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SHORT COMMUNICATION

Treatment and control of mycoplasma contamination in *Plasmodium falciparum* culture

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Abstract A comparative efficacy of four antibiotics, plasmocin (macrolid), Biomyc-1, -2, (tetracycline), and Biomyc-3, and Mycoplasma Removing Agent (quinolone derivatives) was determined for elimination of mycoplasma from *Plasmodium falciparum* culture. Presence of mycoplasma was detected using enzyme-PCR-based mycoplasma detection kit and survival of malaria parasite was determined in Giemsa's stained smear made from treated and untreated cultures. It was observed that a combination of Biomyc-1 and -2 killed malaria parasites within 24 h, whereas plasmocin and Biomyc-3 caused slow death of malaria parasite stretched over a period of 6 days. The only compound which did not kill malaria parasite and eradicated mycoplasma from *P. falciparum* culture was observed to be MRA.

Introduction

Mycoplasma are 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 in Edinburgh, UK (Turrini et al. 1997). 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, can pass through 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 making 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, and 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 -3, and Mycoplasma Removing Agent (quinolone derivatives) for elimination of mycoplasma from *P. falciparum* culture.

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Materials and methods

The long-term in vitro culture of 3D7 strain of *P. falciparum* was maintained in RPNI medium supplemented with 10% fetal bovine serum as described by Srivastava and Puri (2004).

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

1. Biomyc-1 was used at 10 μg/ml concentration for 3 days followed by Biomyc-2 at 5 μg/ml concentration for 4 days
2. Biomyc-3 was used at 0.5 μg/ml concentration for 7 days
3. Plasmocin was used at 12.5 μg/ml concentration (i.e., 1:2,000 dilution of the 25 mg/ml stock solution) for 14 days
4. MRA was used at 0.5 μg/ml concentration for 7 days

Monitoring of parasitemia To monitor the development of parasites, a drop of culture from the settled layer of red blood cells (RBCs) was placed on a clean, grease-free glass slide and a thin smear was drawn. The smear was allowed to air dry, fixed in methanol for 10 s, and stained with Giemsa's stain for 20–30 min. The smears were examined under a light microscope (100× oil immersion objective). Approximately 1,000 RBCs were scanned in about ten fields and percent parasitemia was calculated and plotted.

Dilution of culture In case of MRA-treated and untreated cultures, fresh human RBCs were added to dilute running culture on days 4, 7, 10, and 12 containing approximately 6% parasitemia.

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

Identification of mycoplasma Presence of mycoplasma was ascertained using enzyme-PCR-based mycoplasma detection kit (Biological Industries).

Results and discussion

The growth profile of *P. falciparum* as observed during and post treatment with plasmocin, Biomyc-3, and MRA in a mycoplasma-free and a contaminated culture is shown in Figs. 1 and 2, respectively. It is evident from Fig. 1 that, in mycoplasma, free malaria parasite culture percent parasitemia in cultures treated with plasmocin or Biomyc-3 increased slowly up to 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 parasitemia similar to the untreated culture. The fall in percent parasitemia 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) parasitemia in four 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 parasitemia since 24 h of treatment and percent parasitemia reached to more than 7% on day 3 post commencement of treatment and kept on increasing

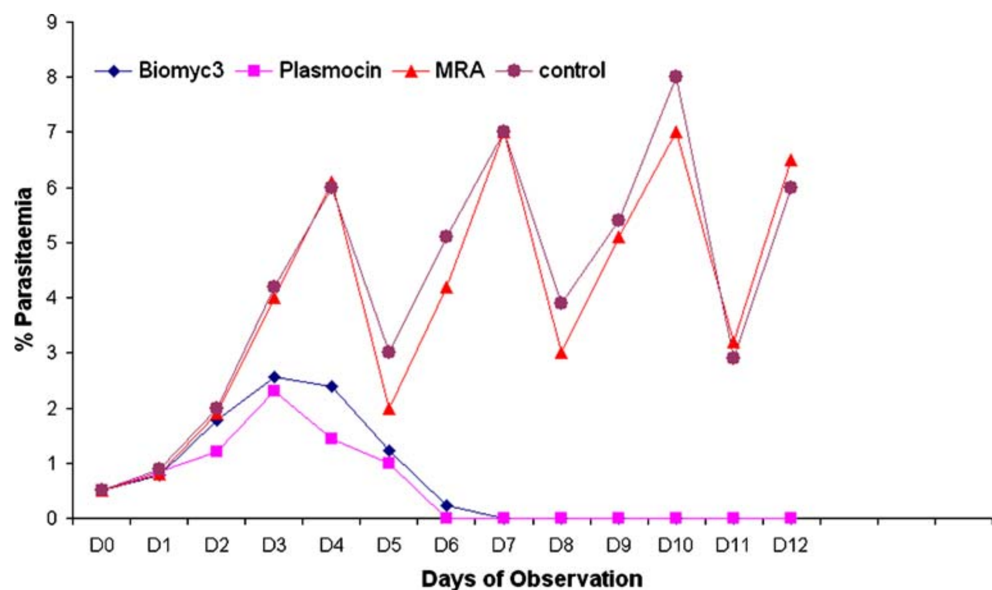

Fig. 1 Growth profile of *Plasmodium falciparum* in mycoplasma-free culture during and post treatment with anti-mycoplasma agents

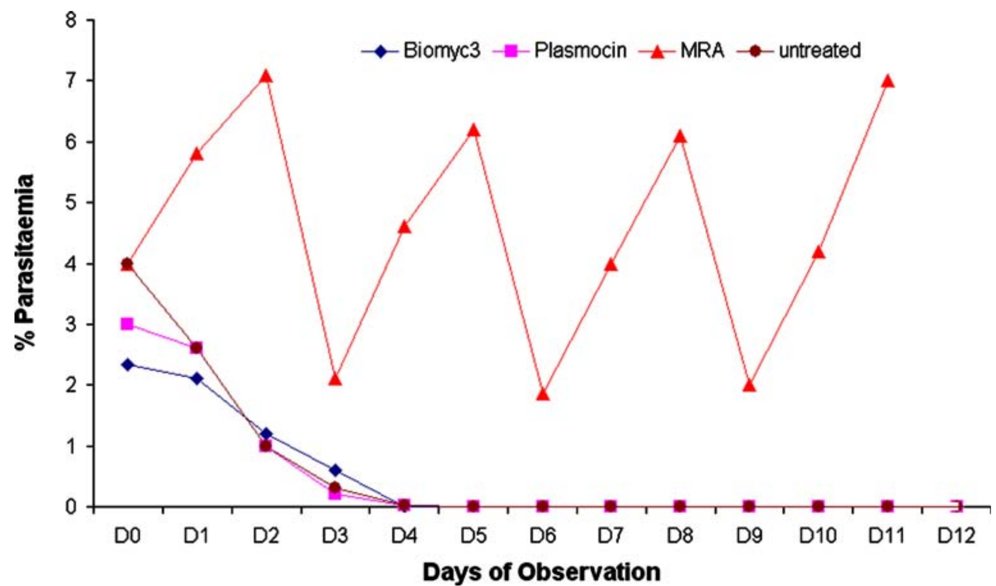

Fig. 2 Growth profile of *Plasmodium falciparum* in mycoplasma-contaminated culture during and post treatment with anti-mycoplasma agents

thereafter. The control culture which did not receive any treatment died due to increased mycoplasma contamination.

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 h of treatment. Whereas in contaminated and untreated culture, pH remained unchanged during the observation period.

Identification of mycoplasma Using a commercial PCR detection kit, a single band of 290 bp was detected in contaminated culture. No band could be seen in culture treated with MRA as well as in mycoplasma-free culture (data not shown).

Mycoplasma is a microorganism (300–800 nm diameter), members of the class Mollicutes, and is the smallest and simplest prokaryotes capable of self-replication. They differ from other bacteria in lacking a cell wall (Razin and Barile 1985).

Sources of mycoplasma contamination include laboratory personnel, reagents, and mycoplasma-contaminated cell lines (WO/2007/033171). The published incidence of mycoplasma-infected cell cultures has ranged from 4% to 92%. 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 (Helgason 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, as most of the antibiotics possess anti-plasmodial activity, they cannot be used in *P. falciparum* culture (Gingras and Jensen 1992; Watt et al. 1992; Pradines et al 2001; Basco and Le Bras 1993; Yeo and Rieckmann 1994). On the other hand, tylosin (Sigma Chem. Co., St. Louis, USA) and enrofloxacin (Baytrii, Bayer, Germany) at intermediate concentrations neither kill malaria parasite nor eliminate mycoplasma permanently whereas at highest concentration they eliminate mycoplasma permanently but kills malaria parasite too. Similarly, plasmocin (macrolid) and Biomyc- 1/-2 (Tetracycline) and Biomyc-3 used in the present study also killed malaria parasite. The only compound which did not kill malaria parasite but killed mycoplasma is found to be MRA, i.e., 4-oxo-quinoline-3-carboxylic acid derivative; it inhibits mycoplasma DNA gyrase. Biomyc-3, an antibiotic belonging to the fluoroquinolone group, also inhibits synthesis of

Table 1 pH of spent culture medium

Days of observation	1	2	3	4	5	6	7	8	9	10
Contaminated and treated culture	6.8	7.0	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
Contaminated and untreated culture	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8
Uncontaminated and untreated culture	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2

the DNA gyrase but is not safe for *P. falciparum.* It can thus be concluded that MRA treatment can cleanse the *P. falciparum* culture but periodical monitoring is mandatory as the mycoplasma 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 cannot be used in routine for prevention of mycoplasma contamination. Thus, there is still a need for an anti-mycoplasma agent both for elimination and prevention of mycoplasma contaminants from long-term culture of malaria parasite.

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References

Basco LK, Le Bras J (1993) Activity *in vitro* of doxycycline against multidrug-resistant *Plasmodium falciparum*. Trans R Soc Trop Med Hyg 87:469–470

Bove JM (1993) Molecular features of Mollicutes. Clin Infect Dis 17 (Suppl):10–31

Drexler HG, Uphoff CC (2002) Mycoplasma contamination of cell cultures: incidence, sources, effects, detection, elimination, prevention. Cytotechnology 39:75–90

Gingras BA, Jensen JB (1992) Activity of azithromycin (CP-62,993) and erythromycin against chloroquine sensitive and chloroquine resistant strains of *Plasmodium falciparum in vitro*. Am J Trop Med Hyg 47:378–382

Helgason CD, Miller CL (2005) Methods in molecular biology—basic cell culture protocols. Humana, Totowa

Loomes LM, Uemura K, Childs RA, Paulson JC, Rogers GN, Scudder PR, Michalski JC, Hounsell EF, Taylor-Robinson D, Feizi T (1984) Erythrocyte receptors for *Mycoplasma pneumoniae* are sialylated oligosaccharides of Ii antigen type. Nature 307: 560–563

Loomes LM, Uemura K, Feizi T (1985) Interaction of *Mycoplasma pneumoniae* with erythrocyte glycolipids of I and i antigen types. Infect. Immun 47:15–20

Pradines B, Rogier C, Fusai T, Mosnier J, Daries W, Barret E, Parzy D (2001) In vitro activities of antibiotics against *Plasmodium falciparum* are inhibited by iron. Antimicrob Agents Chemother 45:1746–1750

Razin S and Barile, MF (eds) (1985) The Mycoplasmas, vol. 4 Academic Press

Roberts DD, Olson LD, Barile MF, Ginsburg V, Krivan HC (1989) Sialic acid-dependent adhesion of *Mycoplasma pneumoniae* to purified glycoproteins. J Biol Chem 264:9289–9293

Sharon Briggs RN, MSN, Garth Nicolson (2005) Mycoplasma Support web site

Srivastava K, Puri SK (2004) *Plasmodium falciparum*: modified medium composition supports continuous cultivation with foetal bovine serum. Experimental Parasitology 108:74–75

Turrini F, Giribaldi G, Valente E, Arese P (1997) Mycoplasma contamination of *Plasmodium* cultures—a case of parasite parasitism. Parasitol Today 13:367–368

Visser I, ter Laak E, Jansen H (1999) Failure of antibiotics gentamycin, tylosin, lincomycin and spectinomycin to eliminate in artificially infected frozen bovine semen. Theriogenology 51:689–697

Watt G, Loesuttivibool L, Shanks GD, Boudreau EF, Brown AE, Pavanand K, Webster HK, Wechgritaya S (1992) Quinine with tetracycline for the treatment of drug-resistant falciparum malaria in Thailand. Am J Trop Med Hyg 47:108–111

WO/2007/033171—Mycoplasma detection method and composition

Yeo AE, Rieckmann KH (1994) Prolonged exposure of *Plasmodium falciparum* to ciprofloxacin increases anti-malarial activity. J Parasitol 80:158–160