Chapter 2

In vitro cultivation of *Plasmodium falciparum*

2.1 INTRODUCTION

Malaria represents the world’s greatest public health problem in terms of number of people affected, levels of morbidity and mortality. The protozoan malaria parasites (*Plasmodium* spp.) are transmitted by infected female mosquitoes when feeding on blood. Parasites soon enter liver cells, and after several days of multiplication, are released into the bloodstream where further cycles of asexual reproduction occur, giving rise to the clinical symptoms of malaria. Some erythrocytic parasites will differentiate into presexual forms (gametocytes), which when taken up by mosquitoes in further blood meals, mature into gametes and undergo a sexual cycle. With the eventual release of infective sporozoites into the mosquito salivary glands, the life cycle of the parasite is completed (Phillips. 1983).

Research on the most pathogenic human malaria parasite, *Plasmodium falciparum*, has expanded dramatically in the last thirty five years, not only because of the advent of recombinant DNA technology, but also because of the demonstration in 1976 that the organism could be cultured *in vitro* (Trager and Jensen, 1976). This rendered *P. falciparum* much more accessible as an experimental organism, and since that time, improvements to and simplifications of the original “candle-jar” method have been made (Oslsanya et al., 1981; Zolg et al., 1982; Fairlamb et al., 1985). In some laboratories, continuous culture of *P. falciparum* has been successfully automated to cope with the more repetitive aspects of the procedure, particularly when large amount of parasite material are required (Jensen and Trager, 1979; Ponnudurai et al., 1983). There are many variables involved in culturing, and many factors that contribute to optimal parasite growth. Here we adopted, a relatively simple method of cultivation of asexual stages of malarial parasite developed by Trager and Jensen (1976) which require the minimum components, and not particularly labor intensive.
2.2 PROCEDURE FOR IN VITRO CULTIVATION AND MAINTENANCE OF PLASMODIUM FALCIPARUM CULTURE

2.2.1 Preparation of culture medium for cultivation of Plasmodium falciparum

2.2.1.1 RPMI medium

One packet of RPMI 1640 (containing 25 mM of HEPES buffer, glucose) dissolved in 960 ml of double distilled water. 40 µg/ml of gentamycin sulfate (1.2 ml of Gentamycin/L) was added to it. This solution was passed through a Millipore filter of 0.22 µm porosity and store at 4°C as 96 ml aliquots in glass media bottle.

2.2.1.2 Washing medium (Incomplete medium)

4.2 ml of 5 % sodium bicarbonate (5 gms of sodium bicarbonate was dissolved in 100 ml double distilled water and filtered through a Millipore filter of 0.22 µm porosity and store at 4°C) was added to 96 ml of stock RPMI 1640 media.

Note: The sodium bicarbonate should be added to the RPMI 1640 media only at the time of requirement and should be prepared afresh.

2.2.1.3 Serum preparation

O⁺ blood was collected in centrifuge tube without anticoagulant and kept at 4°C. Next day it was centrifuged at 10000 rpm for 20 min at 4°C. Serum was separated aseptically and kept in aliquots. The serum was inactivated by keeping it at 56°C water bath for half an hour.

Note: After inactivation the serum could be stored in deep freezer at – 20°C / -70°C up to six months.

2.2.1.4 Complete medium

Normal inactivated O⁺ human serum (10 ml) was added to 90 ml of incomplete media.

2.2.2 Preparation of erythrocytes (RBCs) for culture

O⁺ blood was collected in anticoagulant into centrifuge tubes. Then this was centrifuged at 1500 rpm for 10 min at room temperature. Plasma and buffy coat was removed with sterile Pasteur pipette. After this washing media was added, centrifuged at 1500 rpm for 10 min and supernatant was removed. This washing process was repeated thrice. Equal amount of complete media was added to the pellet and stored at 4°C.
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Note: Prepared RBCs can be used up to 14 days for optimum parasite growth.

### 2.2.3 Continuous culture of *Plasmodium falciparum*

#### 2.2.3.1 Initiation of culture

Suspension (50%) of infected cells with complete media (with 15% serum) was prepared. Appropriate amount of uninfected cells were added to get an initial parasitaemia of 0.5 to 1.0% and diluted with complete media to get 5% cell suspension (5% hematocrit). Culture was kept in CO₂ incubator at 37°C.

Note: Hematocrit is important factor for the optimal growth of parasite.

#### 2.2.3.2 Monitoring culture growth

After every 24 hr the media was removed using a sterile Pasteur pipette without disturbing the cells that settled down. Then the cells were mixed without frothing and a drop of blood was placed on the slide and a thin film was made. Fresh complete media (with 10% serum) was added, mixed properly, and kept back in the incubator. Thin film was stained and examined for parasitaemia. It was then compared with initial parasitaemia.

Note: Parasitaemia should not be kept above 2-3% in the culture, it should be subcultured.

**Smear preparation**

Culture (3.0 µl) was placed on a slide toward left side. The cells were smeared across the slide with the help of another slide, to get a thin film and dried the slide at room temperature. Slide was fixed by immersing in methanol for 30 seconds followed by drying. The slides were then stained with Field A stain for 15 seconds and washed with water to remove excess stain. The slide was then dried and consequently stained with Field B stain for 20 seconds and washed with water to remove the excess stain. It was then dried and observed under the microscope.

**Estimation of the percentage of erythrocytes infected (% parasitaemia) with Plasmodium falciparum**

An area of stained thin blood film where the erythrocytes were evenly distributed, was observed using 100 X objective (under oil immersion). Approximately 100 erythrocytes in this area were counted. Without moving the slide, the number of infected erythrocytes amongst the 100 erythrocytes was also counted. The slide was
moved randomly to adjacent fields and counting was continued as mentioned above. An equivalent of 1,000 erythrocytes was counted. The counting was repeated twice for a total examination of three different parts of the slide, i.e., 3 areas 1000 cells. The mean number of infected RBCs per 1,000 RBCs was taken by dividing the infected RBCs by 3.

Calculation

\[ A = \frac{\text{number of infected erythrocytes}}{1,000 \text{ erythrocytes}} \]

Percent infected erythrocytes (% parasitaemia) = \( A/10 \)

Note: If one erythrocyte contains \( \geq 2 \) it is still counted as one infected erythrocyte.

2.2.3.3 Subculturing (Passaging)

Old media was removed and freshly washed RBCs and complete media (with 10 % serum) were added. For example to 20 µl of infected RBCs with around 5 % parasitaemia, 160 µl freshly washed RBCs (i.e. 80 µl RBCs) suspension (concentration of parasitaemia = 1 %) and 2000 µl of complete media was added. Culture was dispensed to more vials.

Note: Culture should not be kept for more than a week in a glass vial, if growth was good, subculture or change the vial.

2.2.4 Synchronization of Plasmodium falciparum

2.2.4.1 Introduction

Malarial parasites in a continuous cycle of development malaria parasites consists of merozoites, ring-stages, trophozoites and schizonts stages. Many species of Plasmodium undergo synchronous development in their natural hosts, but when \( P \) falciparum is grown in in vitro culture this synchrony is quite rapidly lost. Hence, a method for inducing synchrony in cultures of this parasite is a necessary first step. The most important thing for synchronization is to be sure that there should be enough ring-stage parasites. For tighter synchronization, it should be done several times until the ring-stage predominates in the cultures. Sorbitol destroys large parasites (trophozoites and schizonts) in erythrocytes ((Lambros and Vanderberg, 1979).

2.2.4.2 Procedure

Sorbitol (5 %) was prepared in distilled water and passed through a Millipore filter of 0.22 µm porosity. To 1 ml of the culture pellet, 9 ml of sorbitol was added and kept at
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room temperature for 5 minutes. Supernatant was removed by centrifugation and pellet was washed thrice in complete media (Washing procedure same as mentioned for preparation of erythrocytes (RBCs) for culture). Freshly washed erythrocytes were added to the culture to setup new culture as described in section 2.2.3.

**2.2.4.3 Results**

After synchronization, ring-forms of *P. falciparum* persist as rings (Figure 2.1.) in culture for about 18 hr. Twenty-four hours later, the youngest rings became small trophozoites, and the oldest rings became schizonts (Figure 2.2.). Thus, at this stage the culture was still asynchronous to some extent. Approximately 6 h later, the first generation of new merozoites invaded erythrocytes to become young rings. At this point a second sorbitol treatment allowed only these rings to survive and later these rings developed with a high degree of synchrony. Approximately 95% of the parasites were in the ring stage of development at 48 and 96 hr after sorbitol treatment-likewise, a high percentage of trophozoite and schizont stages was observed at 24, 72, and 120 hr. If the culture is not synchronized regularly, culture remains in unsynchronized with mixed stages containing rings, trophozoites and schizonts (Figure 2.3).

**2.2.5 Freezing and thawing of *Plasmodium falciparum***

There are few methods for the cryopreservation of malaria parasite, viz. sorbitol method and glycerolyle method. Parasites frozen according to one method can be thawed using another method’s thawing protocol.

Note: The culture selected for cryopreservation should contain more than 50 % rings stages as higher stages get lysed in due course.

**2.2.5.1 Cryopreservation**

The cryopreservative was prepared by adding 28 ml glycerol to 72 ml of 4.2 % sorbitol in normal saline. This solution was filtered through Millipore filter of 0.22 μm porosity. The infected blood was centrifuged at 1500 rpm for 10 min. The supernatant/plasma was removed. Equal volume of cryoprotectant was added to the cells, distributed in small screw cap vials (cryotubes) and frozen quickly by immersing in liquid nitrogen (-196°C). Vial was labelled with strain, date and % of rings.
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**Figure 2.1:** Ring stage *Plasmodium falciparum*

**Figure 2.2:** Trophozoite stage *Plasmodium falciparum*
2.2.5.2 Revival of cryopreserved parasites

The vial was taken out from the liquid nitrogen and thawed quickly in a 37°C water bath. Culture was then transferred to a centrifuge tube and centrifuged at 1500 rpm for 10 min at room temperature. The supernatant was removed and an equal volume of 3.5 % NaCl was added (drop wise). This suspension was again centrifuged and the supernatant was removed. The pellet was washed twice with complete medium supplemented with 15 % serum. After washing the cryopreserved cells, the culture was initiated by adding fresh washed erythrocytes as follows.

Note: After revival of cryopreserved parasites complete media should be used with 15 % serum while in maintenance of continuous culture complete media with 10 % serum can be used.

References


