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Short communication

Implications of mycoplasma contamination in *Plasmodium falciparum* cultures and methods for its detection and eradication

J. Alexandra Rowe [a,*], Ian G. Scragg [b], Dominic Kwiatkowski [b], David J.P. Ferguson [c], Daniel J. Carucci [d], Christopher I. Newbold [a]

[a] *Molecular Parasitology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK*
[b] *Department of Paediatrics, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK*
[c] *Electron Microscopy Unit, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK*
[d] *Naval Medical Research Institute, 12300 Washington Ave, Rockville, MD, USA*

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Mycoplasma contamination of eukaryotic cell cultures is a common and widely recognised problem, estimated to affect 15–80% of all long term cell lines [1]. Infection with mycoplasmas can lead to a variety of effects such as impaired growth [2], chromosome abnormalities [3], enzyme inhibition [4], proteolytic degradation [5], B-lymphocyte proliferation [6], cytokine induction [7] and superantigen activity [8]. Mycoplasmas are small free-living prokaryotes lacking a cell wall, and they cannot be seen by routine staining procedures such as Giemsa-stained thin films. We have recently discovered that mycoplasmas can infect and survive in long term in vitro cultures of *P. falciparum*. Similar findings have recently been reported elsewhere [9].

Mycoplasma contamination was detected using a commercially available polymerase chain reaction (PCR) method (ATCC, Rockville, MD). Culture supernatants from overnight cultures of the *P. falciparum* clones R29, A4, C18I (derived in Oxford [10]) and 3D7 (gift of Professor David Walliker, University of Edinburgh, UK) were tested as described by the manufacturer. The R29 clone gave two PCR products (236 and 290 bp; Fig. 1(A)) indicating the presence of two species of mycoplasma. A4 and C18I gave a 290 bp product

Abbreviations: PCR, polymerase chain reaction; MRA, mycoplasma removal agent; TNF, tumour necrosis factor.

* Corresponding author. Tel: +44 1865 222302; fax: +44 1865 222444; e-mail: arowe@worf.molbiol.ox.ac.uk

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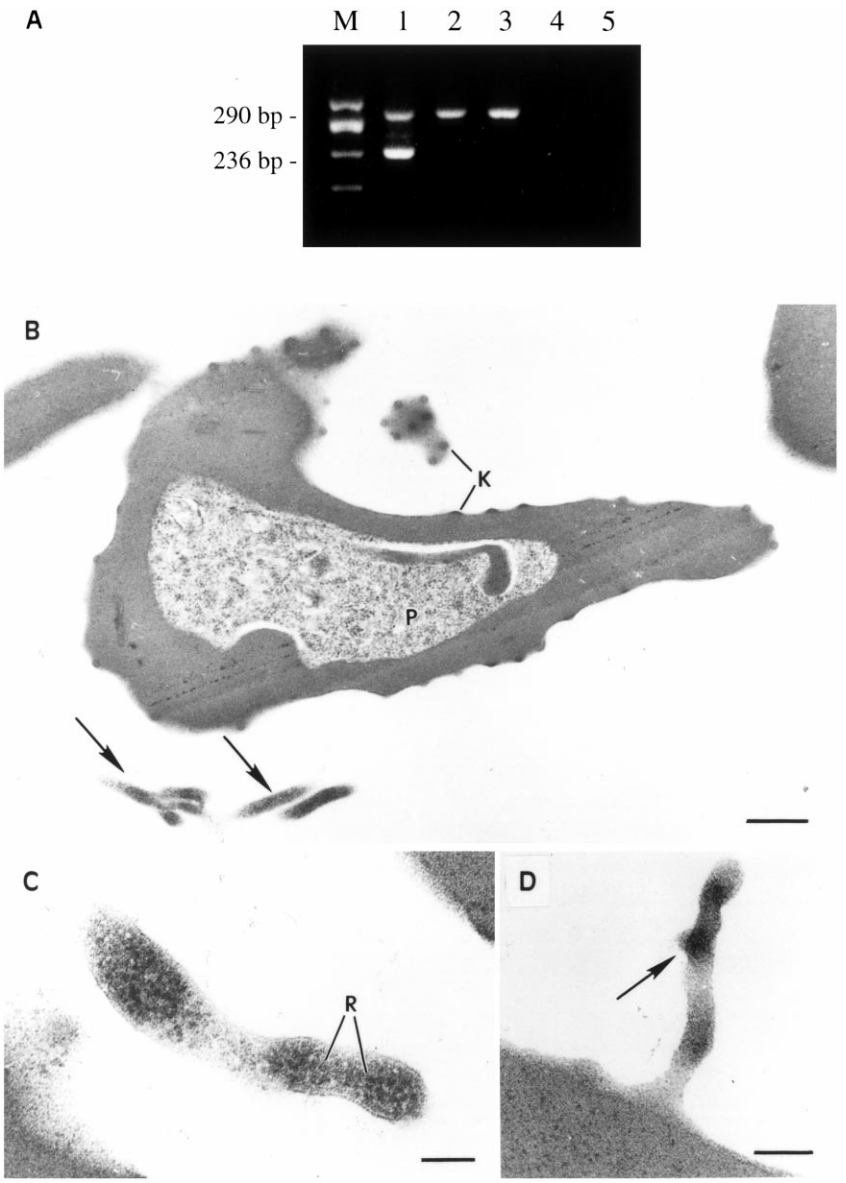

Fig. 1. (A) Detection of mycoplasma in *P. falciparum* culture supernatants. Medium (5 μl) from an overnight culture was tested in a 50 μl PCR reaction with degenerate oligonucleotide primers to the mycoplasma 16–23S ribosomal operon space region as described by the manufacturer (ATCC, Rockville, MD). A mycoplasma detection kit (Stratagene, Cambridge, UK) gave similar results (not shown). PCR products were run on a 3% MetaPhor agarose gel (FMC Bioproducts, Rockland, ME). Lanes: 1, R29; 2, A4; 3, C18I; 4, 3D7; 5, negative control (distilled water). (B) Transmission electron micrograph of the A4 *P. falciparum* clone showing mycoplasma (arrowed) adjacent to an infected erythrocyte exhibiting characteristic knobs (K) at the cell surface. P, parasite. Bar is 0.5 μm. (C) Detail of a longitudinal section through a mycoplasma showing the cytoplasm containing numerous ribosomes (R) and limited by a double membrane. Bar is 100 nm. (D) Detail of the surface of an infected erythrocyte showing a finger-like projection (arrow) that contains fine granular electron dense material. Bar is 100 nm.

indicating contamination with a single mycoplasma species, and 3D7 was negative (Fig. 1(A)). To confirm that the PCR products were a true indication of mycoplasma contamination, and were not due to cross-reaction of the oligonucleotide primers with *P. falciparum* DNA, the PCR products from R29 were cloned into a TA vector (Invitrogen, Leek, Netherlands) and sequenced by standard methods. The 236 bp product was identical with a portion of the 16–23S ribosomal operon spacer region of *M. arginini* (GenBank™ accession number X58560), and the 290 bp product was identical with a portion of the 16–23S ribosomal operon spacer region of *M. orale* (GenBank™ accession number X58556). Restriction digest analysis of the 290 bp PCR product from A4 and C18I showed that these cultures were contaminated with *M. orale* (data not shown). Mycoplasma contamination was confirmed by testing the R29 culture supernatant by DNA fluorochrome assay (using Hoechst stain and a Vero cell indicator cell line) and by direct culture on mycoplasma media (assays carried out by Bionique Testing Laboratories, Saranac Lake, NY). In addition, close examination of transmission electron micrographs of the A4 clone showed the presence of mycoplasmas loosely associated with the surface of infected and uninfected erythrocytes (Fig. 1(B) and (C)). These organisms appear as pleomorphic branching filamentous structures that contain numerous ribosomes and are limited by a double membrane (Fig. 1(C)). However, they should not be confused with finger-like projections from the surface of certain infected erythrocytes which often contain electron dense material similar to that associated with the knobs (Fig. 1(D)).

The R29, A4 and C18I *P. falciparum* clones are members of a clone family derived originally from the IT strain [10]. Testing of other members of the clone family, including stabilates frozen in glycerolyte since 1990, indicated that *M. orale* infection occurred early in the derivation of the clone tree, and had been passed on to all clones. The infection with *M. arginini* was confined to the R29 clone and had been acquired in late 1996 and was not detected in older frozen stocks. Testing of a small number of isolates derived from other laboratories suggests that mycoplasma contamination is a relatively common problem.

To eradicate the mycoplasma infection, the R29 clone was treated for 7 days with 0.5 μg ml^{-1} of Mycoplasma Removal Agent (MRA) (ICN Biochemicals, Thame, Oxon, UK) added to standard culture medium [11]. MRA is a 4-oxo-quinolone-3-carboxylic acid derivative which inhibits mycoplasma DNA gyrase and is bacteriocidal. The medium was changed daily, and regular Giemsa smears showed that *P. falciparum* remained healthy and continued to grow and invade in the presence of drug. Other known anti-mycoplasma agents such as tylosin, erythromycin and ciprofloxacin were tested and found to be toxic to *P. falciparum*. After 7 days of treament with MRA the culture supernatant was negative for mycoplasma by PCR (data not shown). The treated R29 clone was grown under standard conditions (without drug) for a further 12 weeks and tested by PCR every 2 weeks. Both the culture supernatant and a DNA extract of the whole cell pellet remained negative, showing that the mycoplasma infection had been eradicated.

The R29 clone has been carefully characterised in terms of its rosetting and cytoadherence properties (J.A. Rowe, D. Phil. Thesis, University of Oxford, 1994 and [11,12]). It forms rosettes with uninfected erythrocytes, and these rosettes can be inhibited by certain reagents such as sulphated glycoconjugates and antibodies [11]. We have recently shown that erythrocytes with low copy number of complement receptor 1 (CR1) have reduced rosetting with R29, and that soluble CR1 (sCR1) inhibits rosettes [12]. To determine if the presence of mycoplasma had influenced the adhesion properties of this clone, we carried out a series of experiments comparing the MRA-treated (mycoplasma-free) clone with an untreated, mycoplasma-infected subculture of the same clone. The effects of sulphated glycoconjugates (heparin, fucoidan, chondroitin sulphate A; all at 100 μg ml^{-1}), antibodies (CD36 monoclonal antibody OKM5 and MAb 89 to PfHRP1; at 50 μg ml^{-1}) and sCR1 (50 μg ml^{-1}) were tested, and in every case, the rosetting properties of the mycoplasma-free culture did not differ from the mycoplasma-infected culture [11,12]. We also tested the cytoadherence of mycoplasma-free and -infected R29 to CD36 and ICAM-1 in a plate binding assay [10]. Both cultures showed good binding to CD36, and

low-level binding to ICAM-1 (data not shown), indicating that the presence of mycoplasma does not influence cytoadherence to these receptors.

We did, however, find that the previously described ability of R29 to stimulate high levels of tumour necrosis factor (TNF) production from monocytes [13] was due to the presence of mycoplasma contamination. Mycoplasmas are known to be potent inducers of TNF [14], and following MRA treatment of R29 the level of TNF induced by this parasite was greatly reduced (Table 1). Clearly, it is of importance to establish the extent to which mycoplasma contamination has influenced the large body of published data on cytokine stimulation by *P. falciparum* in vitro and by factors purified from cultured parasites.

Another potential hazard of mycoplasma contamination has been noted by one of us. While attempting to clone a cyclase gene from *P. falciparum* using degenerate oligonucleotide primers, a contaminating mycoplasma gene was cloned and sequenced in error. The AT-richness of the mycoplasma genome (61–76% AT content [15]) is similar to that of *P. falciparum*, and makes this a potential pitfall for any researcher working with mycoplasma-contaminated material.

We have therefore shown that mycoplasmas can occur as contaminants of in vitro *P. falciparum* cultures. Testing for mycoplasma can be carried out quickly and easily by PCR, and infection can be eradicated in one week by treatment with MRA. We urge other malaria research workers to test regularly for mycoplasmas and so avoid the possibility of erroneous data resulting from contamination.

Table 1
TNF induction by mycoplasma-free and mycoplasma-infected *P. falciparum* clone R29

Dilution of parasite extract	TNF (pg ml^{-1})[a]	
	Mycoplasma-infected	Mycoplasma-free
1/20	6600	46
1/100	7426	12
1/500	6621	16
1/2500	4086	5

[a] TNF-inducing activity was determined as described in reference [13]. The experiment was repeated a week later with fresh parasite extracts and gave similar results (data not shown).

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