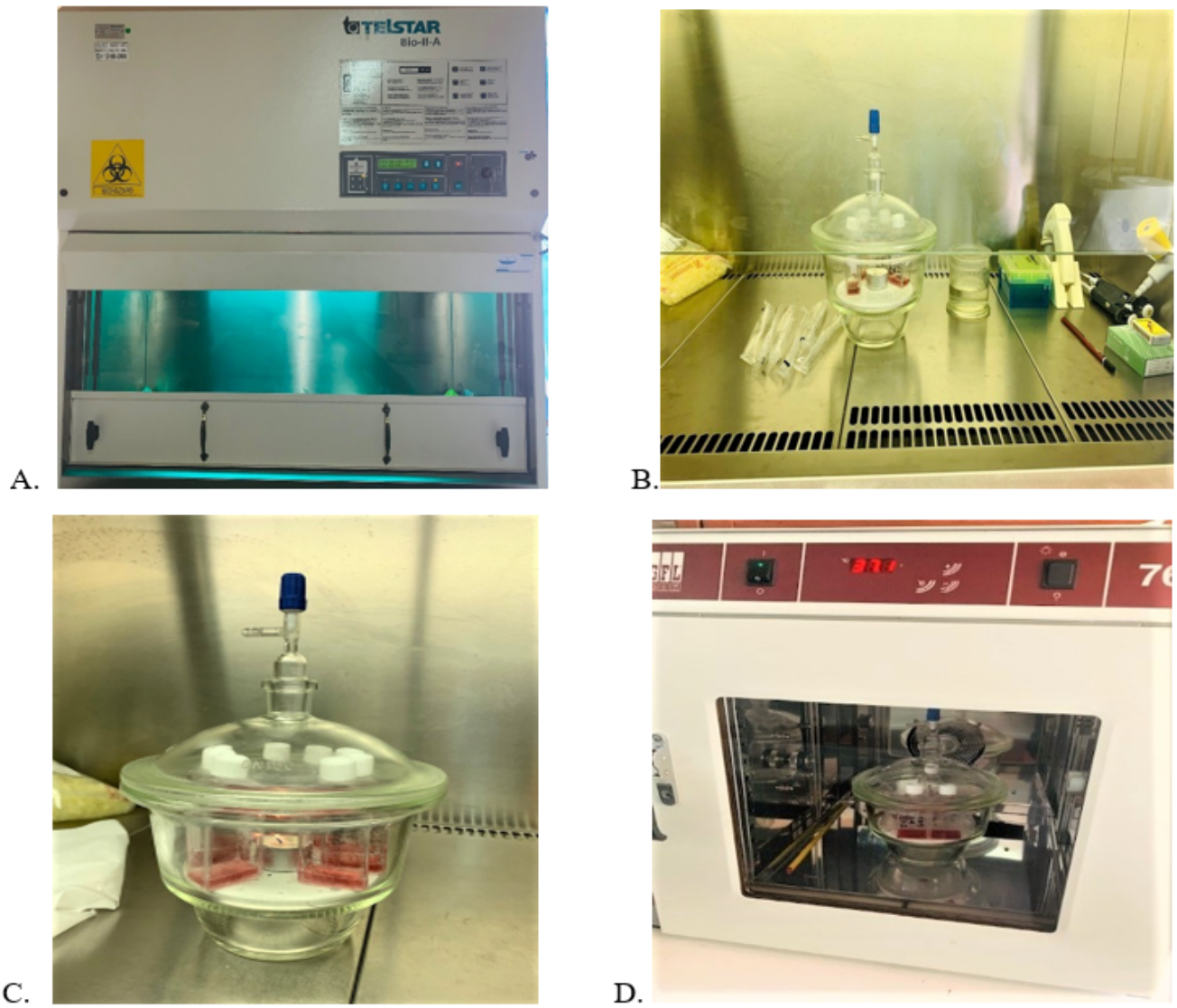

Figure 2. *Laboratory set-up for Plasmodium falciparum culture.* A: Telstar Biosafety Cabinet Class II A with UV light on when not in use. B: Same cabinet when in use. C: Close-up of candle jar with lighted candle. D: Candle jar (flame off) in 37 °C incubator.

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

Strain	*Source*	*Live/frozen*	*Date received*
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
W_2Mef	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 CO_2 incubators with continuous gas flow). By the candle jar method O_2 is 17% and CO_2 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 *in vivo* (parasites/µl)	**Date culture started**	**Initial parasite count %**	**Maximum parasite count %**	**End Date *(In vitro)***	**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 technique[19] 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

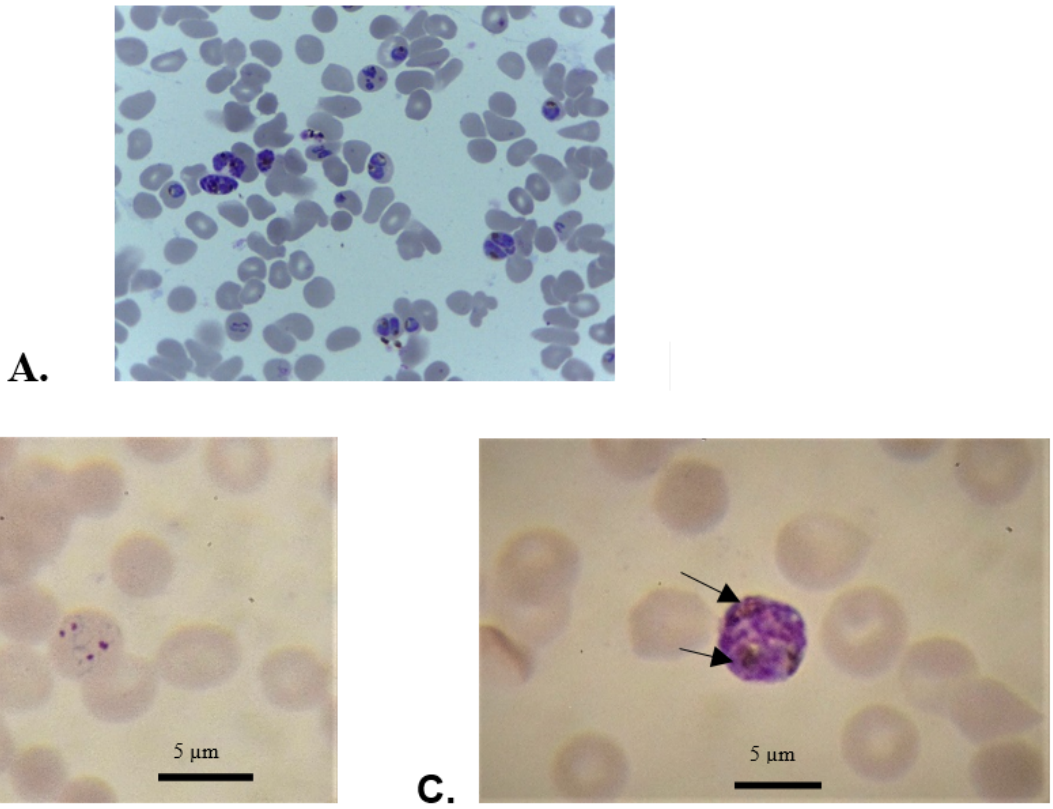

Figure 3. *Microscopic images of Plasmodium falciparum from in vitro cultures* A: Low power view of culture smear: all stages of parasite development are seen. B: High power view: red cell with multiple rings C: Schizont with hemozoin (malarial pigment).

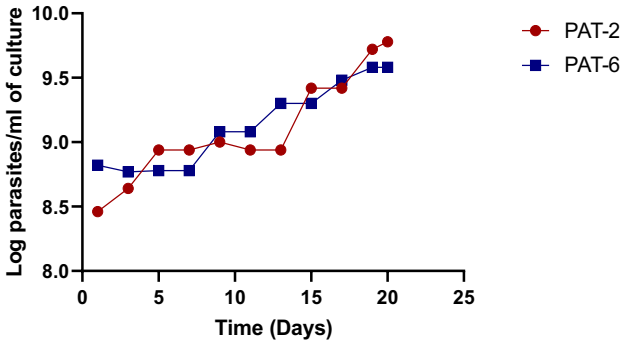

Figure 4. *Exponential growth of Plasmodium falciparum in cultures from clinical isolates.* A mixture of serum/albumax 50:50 was used for culturing. Values on the ordinate axis were calculated from parasite counts on smears, taking into account the dilution of parasitized red cells by non-paraistized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

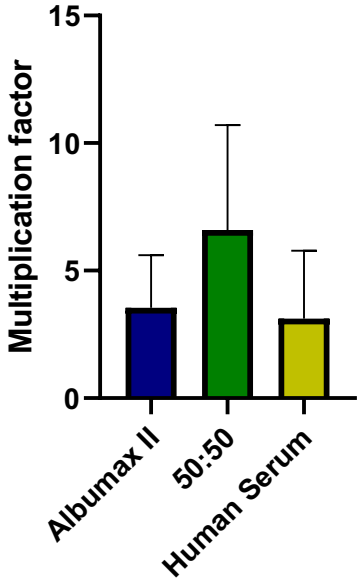

Figure 5. *Supplementation of media with a mixture of human serum and bovine serum optimizes parasite growth.* Parasites were cultured in complete culture medium containing i) Albumax II ii) 50:50 Albumax II and human serum A+ and iii) Human Serum A+. The experiment was performed in two replicates. Paired 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 CO_2 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 -80ºC 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.

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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***