Chapter 8

Treatment and control of Mycoplasma contamination in Plasmodium falciparum culture
8.1 Introduction:

Mycoplasma is common contaminants of cell cultures (Razin and Barile, 1985). The first report of mycoplasma contamination of *Plasmodium* culture was published in 1997 by Turrini et al., who had found Mycoplasma contamination in several strains of *Plasmodium falciparum* directly originating from the Malaria Strain Bank. Mycoplasma does not grow in mammalian erythrocytes, but they attach to human erythrocytes (Loomes et al. 1984) by adhering to sialic acid containing proteins (Roberts et al. 1989) and glycolipids (Loomes et al. 1985). Mycoplasma are very small, pass through the 0.1 μm pore-size membrane filters, have no cell wall and are invisible in smears of malaria cultures stained by Giemsa or Acridine Orange. The low Mycoplasma contamination had no inhibitory effect on parasite invasion or maturation and for this reason their frequent presence in parasite cultures may easily escape notice. But in the presence of heavy contamination of mycoplasma, within 2-4 weeks, malaria parasites do not survive. Thus Mycoplasma contamination is a major hindrance for long term *In vitro* cultivation of *P. falciparum* and may be a source of artefactual results in several ways. Metabolic pathways, specific enzymes or products of Mycoplasma origin may be falsely attributed to *P. falciparum* and the AT-richness (61-76%) of the mycoplasma genome is similar to that of *P. falciparum* (Bove, 1993) thus makes this a potential pitfall for any researcher working with mycoplasma-contaminated material.

Mycoplasma is typically resistant to antibiotics such as penicillin and streptomycin and antibiotics such as gentamycin, tylosin, lincomycin & spectinomycin do not eliminate Mycoplasma contamination (Visser et al., 1999). Three Classes of antibiotics i.e. tetracycline, macrolids and quinolones have been shown to be highly effective against Mycoplasmas, both in human/veterinary medicine and in cell culture. So far, no efforts have been made to observe the usefulness of these antibiotic classes for elimination of mycoplasma from *P. falciparum* culture as most of the
antibiotics possess antimalarial activity. The present study was planned to determine a comparative efficacy of Plasmocin (macrolid), Biomyc-1, -2 (Tetracycline) and Biomyc-3, & Mycoplasma Removing Agent (quinolone derivatives) for elimination of mycoplasma from *P. falciparum* culture.

### 8.2 Materials and Method

#### 8.2.1 Parasite

Laboratory maintained Chloroquine sensitive strain 3D7 of *P. falciparum* was used for the study.

#### 8.2.2 Preparation of incomplete medium

IRPMI was prepared as mentioned in chapter 1.

#### 8.2.3 Serum

Commercially available FBS was used during study.

#### 8.2.4 Preparation of complete medium

CRPNI was prepared as mentioned in chapter 1.

#### 8.2.5 Preparation of RBCs

RBCs were prepared as mentioned in chapter 1.

#### 8.2.6 Staining procedure of blood smear

Blood smear was stained with geimsa stain as mentioned in chapter 1.

#### 8.2.7 Antibiotics tested

- **Biomyc 1, 2 and 3**: Commercially available (Biological Industries).
- **Plasmocin**: commercially available (InvivoGen)
- **MRA**: Commercially available (MP Biomedical)

#### 8.2.8 In vitro cultivation:

Culture of 3D7 was maintained in RPNI-F at 37°C in a CO₂ as mentioned in chapter 1.

#### 8.2.9 Experimental setup:

Effect of four antibiotics- Plasmocin (InvivoGen, San Diego, CA), Biomyc-1/-2, and -3 (Biological Industries) and Mycoplasma Removing Agent (MRA) (MP Biomedicals) was observed on the growth profile of *P.*
*Plasmodium falciparum* in mycoplasma contaminated and uncontaminated cultures. The doses and duration of treatment remained same as recommended i.e.

1- Biomyc 1 was used at 10µg/ml concentration for 3 days followed by Biomyc 2 at 5 µg/ml concentration for 4 days

2- Biomyc 3 was used at 0.5µg/ml concentration for 7 days

3- Plasmocin was used at 12.5µg/ml concentration (i.e. 1:2000 dilution of the 25mg/ml stock solution) for 14 days.

4- MRA was used at 0.5µg/ml concentration for 7 days.

Experiment was setup in 24 well plate at 0.5%P and 6%Hct. Each well receives 2ml culture suspension. Different antibiotics at desired concentration were added to respective wells in duplicate. Three such replicates were carried out. Spent culture medium was replaced on every 48hrs from each well. Blood smears were prepared from each well for assessment of parasitaemia. In case of MRA treated and untreated cultures fresh human RBCs were added to dilute running culture on day 4, 7, 10 and 12 containing approximately six percent parasitaemia.

**Assessment of pH**

The pH of spent culture medium was monitored using pH meter.

**Identification of Mycoplasma**

Presence of Mycoplasma was ascertained using Enzyme-PCR based Mycoplasma detection kit (Biological Industries) using following protocol.

**a) Test sample Preparation**

1ml of culture supernatant was transferred into a sterile 2ml eppendorf tube. It was centrifuged at 250 x g briefly to pellet cellular debris. Supernatant was transferred into a fresh sterile eppendorf tubes and centrifuged at 15,000-20,000 x g for 10 minutes to sediment mycoplasma. Supernatant was discarded carefully and the invisible pellet was re suspended with 50µl of buffer solution and mixed thoroughly with a micropipette. Suspension was heated to 95°C for 3 minutes. Test sample was used for PCR.
**b) PCR Amplification:**

Size of DNA fragments were amplified by using specific primers of 270bp provided in the kit. Reaction mixture as mentioned below was prepared in sterile PCR tubes:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>35 µl</td>
</tr>
<tr>
<td>Reaction Mix.</td>
<td>10 µl</td>
</tr>
<tr>
<td>Test Sample</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

Tubes were placed in a DNA thermal cycler programmed as following:

- **94°C**: 30 sec
- **94°C**: 30 sec
- **60°C**: 120 sec
- **72°C**: 60 sec
- **94°C**: 30 sec
- **60°C**: 120 sec
- **72°C**: 5 min.

35 cycles

These amplified products were identified using slab gel electrophoresis.
8.3 Results

Fig. 1 Growth profile of *Plasmodium falciparum* in mycoplasma free culture during and post treatment with anti-mycoplasma agents.

Fig. 2 Growth profile of *Plasmodium falciparum* in mycoplasma contaminated culture during and post treatment with anti-mycoplasma agents.
The growth profile of *P. falciparum* as observed during and post treatment with Plasmocin, Biomyc 3 and MRA in mycoplasma uncontaminated and a contaminated culture is shown in Fig.1 and 2 respectively. It is evident from fig. 1 that % parasitaemia in cultures treated with Plasmocin or Biomyc 3 increased slowly upto day 3 of treatment after which it declined continuously and no parasite was seen after day 6 of treatment. On the other hand culture treated with MRA showed continuous rise in percent parasitaemia similar to the untreated culture. The fall in percent parasitaemia on days 4, 7, 10 and 12 as shown in Fig.1 is due to dilution of culture with fresh human RBCs.

In mycoplasma contaminated culture (Fig.2) the initial (day 0) parasitaemia in 4 different subcultures varied between 2.3 and 4%. It was observed that treatment with Plasmocin or Biomyc 3 did not make any improvement in parasite growth whereas culture treated with MRA showed increase in parasitaemia since 24hrs.of treatment. The percent parasitaemia reached to more than 7% on day 3 post commencement of treatment and kept on increasing thereafter.

The pH of spent culture medium obtained from mycoplasma contaminated and uncontaminated, treated and untreated culture is depicted in Table 1. pH of culture medium was not altered with low contamination whereas with very high mycoplasma contamination minimum pH was observed to be 6.8 which increased to 7.2 within 24 hrs. of treatment. Whereas in contaminated and untreated culture pH remained unchanged during the observation period.

**Table 1**- pH of spent culture medium

<table>
<thead>
<tr>
<th>Days of observation</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contaminated and treated culture</td>
<td>6.80</td>
<td>7.00</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Contaminated and untreated culture</td>
<td>6.80</td>
<td>6.80</td>
<td>6.80</td>
<td>6.80</td>
<td>6.80</td>
<td>6.80</td>
<td>6.80</td>
<td>6.80</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Uncontaminated and untreated culture</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.20</td>
<td>7.2</td>
<td>7.2</td>
</tr>
</tbody>
</table>
**Identification of Mycoplasma**

Presence of mycoplasma was detected using commercial PCR detection kit. Single band of 270 bp was detected in contaminated culture. No band could be seen in culture treated with MRA as well as in Mycoplasma free culture (Fig. 3).

![270 bp band](image)

**Fig. 3:** Detection of Mycoplasma in treated and untreated culture

Lane 1: Mycoplasma free culture  
Lane 2: Culture treated with MRA  
Lane 3: Mycoplasma contaminated culture  
Lane 4: Positive control

**8.4 Discussion**

Mycoplasma is a microorganism (300-800nm diameter), members of the class Mollicutes, and is the smallest and simplest prokaryotes capable of self replication. They may cause respiratory and urinary tract infections, and differ from other bacteria in lacking a cell wall. (Razin and Barile, 1985)
The published incidence of mycoplasma infected cell cultures has ranged from 4 to 92%.

Mycoplasma contamination usually does not produce visible changes in cell culture medium despite the fact it can reach titers of 10^8 per milliliter. Sources of mycoplasma contamination include laboratory personnel, reagents, and Mycoplasma contaminated cell lines (WO/2007/033171). It has been reported that Mycoplasma contamination passed on across generations and persisted in spite of freezing in liquid nitrogen and subsequent thawing. Storage in liquid nitrogen might be one of the potential contamination sources of cell cultures with mycoplasmas. Once introduced into the nitrogen, mycoplasma could persist in the tank for an indefinite time, not proliferating, but being able to contaminate cell cultures stored in the liquid phase of the nitrogen (Helgaso and Miller, 2005). Mycoplasmas do not gain energy by oxidative phosphorylation, but from fermentative metabolism of diverse nutrients. This can lead to an alteration of the pH value as has been observed during the present study.

The commonly used antibiotics such as Doxycycline, Ciprofloxacin, Azithromycin, Minocycline, Clarithromycin, erythromycin and Levaquin can eradicate Mycoplasma (Drexler and Uphoff, 2002) however due to their antiplasmodial activity these antibiotics can not be used in *P. falciparum* culture. On the other hand Tylosin (Sigma Chem. Co., St Louis, USA) and enrofloxacin (Baytrii, Bayer, Germany) when used at intermediate concentrations did not kill malaria parasite but did not eliminate Mycoplasma permanently whereas at highest concentration though eliminate Mycoplasma permanently kills malaria parasite too. Similarly Plasmocin (macrolid), Biomyc-1/-2 (Tetracycline) and Biomyc-3 used in the present study also killed malaria parasite. The only compound which killed Mycoplasma but did not kill malaria parasite is found to be MRA i.e. 4-Oxoquinoline-3-carboxylic acid derivative, it inhibits mycoplasma DNA gyrase. Biomyc-3, an antibiotic belonging to the Fluoroquinolone group also inhibits synthesis of the DNA gyrase but is not safe for *P. falciparum*. It can thus be concluded that MRA treatment can cleanse the infected cultures, periodical monitoring is mandatory as the organism is a slow-grower with the ability to
change its cellular makeup with every cell division (Sharon Briggs et al. 2005). Under these circumstances reappearance of infection remains a continuous threat and MRA can not be used in routine for prevention of Mycoplasma contamination. Thus there is a need for an anti mycoplasma agent both for elimination and prevention of Mycoplasma contaminants in long term culture of malaria parasite.