## Original article

# Establishment of two lines of Ethiopian isolates of *Plasmodium falciparum in vitro*

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**Abstract**: An attempt was made to establish Ethiopian isolates of *Plasmodium falciparum* in tissue culture flasks. Two lines, FCA-1 and FCA-2, were derived from two patients infected with falciparum malaria in North and South Shewa, Ethiopia, respectively. Parasites were initiated into culture in tissue culture flasks. Both lines grew very slowly for the first four weeks but increased their multiplication rates and became established between the 5th and 7th weeks in culture. Both lines produced gametocytes. Infected red blood cells cryopreserved after five weeks were easily recovered. Sufficient parasite materials have been preserved in liquid nitrogen for later use and/or for supply to researchers in other laboratories. [*Ethiop. J. Health Dev.* 1997;11(3):235-239]

**Introduction**

The *in vitro* culture system for continuous cultivation of human malaria parasite, *Plasmodium falciparum*, was standardized in 1976 (1, 2). This has contributed much to the investigation of new approaches to malaria chemotherapy, immunology, biochemistry, molecular biology, and vaccine development, in the past twenty years. The first continuous cultures of *P.falciparum* were established from the blood of an *Autus trivirgatus* monkey infected with *P.falciparum* by Trager and Jensen (1). Later this *in vitro* method was adopted for the establishment of new lines of *P.falciparum* directly from human infections (3).

Several workers have shown that the availability of *P.falciparum* from continuous cultures is extremely useful in the screening of new antimalarial drugs (4); in investigations of the mechanisms involved in development of drug resistance (5); and in studies of drug susceptibility and resistance of *P.falciparum* in endemic areas (6). *In vitro* establishment of isolates of *P.falciparum* from different endemic areas of Ethiopia is important in this regard. In this study we report establishment of

**Methods**

*Medium*: The medium was prepared according to the protocol developed by Trager and Jensen (1). That is, a stock solution was prepared by the addition of 10.4 g of RPMI-1640, 5.94 g of HEPES powder and 0.9 ml of gentamicin (10 mg/ml) to 960 ml of distilled water. The solutions were allowed to dissolve for at least 4 h on a magnetic stirrer and filtered using 0.22 micron millipore filter. This can be used for washing cells. The medium was supplemented by the addition of sterile 5% Sodium bicarbonate at the concentration of 4.2 ml to 96 ml of the RPMI-HEPES solution and 15% heat inactivated human type AB+ serum.

*Blood cells and serum*: Blood cells (O+) and serum (AB+) were obtained through the kind cooperation of the Ethiopian Red Cross Society.

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two lines of Ethiopian isolates of *P.falciparum* from North and South Shewa zones.

*Blood cells*: The blood cells were transferred aseptically into 50 ml centrifuge tubes and stored

at 4 °C. To prepare cells for culture, 20 ml of stored blood cells was washed twice by centrifugation (2000 rmp, 10 min) in 2-3 volumes of stock RPMI solutions. The supernatant and buffy coat were removed. This wash was repeated once in complete medium. The cells were finally resuspended in an equal volume (50% cell suspension) of complete RPMI-1640 medium and stored at 4°C.

*Serum*: The serum was obtained from volunteers with no malaria history and who had not taken antimalarial drugs for the last three months at the time of collection. The serum used in the culture was heat inactivated (56°C, 40 min) and centrifuged (3500 rpm, 20 min) to remove the clotting factor. It was then filter-sterilized (0.45 micron) and stored at -20°C. The serum was thawed and kept at
4°C before use.

*Parasites*: The blood samples infected with *P.falciparum* were collected at the Tekelehaimanot Clinic, Addis Ababa, in April, 1994. After confirmation that patients were infected with *P.falciparum* mono-infection from microscopic examination of stained thick blood films, parasitemias of the patient's blood were determined by counting the infected RBCs on stained thin blood films. Blood samples were obtained just before the patients were treated with antimalarial drugs. About 4 ml venous blood was withdrawn from each patient in a sterile heparinized venoject. The samples were taken to the laboratory (AHRI) in 1-2 h time and immediately washed as described for the blood cells above. Sufficiently washed type O+ RBC were added to yield a parasitemia of 0.2-0.5% in preparation for addition to culture.

*Culture maintenance*: *P.falciparum* isolates were cultured continuously according to Trager and Jensen (1), with modification using tissue culture flasks as described by Desjardins et al. (4) at the laboratory of AHRI. Parasites were grown in 50 ml tissue culture flasks, each containing 10 ml of complete medium and about 0.5 ml of diluted parasitized blood to give a final blood cell suspension of approximately 5% with an initial parasitemia of 0.2-0.5%. The flasks were set in a $CO_2$ (5%) incubator at 37° C. Complete RPMI-1640 medium was usually changed every 24 h and every 12 h when the parasitemia was greater than 5%. Thin blood films were made every 48 h and stained with Giemsa. The cultures were diluted by adding washed human red cells (O+) every four days and when the parasitemia was greater than 5% to reduce the parasitemia back to 0.2-0.5%.

**Results**

As suggested by Jensen and Trager (3), the system of designation for culture lines established in our laboratory was: FC to indicate *P.falciparum* cultures followed by a letter to indicate the institution where the line was initiated in culture, followed by a number to indicate the chronological order in which lines were placed in culture. The lines we established were initiated at Armauer Hansen Research Institute (AHRI) on the same day on April 7, 1994. So they are designated as FCA1 and FCA-2. FCA-1 was collected from a girl (age 25) who contracted the disease from DebreBerhan (lowland) and FCA-2 from a boy (age 21) who contracted the disease from Ziway, Ethiopia.



Table 1: **Early *in vitro* development of Ethiopian isolate of *P.falciparum*, line FCA-1 in tissue culture flasks[a]**

| Days *in vitro* | Parasite stages per 10,000 erythrocytes[b] | | | | | |
|---|---|---|---|---|---|---|
| | R | T | 2N | >2N | Total | Rate of increase |
| 0 | 50 | 0 | 0 | 0 | 50 | |
| 2 | 8 | 15 | 9 | 4 | 36 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4 | 0 | 18 | 6 | 10 | 34 | |
| 6 | 12 | 14 | 4 | 16 | 46 | 1.4 X |
| 6[c] | 4 | 7 | 0 | 4 | 15 | |
| 8 | 8 | 25 | 3 | 8 | 24 | 1.6 X |
| 10 | 9 | 13 | 10 | 12 | 38 | 1.6 X |
| 12 | 24 | 16 | 9 | 17 | 66 | 1.7 X |
| 12[c] | 10 | 8 | 3 | 5 | 26 | |
| 14 | 16 | 10 | 8 | 9 | 43 | 1.7 X |
| 16 | 22 | 28 | 11 | 17 | 78 | 1.8 x |
| 16[c] | 6 | 8 | 3 | 5 | 22 | |
| 18 | 9 | 12 | 5 | 14 | 40 | 1.8 X |
| 20 | 13 | 36 | 12 | 20 | 81 | 2.0 X |
| 20[c] | 3 | 6 | 7 | 3 | 19 | |
| 22 | 8 | 13 | 0 | 15 | 36 | 1.9 X |
| 24 | 10 | 23 | 14 | 19 | 66 | 1.8 X |
| 24[c] | 0 | 7 | 4 | 6 | 17 | |
| 26 | 9 | 15 | 0 | 12 | 36 | 2.1 X |
| 28 | 28 | 32 | 3 | 18 | 79 | 2.3 X |

[a] Culture was begun on 7 April 1994 [b] R, rings; T, Trophozoites; 2N, binucleated stage; > 2N, Schizonts with more than two nuclei. Mean of 3 different culture flasks. [c] Count after the addition of fresh erythrocytes, mean of 3 seprate counts.

Tables 1 and 2.- show the development *in vitro* of FCA-1 and FCA-2 lines of *P.falciparum*, respectively, for the first 28 days. Both lines were initiated into culture at the same time with an initial parasitemia of 0.5% for FCA-1 and 0.6% for FCA-2, and were handled in exactly the same way using the same medium, cells and serum.

As illustrated in Tables 1 and 2, the early developmental rate of FCA-1 and FCA-2 is almost the same: the initial parasitemia dropped to 0.34 and 0.30%, respectively, in the first two cycles (96 h), but the parasitemia increased two days later (3rd cycle), at which time parasites were diluted with fresh red cells (O+) reducing the parasitemia to 0.15% (FCA-1) and 0.17% (FCA-2). Although there was an increase in the rate of multiplication, both lines grew at a slow rate for the first 28 days.

Table 2: **Early *in vitro* development of Ethiopian isolate of *P.falciparum*, line FCA-2 in tissue culture flasks**[a]

| Days *in vitro* | Parasite stages per 10,000 erythrocytes[b] | | | | | |
|---|---|---|---|---|---|---|
| | R | T | 2N | >2N | Total | Rate of increase |
| 0 | 62 | 0 | 0 | 0 | 62 | |
| 2 | 6 | 18 | 6 | 8 | 38 | |
| 4 | 6 | 16 | 3 | 5 | 30 | |
| 6 | 15 | 12 | 5 | 9 | 41 | 1.4X |
| 6[c] | 6 | 4 | 2 | 5 | 17 | |
| 8 | 11 | 8 | 0 | 7 | 26 | .5 x |
| 10 | 14 | 10 | 5 | 9 | 38 | 1.5x |
| 12 | 19 | 22 | 8 | 14 | 63 | 1.7x |
| 12[c] | 6 | 10 | 2 | 4 | 22 | |
| 14 | 10 | 14 | 4 | 8 | 36 | 1.6 x |
| 16 | 20 | 16 | 7 | 19 | 62 | 1.7x |
| 16[c] | 4 | 5 | 2 | 8 | 19 | |
| 18 | 11 | 8 | 4 | 10 | 33 | 1.7 x |
| 20 | 16 | 20 | 6 | 16 | 58 | 1.8 x |
| 20[c] | 3 | 6 | 2 | 5 | 16 | |
| 22 | 12 | 7 | 0 | 10 | 29 | 1.8 x |
| 24 | 16 | 11 | 6 | 18 | 51 | 1.8x |
| 24[c] | 3 | 6 | 1 | 4 | 14 | |
| 26 | 8 | 10 | 2 | 6 | 26 | .9 x |
| 28 | 12 | 18 | 0 | 22 | 52 | 2 x |

[a] Culture was begun on 7 April 1994 [b] R, rings; T, Trophozoites; 2N, binucleated stage; > 2N, Schizonts with more than two nuclei. Mean of 3 different culture flasks. [c] Count after the addition of fresh erythrocytes, mean of 3 separate counts

Developmental rate after seven weeks and 4 months is presented in Table 3, indicating an improved rate of multiplication for both FCA-1 and FCA-2 after 7 weeks in culture. The multiplication rate of FCA-1 (26x) was relatively higher than that of FCA-2 (14x). Interestingly FCA-2 (7 fold per cycle) increased its rate of multiplication after 4 months and exceeded the rate of multiplication of FCA-1 (6.8 fold per cycle).

In general, both lines became established by increasing their rate of multiplication significantly from 26x (FCA-1) and 14x (FCA-2) in two cycles (96 h) after 7 weeks to 6.8x and 7x per cycle respectively after 4 months, but growth of the asexual blood stages was not synchronous (Tables, 1,2 & 3). Gametocyte production was evident in both lines starting from one week in *in vitro* culture, the percentage (0.01%) of gametocyte was, however, very low.

Table 3: **Developmental rates of two lines of Ethiopian isolates of *P.falciparum* in tissue culture flasks - line-FCA-1 and FCA-2 after 7 weeks and after 4 months *in vitro***

| Strain | Parasite stages per 10,000 erythrocytes[a] | | | | | |
|---|---|---|---|---|---|---|
| | R | T | 2N | >2N | Total | Rate of increase after 2 Cycles (96 hr) |
| **FCA-1** | | | | | | |
| 7 weeks in vitro | | | | | | |
| Mean[b] of "0" time count | 6 | 12 | 5 | 15 | 38 | |
| Mean[c] of 96 h count | 346 | 70 | 65 | 02 | 983 | 26 X |
| 4 months in Vitro | | | | | | |
| Mean[b] of "0" time count | 8 | 7 | 2 | 4 | 21 | |
| Mean[c] of 96 hr count | 425 | 375 | 36 | 102 | 965 | 46X |

| FCA-2 | | | | | | |
|---|---|---|---|---|---|---|
| 7 weeks in vitro | | | | | | |
| Mean[b] of"0" time count | 14 | 12 | 7 | 20 | 53 | |
| Mean[c] of 96 h count | 285 | 138 | 62 | 276 | 761 | 14X |
| 4 months in Vitro | | | | | | |
| Mean[b] of "0" time count | 6 | 8 | 0 | 4 | 18 | |
| Mean[c] of 96 hr count | 372 | 410 | 22 | 94 | 898 | 50X |

[a] R, rings; T, Trophozoites; 2N, binucleated stage; > 2N, Schizonts with more than two nuclei.

[b][c] Means of 3 different culture flasks.Mean of 3 separate counts.

**Discussion**

There is a considerable number of culture- adapted lines of *P.falciparum* now available for research purposes, including research in malaria chemotherapy (1, 3, 5, 6, 7). The successful establishment of two lines of Ethiopian isolates of *P.falciparum* we are reporting here may also be relevant as an additional source of culture-adapted parasite materials in this regard.

The successes and disappointments in an attempt to cultivate new strains of *P.falciparum* were discussed by Jensen and Trager (3).

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In the present work we have attempted to establish a large number of isolates from different endemic areas of Ethiopia. However, in the process, we lost a lot of our cultures mainly due to contamination. But we have been able to establish two lines (FCA-1 and FCA-2) of Ethiopian isolates of *P.falciparum* from two different zones. The early and late development of our culture lines was not different from that described by Trager and Jensen (3). Gametocytes were freqently and routinely seen in asexual stage cultures of freshly isolated *P.falciparum* (8). However, considerable varations have been found among isolates of *P.falciparum* in their capacity to produce gametocytes during *in vitro* cultures (9). Gametocyte production in such culture system is believed to be induced due to lack of nutrients and accummulation of metabolic waste products. Thus the low level of gametocytes in our culture system could be due to more frequent change of medium and dilution of cultures with fresh RBCs.

Parasite materials frozen after five weeks in culture were found to be recoverable. It was observed that reducing the haematocrit to about 3-4% is necessary when retrieving frozen parasite materials in preparation for culture. It has been suggested that some human sera, especially in areas endemic for malaria, are unsuitable for use in *P.falciparum* cultures (10). Thus, the interview approach used to avoid the presence of immune serum in our culture may be of value in this regard. Enough copies of the parasite materials have now been preserved in liquid nitrogen for later use or supply to workers in other laboratories. We suggest the establishment of new isolates from different endemic areas since studies of their drug response characteristics could play an important role to fully understand the malaria problem in these areas.

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