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Achieving *in vitro* gametocytogenesis of *Plasmodium falciparum* in optimal conditions: A review

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Abstract
Background: Globally, *in vitro* culture of all life stages of *Plasmodium falciparum*, existing in the human hosts,is being successfully accomplished under disparate environmental conditions for a variety of studies.
Methods: It is universally accepted that culturing of the parasites in a laboratory is challenging being expensive and resource intensive besides the risk of contamination. Different laboratory conditions give varying results in terms of development of mature gametocytes. We have reviewed globalpublished literature on these conditions and analysed them in-depth.
Results: Of all the factors analysed, the level of initial parasitemia appears to be most critical in obtaining maximum yield of mature gametocytes.
Conclusion: This review explores the key factors involved in lab culture of the gametocyte and its finding can assist in optimising the lab resources for maximal gains. Suggestions have also beenmade on the plausible areas of future research in *Plasmodium falciparum* gametocytogenesis.

Keywords: gametocytogenesis, *in-vitro* culture, *plasmodium falciparum*, parasitemia, gametocytaemia, malaria

1. Introduction

The causative organism of human malaria is a protozoan of genus *Plasmodium*. There are currently five known species of *Plasmodium* which cause malaria in humans. These are *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale and P. knowlesi*. Among these *Plasmodia*, *in-vitro* culture of only *P. falciparum* has been successfully achieved. These include the sporogonic stages in the invertebrate host and schizogonic stages (pre erythrocytic and erythrocytic stages) in the human host [1-5]. The gametocytogenesis inititates in the human hosts. Although imperative for genetic continuity of species, there appears to be reluctance in *Plasmodium* for transitioning of its asexual stage to the sexual as is evident from the *in vitro* studies.

All human *Plasmodia* have a human and an *Anopheles* host to complete their life cycle. The *Plasmodium* enters human host as a sporozoite by a bite of an infected female anopheles mosquito. Sporozoites infect liver parenchymal cells and mature into schizonts (Pre erythrocytic schizogony). Pre erythrocytic schizogony culminates in the production of a large brood of merozoites which are released into the bloodstream. When merozoites infect RBCs, the ring form of trophozoites are formed which develop into schizonts. When an infected RBC ruptures, it releases merozoites from the schizont along with hemozoin. The clinical manifestations of malaria are because of the blood stages of the parasites. The merozoites when released by synchronous burst of innumerable schizonts along with accompanied debris trigger the malaria symptoms [6].

Under certain stressful conditions, the merozoite stage undergoes an alternative mechanism of development leading to differentiation into sexual stages of parasite instead of producing the usual asexual forms. The process of development of sexual stages of parasite is called gametocytogenesis. It leads to the production of male and female gametocytes. When a mosquito takes a *Plasmodium* infected human blood meal, it ingests these intra erythrocytic gametocytes which mate in mosquito midgut initiating the sporogonic cycle.

Gametocytes round up inside the erythrocytes and get activated before emerging out. During activation in the mosquito midgut, each female gametocyte develops into a female gamete (macrogamete) after discarding 3 polar bodies while each male gametocyte forms eight microgametes by a process called exflagellation. The micro and macro gametes are both haploid and they fertilize to produce a zygote within 20 minutes post activation. The zygote is initially diploid but undergoes meiosis without cell division to form a tetraploid zygote. It matures into an elongated ookinete in the midgut of the female mosquito within next 24 hours. The ookinete crosses the peritrophic membrane and converts into an oocyst under the basal lamina of the midgut. Sporozoites are released when a mature oocyst ruptures. These motile sporozoites reach salivary glands of mosquito and are inoculated into a human host via an infected mosquito bite [Fig-1].

Throughout this cycle involving both vertebrate host and invertebrate vector, the *Plasmodium* evolves through several forms. However the ring form of trophozoites is at the crossroads of evolution of the species, as at this stage the choice of asexual or sexual route by the parasite is made. The factors which lead to sexual differentiation are multiple and under scientific scrutiny. Much of the available scientific

evidence is based on *in vitro* cultivation and the factors leading to successful production of gametes.

Here we compare and contrast the available scientific evidence on factors responsible for *in vitro* sexual differentiation from asexual stages and discuss protocols used to successfully induce gametocytogenesis and maintenance of gametocytes of *P. falciparum*.

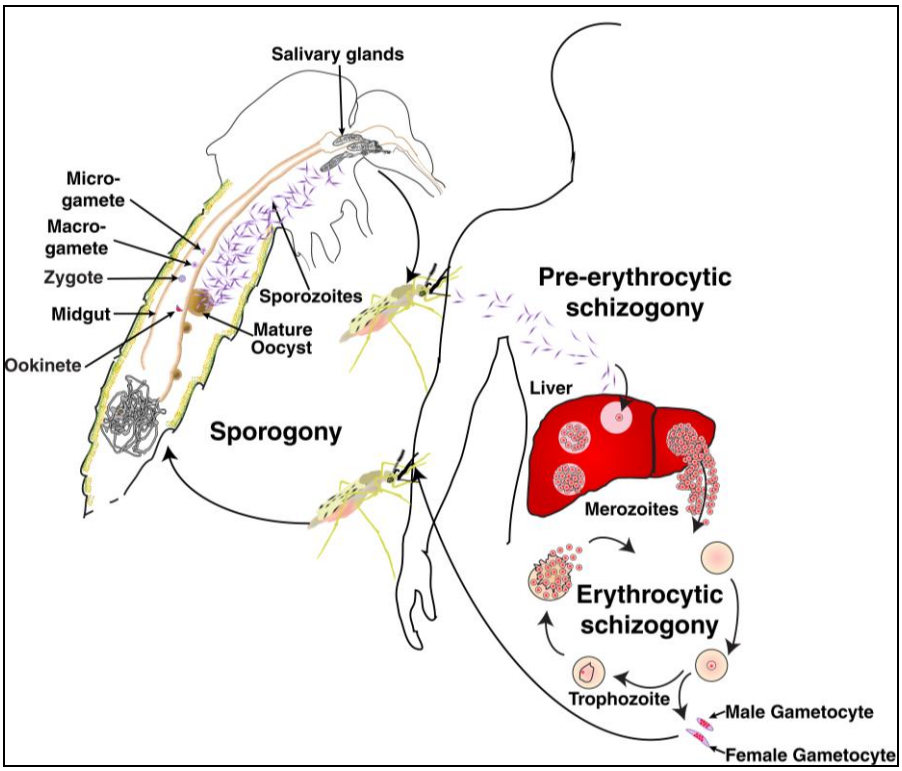

Fig 1: The complex life cycle of *Plasmodium falciparum* involves both asexual and sexual phases. The asexual phase is completed in human host whereas mosquito host is required for consummation of sexual phase and completion of sporogony of *Plasmodium* life cycle.

2. Methods

All scientific methods and protocols used globally over the last 40 years were reviewed with an aim to identify the methods being used consistently for *in-vitro P. falciparum* gametocyte culture. All published database in Pub Med, concerned chapters in textbooks, CDC, WHO websites and protocols being used operationally in various institutional labs were reviewed thoroughly. Besides, practical aspects of parasite culture were studied in depth for several months at the malaria parasite bank of National Institute of Malaria Research (Indian Council of Medical Research) New Delhi. Innumerable formal and informal discussions with experts in the field were held for the study purpose.

It is a scientific enquiry into appropriateness of current methods and recommendation towards adopting more accurate techniques and best practices leading to saving of time and resource and achieving far higher outputs in a sustainable manner with an assumption that the probability of obtaining viable gametocytes in optimum numbers in any culture cycle is increased many folds if a combination of certain *in vitro* factors is preferred over certain other time consuming and cumbersome protocols.

3. Results

3.1 *In vitro* factors affecting gametocytogenesis

The epidemiology of gametocytogenesis appears to be multifactorial and complex. The factors which tilt balance of development in favour of sexual differentiation are host, parasite and environment related. The host and parasite factors are largely not modifiable as opposed to environmental factors that can be manipulated to attain most favourable *ex vivo* conditions for optimal results.

3.2 Level of parasitemia

Trager and Jensen in 1976, for the first time showed that *P. falciparum* gametocytes could be produced and maintained *in-vitro* for long periods of time. They heralded a new era in lab based scientific research on human malaria [11]. Carter and Miller in 1979, described the impact of changes in the parasite culture environment on gametocytogenesis. They attributed the decline in conversion rate of ring form to gametocytes to the low parasitemia due to dilution of fresh cells at the time of establishment of new culture. Undiluted cultures gave higher gametocyte yield. However, gametocyte production was restored 7 days post dilution. Also, it was claimed that ageing of RBCs at 37 degree Celsius played no role in inducing or maintaining gametocytogenesis [12]. The commitment towards gametocytogenesis is increased on reduction of erythrocyte concentration for part culture duration and also when mature gametocytes are seeded for new culture [7, 8]. As per Trager and Gill, the presence of immature erythrocytes (reticulocytes) leads to improved gametocytogenesis as compared to mature

erythrocytes [16].

The levels of parasitaemia both initial and subsequent, has proved to be a critical factor contributing to not only successful initiation of gametocyotogenesis but also to the maintenance of high levels of gametocytemia over next 2 to 3 weeks of culture. High parasitemia *in vitro* acts by simulating the natural host physiological conditions of raised levels of stress leading to preference of sexual differentiation over asexual forms. Several researches have conclusively proved that high parasite load coupled with decreased haematocrit concentration exacerbate gametocytogenesis [7-10]. The mature stage V gametocytes is reportedly being produced most successfully in response to a drop in haematocrit to less than 0.5% before complete maturation of the gametocyte and to withholding of any addition of fresh cells for at least 2--3 weeks during maintenance of culture [12, 13].

Carter and Miller chose the starting parasitemia in daughter culture to be 0.1%. More recently Bounkeua, Li *et al.* recommend the dilution of asexual culture at 8-10% parasitemia to get highest yields [12, 17]. As per Roncales *et al*., the best initial parasitemia is 0.5% for 3D7 and Dd2 strains and 0.75% for NF54 strain at 2% haematocrit level giving a 1.2% gametocytemia [14]. Ghosh *et al*. have taken 0.5% initial parasitemia for NF54 strain at 5% haematocrit level [15]. Ifediba *et al.* initiated all cultures at 0.2% parasitemia in Honduras and FCN2 strains and 12% haematocrit which was reduced to 6% on day 7. They reported obtaining gametocytemia ranging from 2.3 to 2.9 ± 0.6% depending on the strain used [7]. Schuster claims that lower the initial parasitemia, greater will be the increase in number of total parasites during the course of growth in the culture [5].

3.3 Synchrony in culture medium

Synchrony is described as the presence of same stage of parasite population in its life cycle at a given point of time in an infected host blood sample. *In vivo P. falciparum* exhibit a 48 hours synchrony in response to human circadian rhythm [5]. There appears to be an almost imperative requirement of artificially imposing synchrony *in-vitro* culture of parasite in order to successfully induce and harvest large number of gametocytes of a particular stage in response to stress while obliterating the unwanted stages. Various authors have used different methods to achieve synchrony. Sorbitol has been used extensively for this purpose. 5% sorbitol selectively lyses erythrocytes infected with trophozoite and schizont stages while the ring stage remains unaffected [18, 19]. It is a function of permeability of cell membrane of infected RBCs [20].

Roncales *et al.* used 5% sorbitol in two serial treatments (48 hours each) to ensure highest ring stages in culture thus raising the percentage conversion rates of rings to gametocytes. Sorbitol treatment was repeated on day 9, 10 and 11 and remaining asexual forms were also removed. They report a gametocytemia of 1- 1.2 % on day 14 and a conversion rate of 11-23% [14]. Chavalitshewinkoon-Petmitr *et al.* also report successful use of sorbitol to achieve synchrony [21].

As per Jensen *et al*., gelatin when mixed with RPMI 1640 causes sedimentation of infected erythrocytes containing ring stages and uninfected erythrocytes whereas cells with late stage remain in suspension. This method can raise infected cells enriched with trophozoite and schizonts to a parasitemia of 75%. These cells, if allowed to grow and invade fresh RBCs and then treated with 5% sorbitol to remove late stages will give a tightly synchronised culture within a matter of few hours [22].

Similar results can be achieved by sedimentation in plasmagel. Red cells infected with late stage do not form rouleaux in plasmagel and thus can be separated in suspension and used to reinvade fresh cells and produce synchronous culture [23]. N-acetyl glucosamine (GlcNAc) is a selectively toxic medium for trophozoites and schizonts while ineffective towards gametocytes in culture [19].

Fivelman *et al.* kept asexual culture at 1 and 5% parasitemia at 3% haematocrit with sub-culturing done at trophozoite or schizont stage to maintain synchronicity of culture [19]. They controlled the point of stress induction carefully with constant monitoring of stages and used 5% D-sorbitol in water to select ring stage parasite. Synchronised gametocytes were induced by a sudden increase in haematocrit of a ring stage culture while keeping partially spent medium as a stimulus to growth. The point of induction was decided when ring stage culture contained 8-10% parasitemia at 3% haematocrit. Levels beyond 12% may hamper asexual development. They also mention that the quantitative change of spent medium should depend on ring stage parasitemia. This group also claims that culture with 8-10% rings produced a combination of highest achievable gametocytemia and lowest cell death rate [19].

3.4 *Plasmodium* Strain variation

Different isolates of *Plasmodium* have different conversion rates just as different clones of same isolate also show variable results [24, 25]. Different strains under similar *in-vitro* conditions have also shown variable rates of gametocytemia. Roncales *et al*. achieved highest gametocytemia with NF54 strain as compared to 3D7, Dd2, FCR3, HB3 and W2 strains that they studied under similar lab conditions [14]. Bennett *et al*. also concluded that gametocyte production is clearly strain dependent [26]. It has been observed by several authors that recently isolated strains give better output as compared to those which have undergone repeated culture and sub-culture over a long time and reared in lab hosts [5, 27-29]. Conversely, *Plasmodium* 3D7 strain has been widely used in lab experiments and has shown a stable rate of gametocyte production almost indefinitely.

Ono *et al*. however report re induction of gamete production in lines of *P. falciparum* after a gap of several years in continuous culture. The reduced sensitivity to growth environment is overcome by artificial induction [24, 30, 31]. Cryopreservation of isolates can however prevent this loss completely. Thus instead of maintaining the strain by sub-culturing, cryopreservation may be resorted to as a better alternative. Ponnudurai *et al*. concluded that cryopreserved NF54 strain retained its capacity to produce gametocytes completely. Similar results were observed in case of *P. berghei* culture as well [29, 32, 33].

Genetic variation due to chromosomal deletion in certain lines cultured in lab leads to irreversible damage and loss in sexual potential [32]. Population dynamics studies reveal fluctuating response of serotypes in time and space which is modulated largely by immune status and response of host mechanisms [33].

3.5 Role of Hypoxanthine and Xanthurenic acid

Ifediba and Vanderberg proved that hypoxanthine (50µg/ml) when added in the culture medium permitted production of mature infectious *P. falciparum* gametocytes on a regular basis. Whereas, in the control cultures the gametocytes though appeared morphologically normal but failed to infect mosquitoes. The gametocyte enhancing role is derived from hypoxanthine, the main pregenous purine utilised by the parasite. The RPMI 1640 culture medium alone is incapable of fulfilling the nutritional requirements of *Plasmodium* gametocytes for extended periods of 2-3 weeks and thus requires additional growth factors for optimum gametocyte development [7].

Gregory *et al.* claim that Xanthurenic acid, which is a product of tryptophan catabolism, activated gametocytogenesis of *P. falciparum* and *P. gallinaceum* at a concentration ≤0.5µM in saline at a pH of 7.4 and proposed this be known as Gamete Activating Factor (GAF). Xanthurenic acid is found in high quantities in mosquito's head and gut and is utilised in the metabolism of eye pigment [34].

The stimulation of membrane associated guanyl cyclase activity via parasite cyclic guanyl mono phosphate (c- GMP) signalling pathway by xanthurenic acid results in exflagellation in the human malaria parasite [35]. Ghosh *et al.* demonstrated increased level of exflagellation in *P. falciparum* cultures which either used 0.2µM xanthurenic acid as a supplement or due to the presence of xanthurenic acid in the medium containing pupal extracts [15, 36].

Bhattacharya *et al* based on their work on 3D7, 7G8 and W2 strains of *P. falciparum* claim that xanthurenic acid is not only an inductor of exflagellation but also a promoter of infectivity at an optimum concentration of 100µM in the membrane feeding assay. Few researchers have also implicated the role of ammonium carbonate, bicarbonate and c-AMP in raising levels of gamete formation in varying degrees. However substantial evidence is still lacking in this regard [30, 37, 38].

3.6 Role of Lysed Uninfected Erythrocytes (LUE)

There has been renewed interest lately in the use of lysed uninfected erythrocytes in the culture medium to increase gametocytemia. An increase in the rate of gametocyte production on addition of infected as well as uninfected erythrocytes lysate is reported by several authors [24, 39, 40]. Roncales *et al.* have given an optimised protocol including two key factors for enhanced gametocytemia. One of them includes addition of LUE and the other complete elimination of asexual stages [14]. While measuring infectivity of *in-vitro* generated ookinetes, Ghosh *et al.* have recommended the use of human RBC lysate in a medium which gave highest yield of oocysts *in-vitro*. The other variables in the experiment were temperature of less than 24º C and use of human serum and pupal extract [15]. It appears that the factors in RBC lysate enhanced functional capabilities of gametocytes to produce ookinetes. In normal physiological condition also erythrocyte sequestration and lyses due to high asexual load is followed by gametocyte generation and beginning of sexual cycle inside the host.

3.7 Role of PCO_2, pH and Temperature in Exflagellation

pH, CO_2 and temperature need to be stabilised at optimum levels to gain maximum yield of gametocytes as they are very sensitive to any changes in their environment and both subnormal as well as increased levels of these factors individually or collectively are capable of either non initiation of gametocyte generation or poor maintenance of their culture. Human blood has a pH of 7.4 which may be a major factor in keeping the gametocytes inactive in vivo. Several suspended animation solutions are being used to keep *Plasmodium* gametocytes inactive in external environment. These contain a bicarbonate free atmosphere at pH 7.4.

Ogwang *et al.* noted that an increase in pH to 7.9 or addition of sodium bicarbonate induced exflagellation in the culture of NF54 and K67 strains of *P. falciparum*. Both factors in conjunction gave lesser exflagellation rate as compared to when used alone. However similar conditions at a temperature either above 30º C or room temperature failed to induce gametocyte activation. Thus they concluded that temperature alone is sufficient to keep gametes in an inactive state. Temperature constraints may vary for other *Plasmodium* species like *P. berghei* and *P. gallinaceum* [41-45]. Carter and Beach washed *P. falciparum* gametocytes in human serum at pH 8 and induced exflagellation successfully. Thus pH is an additional signal inducing gametocyte activation [46, 47].

Ghosh *et al.* emphasized that exflagellation normally occurred successfully in culture by lowering temperature from 37º C to 22º C even in the absence of any other gametocyte inducing factors. However the levels were lower as compared to those cultures where additional inducing agents like xanthuric acid were used [15].

Generally gametocytes prefer a low oxygen atmosphere for growth coupled with higher carbon dioxide levels and predominantly rich in nitrogen. An atmosphere ranging from 7% O_2, 3% CO_2 and 90% N_2 created by Carter and Miller to that of 3% O_2, 2% CO_2 and 95% N_2 by Ifediba *et al*. has been used in the past [7, 12]. More recently, several authors have reported using a combination of 5% O_2, 5% CO_2 and 90% N_2 [15, 17]. It is not clear whether these changes enhanced the yield of gametocytes.

3.8 Sex ratio of gametocytes

There is normally a female bias to sex ratio of *Plasmodium* gametocytes in vertebrate host. This is essential to balance out greater number of male gametes as compared to female gametes per gametocyte (8:1 ratio) [49, 50]. The rate of transmission of infection is highly affected by sex ratio and is also dependent on gametocyte density. At low density the male sex ratio plays a decisive role and controls the overall transmission efficiency [50]. In contrast, with an increase in gametocyte density sex ratio in favour of female prominently determine overall fertility. Mitri *et al.* demonstrated this by carrying out 148 mosquito infections over 2 yrs on 95 independent cultures of *P. falciparum* NF-54 isolate [51]. They decided the cut off for high versus low infectivity at 24000 gametocytes/µl. They also reported that any further increase in gametocyte density did not significantly increase the infection rate [51].

Sex ratio is a reasonable predictor of proportion of infected mosquitoes and achievable oocyst load. Infectivity is found to

be directly proportional to increase in male ratio to 50%. Robert *et al.* experimentally fed insectary reared *An. gambiae* with human volunteer's blood infected with *P. falciparum* carrying gametocytes and found that the mean proportion of male gametocytes was 0.217 (3.6 females per male) [52]. In the experiment conducted by Ghosh *et al.*, in the beginning of gametocyte genesis, the F:M sex ratio was 3 which declined to 2 within two days and to 1.9 by day 18 [15].

In addition to gametocyte density, the importance of fertility insurance cannot be ruled out in case of low gametocyte viability and poor efficacy during infection. Fertility insurance has been defined as the surety that each female gamete will surely be fertilized by a male gamete. It gives evidence in support of sex selection by parasite in response to environmental conditions to ensure fertilisation [53-56].

The sex ratio of gametocytes which is in favour of female sex found normally in the field condition is lower than that which gives rise to greatest level of infectivity experimentally. This appears to be a via media for maintaining ecological balance. In *P. falciparum* commitment to sexual differentiation is known to occur at schizont stage [48].

4. Discussion and Conclusion

In vitro gametocytogenesis of *P. falciparum* is vital in various research studies viz., to understand initiation of sporogonic processes in vectors, *Plasmodium* infection biology, vector immunity, sporozoite production and harvesting, and to investigate molecular targets of transmission blocking antimalarials and vaccine on gametocytes. Hence an insight in to optimal conditions that ensure continuous and robust production of healthy gametocytes is highly beneficial. Hence in this review effort has been made to review literature and pinpoint optimal conditions that govern gametocytogenesis. This exercise we believe can cut costs of gametocyte production by making necessary adjustments in factors affecting gametocyte production based on reviewed literature.

A gist of protocols used by various researchers resulting in successful *Plasmodium falciparum* gametocyte initiation and maintenance in lab over last 35 years is presented as ready reckoner (Table-1). This analysis is intended to provide researchers with a clearer picture regarding the major factors influencing the generation of healthy gametocytes in a sustainable manner. The most consistently significant aspect appears to be the level of parasitemia used to kick-start the gametocyte culture.

Akompong *et al.* demonstrated gametocytocidal activity of riboflavin against growth of *P. falciparum*, 3D7 strain cultured under standard lab conditions with low initial parasitemia of 0.2% without the use of any other modifying factor yielded mature gametocytemia as high as 9.6% on day 17 of culture [59]. It would also be worthwhile to take in to consideration protocols wherein the generation time of mature gametocytes has been reduced significantly to almost 96 hrs (26).

P. falciparum NF 54 has been universally acknowledged as an efficient producer strain of high levels of infective gametocytes and most investigated. Variations in haematocrit ranging from 2-12% have not been shown to change the final percentage of mature gametocytes to any large extent. Almost all protocols surfed have utilised fresh RBCs to begin with and the maintenance period ranged from 15-18 days. Another interesting factor appears to be the use of hypotonically shocked RBCs with 40% depleted Hb to initiate the culture. It gave higher gametocytemia as compared to another protocol which used both sorbitol synchronisation and LUE in the same experiment (14). Xanthurenic acid is an inductor of exflagellation at an optimum concentration and is a worthwhile subject for further investigation regarding its role as a promoter of infectivity. Out of all the environmental factors considered in this review, optimum temperature and pH appear to be most decisive in induction of exflagellation. Sex ratio, as a predictor of infectivity, appears to be limited in its role till 50% percent of male ratio is achieved. It can be proposed as one of the surrogate marker of infectivity of the culture in general. Initial haematocrit level, synchronisation and other additional factors together support the process synergistically. However individual contribution of all these factors towards achieving a high mature gametocytemia is a subject of further study.

5. Competing Interests

The authors declare that they have no competing interests.

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7. Authors' Contributions

AK and RD conceived, planned the study. RD reviewed the literature and wrote the manuscript. AKM and RD compiled the data. AK and CRP provided critical inputs and revised the manuscript. All authors read and approved the final manuscript.

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Table 1: A comparative analysis of various protocols used for parasite culture in different studies.

Protocol	Initial Parasitemia	Initial Haematocrit	Initial RBC taken	Day of dilution of culture	Maintenance period	Synchronisation	Strain used	Gametocytemia	Reference
Richard Carter & Louis H. Miller (1979)	0.10%	2%	fresh	8th day	18 days with daily media changes	None	Field Isolates from Africa	max of 16% on day 12-13 falling to 10%on day 18	[12]
Ifediba and Vanderberg (1981)	0.20%	12%	fresh	7th day (12 to 6%)	12-14 days with daily changes	None	Honduras & FCN-2	1.4 - 2.9%	[7]
Ponnudurai et.al. (1982)	0.10%	8-10%	fresh, washed	7th day	15-20 days	None	Lab isolates	3%	[32]
Schneweis (1991)	0.5-1%	10%	fresh	3rd day	Daily changes	LUE(Lysed Uninfected Erythrocytes) @ 20% added on day 3 of culture	NF 54	2.0% on day 16	[42]
Chutmongkonkul (1992)	0.75%	2%	fresh		The medium was changed on day 4 and daily from days 6 to 15.	On day 5, 40 µL of pyrimethamine (24.87 ng/mL) was added to eliminate asexual stages	NF 54		[60]
Akompong (2000)	0.20%	6%	fresh	3rd day (3-6%)	18 days with daily media changes	None	3D7	9.6-9.2% on day 15-17 of stage IV and V	[59]
Chavalitshewinkoon-Petmitr (2001)	1%	2%	Treated with PIGPA solution (50 mm sodium pyruvate, 50 mm inosine, 100 mm glucose, 500 mm disodium hydrogen phosphate and 5 mm adenine in 0.9% (w/v) NaCl, pH 7.2 and 50 mg/l of hypoxanthine)	4th day	15 days with change on 4,6 and daily from 8-15days	two serial sorbitol treatments initially. On days 9, 10, and 11, 2.5 pellet volumes of 5% (w/v) sorbitol solution were added for 5 minutes once a day	Multi Drug Resistant KT1 &KT3 isolates (Thailand) in culture for 5 yrs.	3-4% on day 11-15 of stage III& IV	[21]
Bennett (2005)	1.0%	2%	hypotonically shocked RBCs with 40% depleted Hb	results observed at 96 hrs	4 days with daily changes	Alanine treatment	chloroquin sensitive GC-03 (Progeny of a genetic cross between HB3 & Dd2)	2.3 -2.9 @ 96 hr	[26]
C. Mitri, I. Thiery *et al*. (2009)	0.50%	7%	fresh	Not documented	14 days	none	NF 54	2.3% on day 14	[53]
Anil K Ghosh& Marcelo Jacobs-Lorena (2010)	0.50%	5%	fresh	Not documented	18 days with daily media changes	none	NF 54	2-3% on day 18	[15]
Roncales M. (2012)	0.75%	2%	fresh	Not documented	medium changed on day 4,6 and 8-15	5% sorbitol twice @48 hrs interval and on 9,10,11 days. Also addition of LUE on day 4 at 20% conc.	NF-54	1.18% on day 15 of stage IV &V	[14]

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