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ORIGINAL ARTICLE

Flow cytometric method for quantifying viable *Mycoplasma agassizii*, an agent of upper respiratory tract disease in the desert tortoise (*Gopherus agassizii*)

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Abstract

Aims: *Mycoplasma agassizii* can cause upper respiratory tract disease in the threatened desert tortoise of the Southwestern United States. Two technical challenges have impeded critical microbiological studies of this microorganism: (i) its small size limits the use of light microscopy for cell counting and (ii) its extremely slow growth in broth and agar cultures impedes colony counting. Our aim was to develop a rapid and sensitive flow cytometric method using a vital fluorescent dye to enumerate viable *M. agassizii* cells.

Methods and Results: Here, we demonstrate that the nonfluorescent molecule 5-carboxyfluorescein (5-CF) diacetate acetoxymethyl ester penetrates *M. agassizii* cell membranes and it is converted in the cytoplasm to the fluorescent molecule 5-CF by the action of intracellular esterases. Labelled mycoplasma cells can be easily detected by flow cytometry, and cultures with as few as 100 viable mycoplasma cells ml^{-1} can be labelled and counted in less than 1 h. Experiments using temperature-induced cell death demonstrated that only viable *M. agassizii* cells are labelled with this procedure.

Conclusions: A rapid and sensitive flow cytometric technique has been developed for enumerating viable *M. agassizii* cells.

Significance and Impact of the Study: This technique should facilitate basic immunological, biochemical and pharmacological studies of this important pathogen which may lead to new diagnostic and therapeutic methods.

Introduction

The Mojave population of the desert tortoise (*Gopherus agassizii*) is found in the Mojave Desert, west of the Colorado River in Nevada, Arizona, Utah and California. Encroachment of civilization on desert tortoise habitat, together with periods of severe drought, has placed significant stress on these ancient reptiles. Populations of desert tortoises have declined by as much as 90% in some parts of their range, and infectious diseases are thought to be one of the causes. In particular, many desert tortoises suffer from upper respiratory tract disease (URTD), and the bacterium *Mycoplasma agassizii* has been identified as one of the causative agents (Jacobson *et al.* 1991; Brown *et al.*1994, 2001).

Mycoplasma agassizii is a pleiomorphic mycoplasma with an average diameter of less than 300 nm that prevents its visualization by light microscopy. Although *M. agassizii* can be cultured in the laboratory in SP4 medium at a temperature optimum of 30°C, its growth rate is extremely slow and it takes several days to visualize even slight changes in the colour of the medium (colour changing units). Moreover, it takes up to 8 weeks for even small colonies to appear on solid agar (Wendland *et al.* 2007; Hunter *et al.* 2008), and these technical challenges have impeded studies of the immunology, biochemistry and pharmacology of this important micro-organism.

Flow cytometric methods for counting bacteria that provide faster results than culture methods have been

developed previously (Lopez-Amoros *et al.* 1995; Langsrud and Sundheim 1996; Henningson *et al.* 1998; Rattanasomboon *et al.* 1999; Gunasekera *et al.* 2000; Holm and Jespersen 2003). Recently, flow cytometry using the DNA-intercalating fluorophore SYBR Green in combination with the membrane impermeant fluorophore propidium iodide compared favourably with colony counting for determining the numbers and viability of various mycoplasma species (Assunção *et al.* 2005, 2006a,c). We developed an alternative flow cytometric method for identifying and enumerating viable *M. agassizii* cells in broth culture using a single fluorophore.

Carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) is an esterified fluorogenic substrate that has been used widely for assessing esterase activity in both bacterial and mammalian cultures (Rotman and Papermaster 1966; Hoefel *et al.* 2003). The nonfluorescent hydrophobic molecule 5-CFDA-AM is cell permeant because of the acetoxymethyl ester. Once inside the cell, the lipophilic blocking and diacetate groups are cleaved through hydrolysis by nonspecific esterases and the resulting fluorescent 5-carboxyfluorescein (5-CF) is more polar and leaks out of the cells very slowly. We demonstrate that *M. agassizii* has a cytoplasmic esterase that can generate 5-CF from 5-CFDA-AM and these fluorescent molecules remain in the cell and can be used to detect and count viable *M. agassizii* by flow cytometry.

Materials and methods

Mycoplasma culture

Mycoplasma agassizii was purchased from the American Type Culture Collection (ATCC No. 700616) and cultured as described (Brown *et al.* 1994). Briefly, mycoplasma cells were cultured in Spiroplasma medium 4 (SP4) containing PPLO broth (without crystal violet), tryptone, yeastolate yeast extract all from Becton Dickinson (Franklin Lake, NJ, USA), glucose (Fisher Scientific, Pittsburgh, PA, USA), CMRL-1066 medium (ATCC, Rockville, MD, USA), heat-inactivated foetal bovine serum (Hyclone Laboratories, Logan, UT, USA) and phenol red (Sigma Chemicals, St Louis, MO, USA). Mycoplasma cells were grown in suspension cultures using an Innova 4200 incubator shaker set at 90 rev min^{-1} and 30°C.

CFDA-AM staining of *Mycoplasma agassizii* cells

Mycoplasma agassizii cells from broth culture were centrifuged at 9279 ***g*** in an Eppendorf 5417R centrifuge at 4°C for 30 min. All subsequent washes were carried out with phosphate buffered saline (PBS) without Ca^{++}/Mg^{++}. Pellets were resuspended in one-ml PBS; then, the mycoplasma cells were stained with 1 μmol l^{-1} CFDA-AM (Molecular Probes, Eugene, OR, USA) dissolved in dimethyl sulfoxide (DMSO) for 1 h in the dark at room temperature using an end-over-end rocker. Cells were centrifuged and washed as before and then resuspended in 20 μl PBS. Aliquots of 5 μl were placed on glass slides under cover slips and observed through a U Plan Apochromats 60× oil immersion objective lens using an Olympus Fluo View 1000 confocal laser scanning microscope to confirm the uptake of CFDA-AM and conversion to fluorescent 5-CF.

To verify that 5-CFDA-AM-labelling identified only viable cells, we measured the fluorescent intensity of *M. agassizii* cells incubated for 1 h at temperatures ranging from the optimal 30°C to the lethal 60°C using a programmable heating block. After incubation, the cells were centrifuged and washed as previously described. Microorganisms were then stained with either 1 μmol l^{-1} 5-CFDA-AM or 1 μmol l^{-1} SYBR Green (Molecular Probes) as described earlier. Samples were centrifuged and washed as before; pellets were resuspended in 1 ml PBS and analysed by flow cytometry.

Flow cytometry

CFDA-stained mycoplasma cells were analysed using an Epics XL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA) equipped with an air-cooled 488 nm argon-ion laser. Cells were characterized by side-angle scatter, forward-angle scatter and green fluorescence for CFDA (517 nm, FL1 detector). Data were portrayed on a 4-log scale, and CFDA-labelled cells routinely showed a 2-log shift in fluorescence intensity. Absolute cell counts were made using Flow-Check fluorescent beads (Polysciences, Inc., Warrington, PA, USA). To avoid coincidence each sample was tested at several dilutions to maintain a flow rate below 2000 events s^{-1}. Data were analysed using Flo-Jo software (Tree Star, Inc., Ashland, OR, USA).

Results

Vital fluorescence labelling and imaging of *Mycoplasma agassizii* cells

We first wanted to see whether nonfluorescent 5-CFDA-AM molecules would be internalized by intact *M. agassizii* cells and whether this mycoplasma possessed a cytoplasmic esterase that would hydrolyse these molecules to the fluorescent 5-CF product. Confocal microscopy showed that during a 1-h incubation 5-CFDA-AM was internalized and hydrolysed to the fluorescent 5-CF (Fig. 1a). Similar results were obtained with incubations as short as 20 min (data not shown). These data indicate that *M. agassizii* possesses an intracellular esterase or

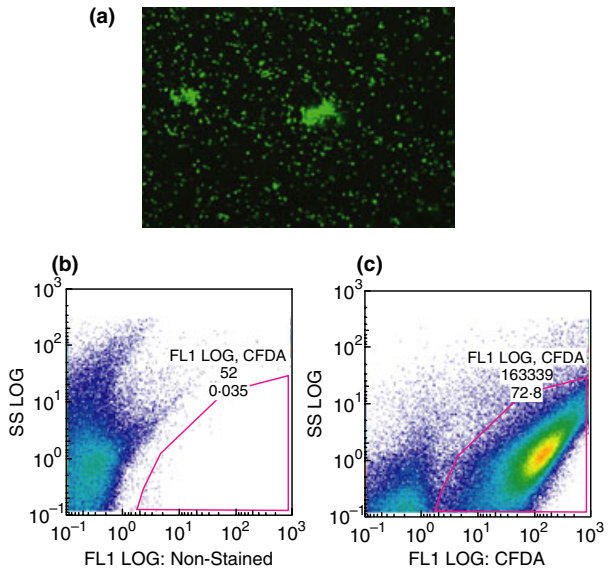

Figure 1 (a) Confocal image of *Mycoplasma agassizii* cells labelled in vitro with 5-CFDA-AM. Flow cytometric dot plots of *M. agassizii* cells before (b) and after (c) staining with 5-CFDA-AM. Unstained mycoplasma had little autofluorescence, but variable side scatter properties consistent with their pleiomorphic shape. A gate was chosen that excluded almost all unstained organisms (inset represents number of micro-organisms and the % of total micro-organisms in this gate). After CFDA-AM staining, 72% of the events were located within this gate; the events located outside of the gate were dead cells and cellular debris. CFDA-AM, carboxyfluorescein diacetate acetoxymethyl ester.

esterases capable of hydrolysing diacetate and acetoxymethyl esters and demonstrate that *M. agassizii* cells can be visualized by fluorescence imaging.

Flow cytometric analysis of fluorescent-labelled *Mycoplasma agassizii* cells

Next we asked whether *M. agassizii* cells labelled with 5-CFDA-AM could be detected by flow cytometry. Unlabelled *M. agassizii* showed low or no autofluorescence (Fig. 1b). These pleiomorphic cells showed some variability in side scatter, but they could be detected as discrete events despite their small size. After labelling with 5-CFDA-AM, 72% of the cells showed a greater than two-log shift in fluorescence intensity (Fig. 1c). Some low fluorescence material remained, which was most likely intact dead cells and debris. We gated on the major population of highly fluorescent cells.

Selective labelling of viable *Mycoplasma agassizii* cells by 5-CFDA-AM

We used the known temperature sensitivity of *mycoplasma* to demonstrate that only viable *M. agassizii* cells were labelled by 5-CFDA-AM. When these organisms were grown for 1 h at temperatures ranging from a permissive 30°C to a lethal 60°C (Assunção *et al.* 2006a), and then labelled with 5-CFDA-AM, a progressive decrease in median fluorescence intensity was observed (Fig. 2a). Almost no fluorescent signal was observed in cells grown at 60°C, perhaps because the intracellular esterases required to convert the 5-CFDA-AM to 5-CF are heat labile. Also, the *M. agassizii* cells grown at 60°C for 1 h did not exhibit growth when returned to a permissive 30°C, indicating that they were not viable. A 1 : 1 mixture of *M. agassizii* cells grown at the permissive 30°C, and the lethal 60°C was prepared, and after labelling with 5-CFDA-AM, two discrete populations of cells in equivalent numbers were observed; one with no fluorescence and the other with high fluorescence (data not shown).

Mycoplasma agassizii cells were incubated for 1 h at the permissive 30°C or lethal 60°C and then labelled with 5-CFDA-AM or SYBR Green, a DNA intercalating dye that labels both live and dead cells. While the 5-CFDA-AM-labelled cells grown at 60°C showed a decrease in fluorescence intensity to near nonstained levels (Fig. 2b), the SYBR Green-labelled cells showed no

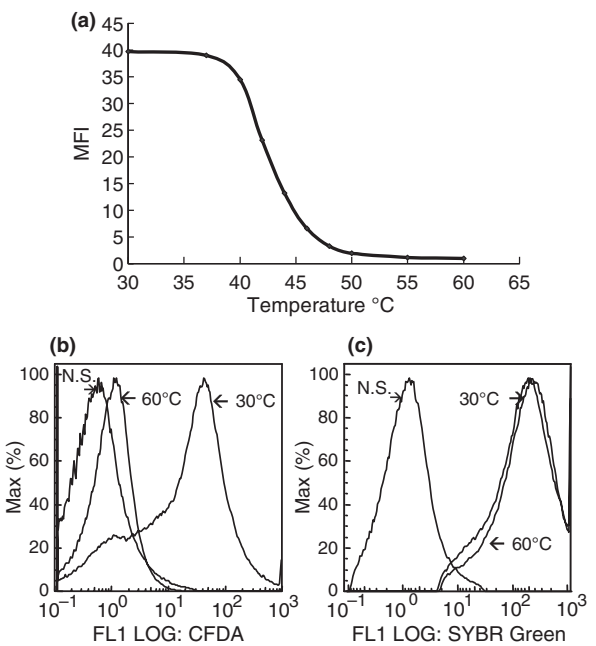

Figure 2 Effect of incubation temperature on viability of *Mycoplasma agassizii* cells. (a) Cultures of 2.0×10^6 mycoplasma cells ml^{-1} were incubated at temperatures ranging from 30 to 60°C for 1 h. Mean fluorescence intensity (MFI) is plotted against incubation temperature. Flow cytometric histograms showing effect of temperature on fluorescence intensity of *M. agassizii* stained with 5-CFDA-AM (b) or SYBR Green (c). Cultures were grown at 30°C or 60°C for 1 h then stained. N.S., nonstained *M. agassizii*; CFDA, carboxyfluorescein diacetate acetoxymethyl ester.

effect of temperature on fluorescence intensity (Fig. 2c). Together these data confirm that the nonfluorescent 5-CFDA-AM is internalized and converted to the fluorescent 5-CF only by viable *M. agassizii* cells.

Assessment of *Mycoplasma agassizii* growth and detection levels in broth culture

The flow cytometric method using 5-CFDA-AM staining was used to evaluate the growth of *M. agassizii* in broth culture (Fig. 3a). As expected, based on previous studies, *M. agassizii* growth was very slow. Although growth curves followed typical kinetics, the doubling time was greater than 72 h. To determine the lowest number of *mycoplasma* cells that can be detected reliably and counted by flow cytometry, serial dilutions of 5-CFDA-AM-stained *M. agassizii* cells were analysed by flow cytometry. We found fewer than 100 *M. agassizii* cells ml^{-1} could be detected by this method (Fig. 3b,c).

Discussion

Despite their small size and slow growth, the mollicute *Mycoplasma hypopneumoniae* has been enumerated using SYBR Green staining and flow cytometry with results comparable to colony counting (Assunção *et al.* (2005). Similar findings were obtained with *Mycoplasma mycoides* subsp. *mycoides* (Assunção *et al.* 2006a,b,c,d). However, because SYBR Green intercalates into the nucleic acid of both live and dead mycoplasma cells (Barber and Fabricat 1971), it cannot be used to determine the number of viable micro-organisms. However, in an *in vitro* study of antibacterial agents on several mycoplasma species, the cell impermeant fluorophore propidium iodide was added to SYBR Green allowing live and dead mycoplasma cells to be distinguished by flow cytometry (Assunção *et al.* 2006a,b). This dual staining procedure worked in principle, although not all antibacterial agents (including known bacteriostatic agents) had the same effect on membrane permeability.

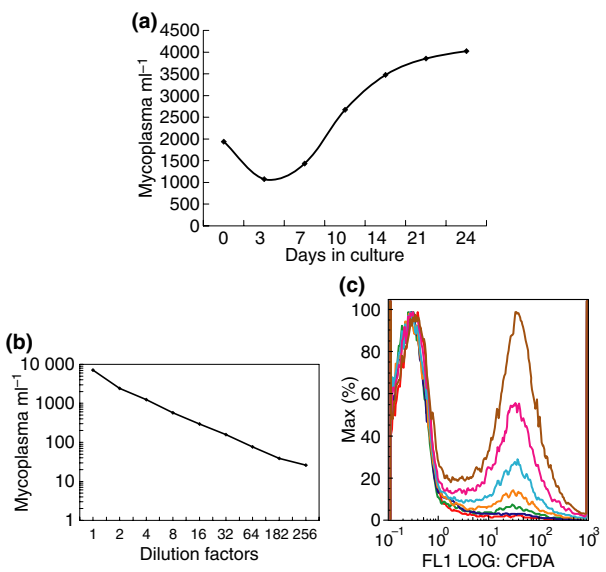

Figure 3 (a) Growth rate of *Mycoplasma agassizii* in broth culture. (b) Plot of viable cell numbers stained with 5-CFDA-AM *vs* dilutions, demonstrating fewer than 100 viable *M. agassizii* ml^{-1} can be detected using flow cytometry. (c) Flow cytometric histogram showing the progressive decrease in viable cell numbers at the same peak fluorescence intensity. The highest peak represents the lowest dilution shown in Fig. 3b, and each lower peak in turn represents a progressively higher dilution of mycoplasma. CFDA, carboxyfluorescein diacetate acetoxymethyl ester.

In contrast to many *mycoplasma* species, *M. agassizii* grows very poorly in standard SP4 medium (Wendland *et al.* 2007; Hunter *et al.* 2008). Indeed, the growth rate is too slow to use colour changing units accurately in broth culture, and it takes 6–8 weeks to observe even small colonies on agar medium. We were unable to find quantitative relationships when using colony counts (data not shown). Moreover, for many experimental purposes, it is desirable to know the number of viable micro-organisms at the time research studies are performed. The purpose of this study was to show that a single fluorescent label can be used to rapidly enumerate viable *M. agassizii* by flow cytometry.

The internalization of nonpolar, nonfluorescent molecules and their conversion by the action of intracellular enzymes (such as esterases) to polar, fluorescent compounds that are retained within living cells has been shown to be a reliable measure of mammalian cell viability (Prosperi *et al.* 1986). Here, we show that nonfluorescent 5-CFDA-AM molecules, once internalized by viable *M. agassizii*, are converted into fluorescent 5-CF molecules through esterase hydrolysis and retained within the cells. By culturing *M. agassizii* organisms at a permissive 30°C and a lethal 60°C, we were able to show that only viable *mycoplasma* are stained with 5-CFDA. In addition, this method allowed for the enumeration of fewer than 100 viable microbes ml^{-1}, which compares to the detection limit reported for various *mycoplasma* species in goat milk (Assunção *et al.* 2007). The ability to detect and enumerate viable *M. agassizii* in broth cultures by 5-CFDA-AM staining allows experiments with these organisms in the laboratory that were previously not possible using conventional culture methods. Further studies are needed to determine if this method can be used to detect mycoplasma infections in the desert tortoise. We are presently working on a combination of the proposed method and specific *M. agassizii* antibodies in a dual fluorescence format.

Acknowledgements

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