[Frontiers in Bioscience 11, 492-497, January 1, 2006]

Quantification of mycoplasmas in broth medium with sybr green-I and flow cytometry

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1. ABSTRACT

Mycoplasmas are the smallest and simplest organisms known. They form a large group of bacteria that can infect humans, animals, and plants. Even though several techniques have been proposed to enumerate mycoplasmas in broth medium, the determination of mycoplasma growth still remains a difficult task. The potential of using flow cytometry (FC) for rapidly estimating several species of mycoplasmas, *M. agalactiae* (Ma), *M. putrefaciens* (Mp), *M. capricolum* subsp. *capricolum* (Mcc), *M. bovis* (Mb), *M. capricolum* subsp. *capripneumoniae* (Mccp) and *M. hyopneumoniae* (Mh) in broth medium was examined. The FC analysis was performed by staining the mycoplasma cells with a fluorescent dye, SYBR green-I (SYBR), and the results were compared with plate count (Colony Forming Units - CFU) or Colour Changing Units (CCU) methods, depending on the mycoplasma species. There was a good correlation between mycoplasma counts determined by FC (cells ml^{-1}) and by traditional plate count (CFU) or CCU methods. A correlation of 0.841, 0.981, 0.960, 0.913, 0.954, and 0.844 was obtained for Ma, Mp, Mcc, Mb, Mccp and Mh, respectively. FC method allowed results in 20-30 min, while 24-72 h was necessary for plate count method and 15 days for CCU method. FC was found to be a very useful, practical and fast technique to count mycoplasmas. These findings suggest that FC can be a good alternative to replace other time-consuming techniques that are currently used to enumerate mycoplasmas in broth medium.

2. INTRODUCTION

Mycoplasmas are the smallest known free-living life forms and they are nearly ubiquitous in both the plant and animal kingdoms as colonizers and pathogens (1, 2).

One of the major issues that remain questionable is the enumeration of mycoplasma cells. Even though several techniques have been proposed to assess the number of cultured *Mollicutes* cells (3, 4, 5, 6, 7, 8) accurate determination of *Mycoplasma* growth still remains a difficult task (7). First, the small cellular dimensions of *Mollicutes* (0.2-0.7µm) preclude total cell counts by light microscopy. Second, many species form microcolonies in broth or adhere to the surface of culture vessels, resulting in underestimated viable cell counts. Third, because of fastidious growth requirements, certain species grow poorly on agar or when highly diluted in liquid medium, leading to further underestimation of cell numbers (5, 7).

In the past, several methods such as Colour Changing Unit (CCU), plate counts [Colony Forming Units (CFU)], turbidity measurements, tetrazolium reduction to formazan, determination of cellular ATP concentrations by luciferin-luciferase luminometry and protein measurements have been proposed to determine growth rates of *Mollicutes* with different results (3, 5, 6, 8, 9, 10). These techniques have as main disadvantages that they are either unacceptable for some mycoplasma species and/or are time-consuming.

There is still a particular need for rapid methods of monitoring growth to enable the harvesting of

mycoplasma cultures at the peak of metabolic activity or viable count (8).

Recently, the use of flow cytometry in combination with fluorescent dyes to enumerate *M. hyopneumoniae* in broth medium gave promising results (11).

Flow cytometry is an optical system which permits rapid and highly sensitive direct estimation of particle or cell numbers present in a suspension (12). It allows the rapid measurement of light scattered and fluorescence emission produced by suitably illuminated cells (13).

The aim of this study was to discriminate and enumerate several species of mycoplasmas, *M. agalactiae* (Ma), *M. putrefaciens* (Mp), *M. capricolum* subsp. *capricolum* (Mcc), *M. bovis* (Mb), *M. capricolum* subsp. *capripneumoniae* (Mccp) and *M. hyopneumoniae* (Mh), in broth medium with Sybr Green I (SYBR) and flow cytometry. Flow cytometry analysis was validated with plate count (CFU) or CCU methods.

3. MATERIAL AND METHODS

3.1. Strains and culture conditions

Type strains of *M. agalactiae* (NCTC 10123), *M. putrefaciens* (NCTC 10155), *M. capricolum* subsp. *capricolum* (NCTC 10154), *M. bovis* NCTC 10131), *M. capricolum* subsp. *capripneumoniae* (NCTC 10192) and *M. hyopneumoniae* (NCTC 10110) were used in this study. Type strains were obtained from The National Collection of Type Cultures (NCTC, United Kingdom). All type strains were propagated in pH broth medium (14), with the exception of *M. hyopneumoniae* which was propagated in Friis broth medium (15), under aerobic conditions at 37ºC. A gyratory shaker (12 rpm) was used to grow *M. hyopneumoniae* cultures. Samples at 0, 6, 12, 24, 48, 72, 96, 120 and 144 h of the culture were taken to perform CCU (5) for *M. hyopneumoniae* cultures and CFU (4) for the rest of the mycoplasmas species. All strains were analysed by flow cytometry at the time-matched samples. All experiments were repeated at least three times on different days. All measurements were performed in triplicate.

3.2. Fluorescence labelling and flow cytometry analysis

Mycoplasma samples (100 µl) were diluted up to 1 ml in saline solution (0.85% NaCl) and stained with SYBR green-I (SYBR) (Amresco, Ohio, USA) at a final concentration of 1:10000 of the commercial stock solution (neither the molecular weight nor the chemical formula are provided by the manufacturer) for 15 min at room temperature in the dark. Saline solution was previously filtered through a 0.2-µm-pore-size filter (Millipore, Billerica, MA). Sample analysis was performed in a Coulter Epics XL-MCL flow cytometer (Coulter, Miami, FL, USA) equipped with an air-cooled 488 nm argon-ion laser. Each cell was characterised by two optical parameters. Side-Angle-Scatter (SSC) and green fluorescence for SYBR (525 nm, FL1 detector) were acquired in a four-decade logarithmic scale. Green fluorescence from SYBR was collected combining a 550 dichroic long filter and a 525 band pas filter. Optical alignment was based on an optimised signal from 10 nm fluorescent beads (Flow-check, Beckman-Coulter Inc., Fullerton, CA, USA). The discriminator was set on green fluorescence (FL1). For absolute counts we used the Coulter Fix Volume System analysis (samples were analysed for 1 min at a rate of 20 µl min^{-1}). Before FC analysis, to avoid coincidence, the samples were diluted between 4 and 4000 times in filtered 0.85% NaCl, in order to maintain the rate below 2000 events per second. The number of cells counted was then converted to cells/ml. Data were analysed with the SYSTEM II software (Coulter, Miami, FL, USA) and the WinMDI software version 2.8 (Joseph Trotter, The Scripps Research Institute La Jolla, CA, USA).

4. RESULTS

In this study, optical parameters (SSC and FL1) were obtained from SYBR-stained mycoplasma cells of the reference strains of Ma, Mp, Mcc, Mccp, Mb, and Mh, grown for different time-periods (0-144 h) and compared with time-matched control samples containing culture medium alone (for clarity only data from 144 h control media are depicted in Figure 1). Results show that mycoplasma cells stained with the nucleic acid-specific SYBR dye were easily distinguished from background noise of broth medium (Figure 1).

Figure 2 shows the different growth rates obtained with the different mycoplasma species studied. A good correlation between cell counts determined by the traditional plate count (CFU) or CCU methods and FC method was observed during the different growth phases of the cultures.

In Figure 2, it can be observed that Ma growth curve reached its peak level at 72h (of the order of 10^9 cells ml^{-1}) and started its senescence phase after 96h, although the values obtained between 24 and 72h were very similar. On the other hand, the growth curve of type strains of Mp, Mb and Mccp simultaneously reached their peak levels at 48h (of the order of 10^9 cells ml^{-1}), but had different stationary and senescent phases. While Mccp and Mb growth curves started the senescence phase at 96h, Mp started to decrease at 120h (Figure 2). Finally, Mcc and Mh growth curves reached peak levels at 24h, but while Mcc counts slightly decreased after 48h, Mh had a stationary phase from 24 to 72h and a marked senescence phase after 96h (Figure 2).

From the data presented in this study, it can be concluded that Mp and Mcc were the strains that better survive in the broth medium since they had only a slight decrease after the stationary phase. on the other hand Ma, Mb, Mccp and Mh growth curves were characterized by a marked decrease in the senescence phase, which indicates a lower survival rate in broth medium (Figure 2). Mh seemed to be the mycoplasma species with the lowest survival rate in broth medium, denoted by the highest decrease of counts (FC or CCU counts) after 72h (Figure 2). This can also be appreciated by the evolution of mycoplasma populations on

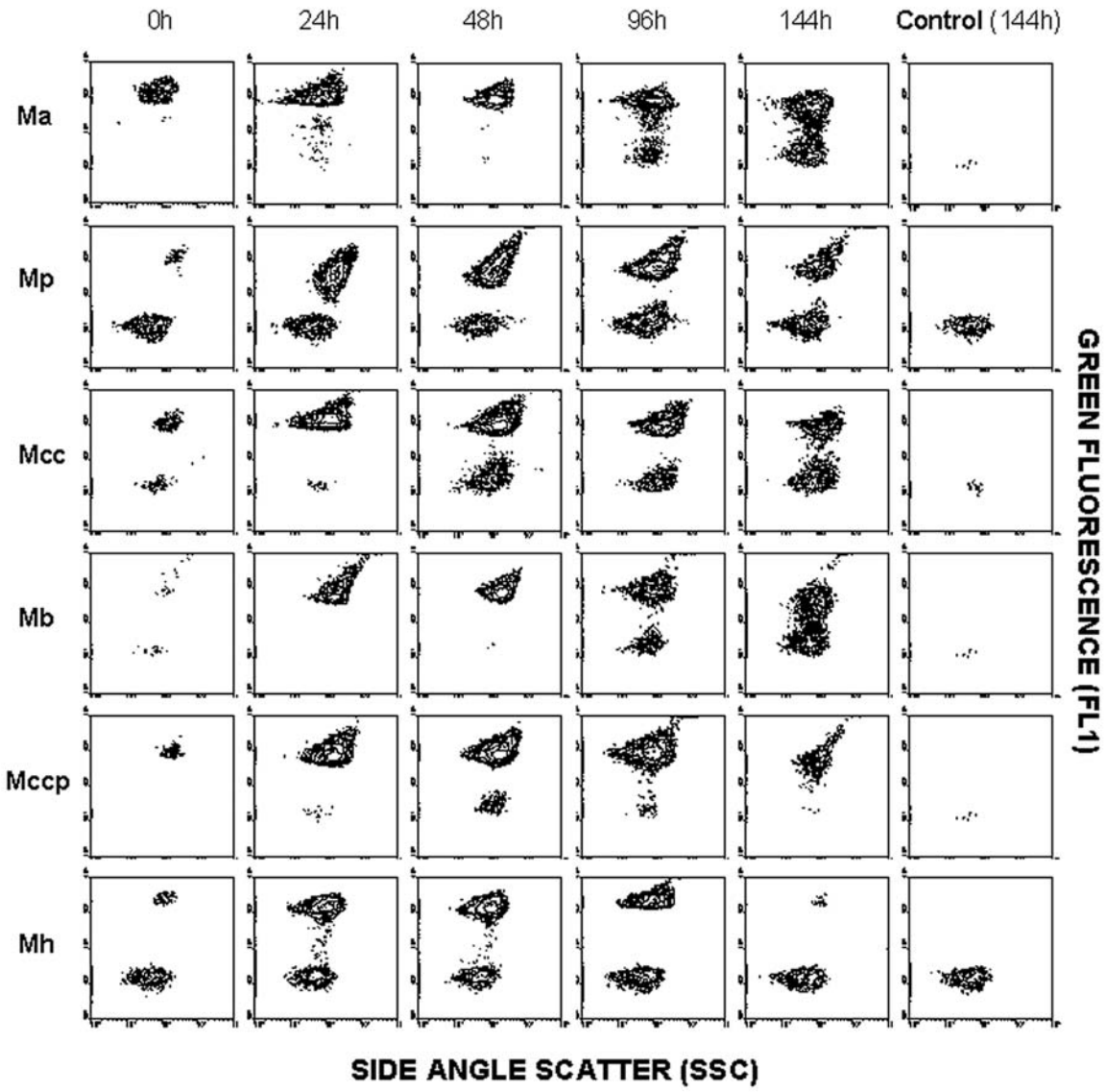

Figure 1. Dual parameter contour plot of Side Angle Scatter (SSC) versus Green Fluorescence (FL1) of Ma (*M. agalactiae*), Mp (*M. putrefaciens*), Mcc (*M. capricolum* subsp. *capricolum*), Mb (*M. bovis*), Mccp (*M. capricolum* subsp. *capripneumoniae*) and Mh (*M. hyopneumoniae*) growth curves. Mycoplasma cells were stained with SYBR. Mycoplasma cells were differentiated from the background noise by the high intensity of green fluorescence. Broth medium was analysed as the time-matched samples (Control). The hours of incubation are indicated in each histogram.

dual parameter histograms of Figure 1. For example, in the histogram which corresponds to the FC analysis of 144h sample, the Mh population has been highly reduced (when compared to the other histograms of the other time-matched samples of this species).

Furthermore, on dual parameter histograms of the growth curves of Ma, Mcc and Mb, a population of lower fluorescence intensity was observed at 96, 48 and 96h, respectively, which was maintained until the end of the experiments (Figure 1).

FC counts correlated well with plate count (CFU) or CCU methods. A correlation (*r)* of 0.841, 0.981, 0.960, 0.913, 0.954, and 0.844 was obtained for Ma, Mp, Mcc, Mb, Mccp and Mh, respectively. Nevertheless, although the mentioned methods correlated well in all phases of the different growth curves, in the senescence phase of Ma, Mb and Mccp growth curves, lower values were obtained with the plate count (CFU) or CCU method with respect to FC method (Figure 2).

Finally, it is important to mention that results obtained with plate count (CFU) method took 24h for Mcc,48h for Mp and 72h for Ma, Mb and Mccp, whereas results with CCU method took 15 days for Mh. In comparison, the FC method took only 20-30 min, including the staining step with SYBR.

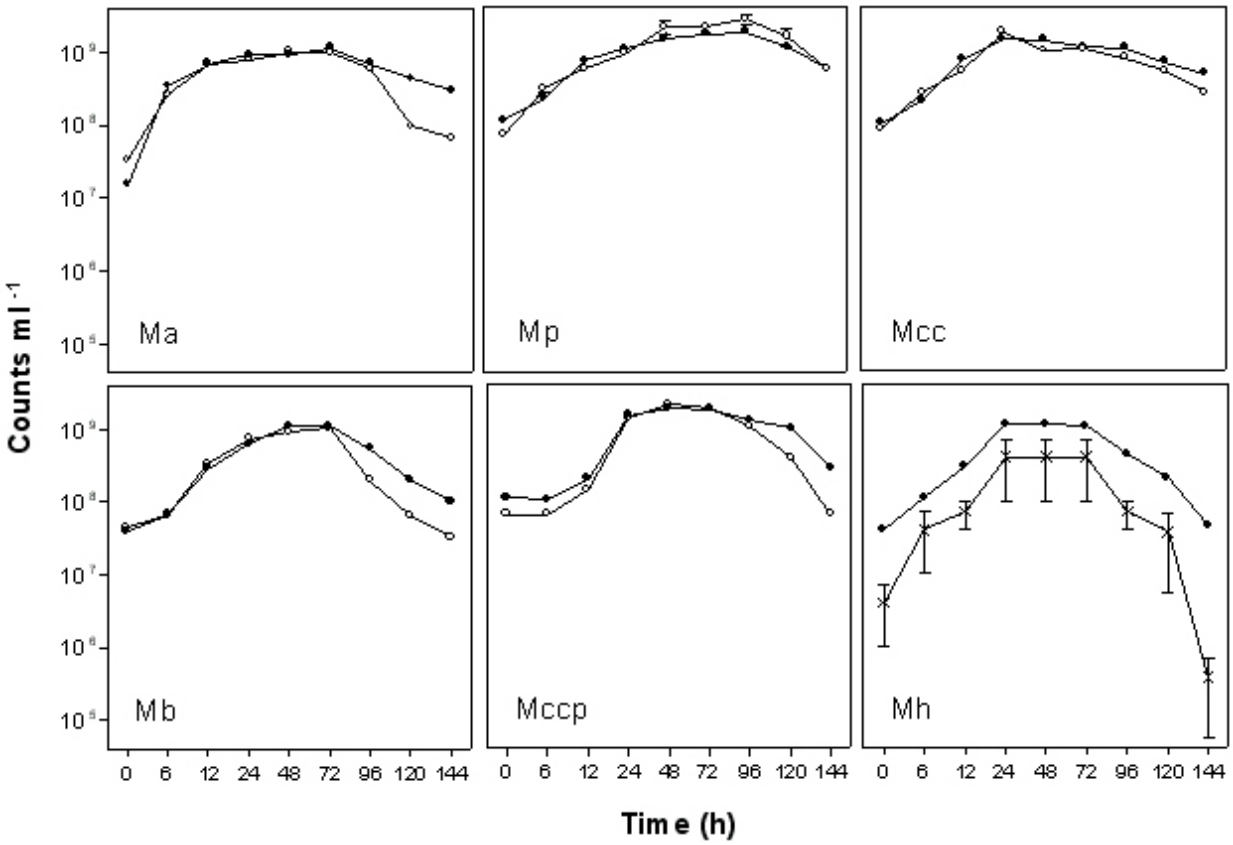

Figure 2. *M. agalactiae* (Ma), *M. putrefaciens* (Mp), *M. capricolum* subsp. *capricolum* (Mcc), *M. bovis* (Mb), *M. capricolum* subsp. *capripneumoniae* (Mccp) and *M. hyopneumoniae* (Mh) growth curves in broth medium measured by Flow Cytometry (cells ml^{-1}), plate counts (CFU ml^{-1}) or Colour Changing Units (CCU ml^{-1}).
FC counts (-• -), CFU (-o-) and CCU (-x-).

5. DISCUSSION

Microbiology is important to both human health and industry, and therefore many methods have been developed to count as well as identify micro-organisms in living organisms and enviromental studies (16). With flow cytometry, one must first differentiate the bacteria from other particles in a sample, which becomes more difficult as the sample contains increasing amounts of inorganic or organic particulates in the microbial size range (17). Microbial detection and discrimination can best be achieved by using DNA specific stains in combination with intrinsic light scatter measurements. Special care must be taken to avoid interference by DNA fragments and micelles that can stain non-specifically (16, 18).

In this study SYBR was able to differentiate the mycoplasma cells of all mycoplasma species studied from the background noise. Furthermore, SYBR alone was able to detect the logarithmic, stationary and the senescence phase of all strains tested. FC counts together with SYBR correlated well with plate count (CFU) and CCU methods, although in the senescence phase of the growth curve of three mycoplasma species (Ma, Mb and Mccp), lower values were obtained with the plate count (CFU) or CCU with respect to FC counts. This can be due to the otherwise so called "viable but not culturable" cells that can remain undetected by growth based methods (16, 19, 20). Classical microbiology states that a cell is said to be viable when it has been shown to reproduce and a demonstration of this has become the "gold standard" for proof of cell viability (16). All microbial systems that rely on cell replication are limited by our own ability to grow bacteria in an artificial environment (16, 18). So, it is possible that the plate count (CFU) and CCU methods, classified here as the "gold standard" methods, are the ones that are giving misleading information (21).

Furthermore, other methods like determination of lactate dehydrogenase (LDH) activity in lysed cells (22) and tetrazolium reduction (8) by *M. mycoides* subsp. *mycoides* small-colony type, also gave lower correlations in the stationary and senescence phases of the growth curves when compared with viable counts (CFU).

Nevertheless, on dual parameter histograms of the growth curves of Ma, Mcc and Mb, a population of lower fluorescence intensity was observed after 96, 48 and 96h, respectively (Figure 1). This is in agreement with other authors who have used SYBR dyes, and have observed that this fluorochrome was able to distinguish between bacterial

cells with high nucleic acid and low nucleic acid contents (20, 23). It was suggested that the nucleic acid content alone could be a good indicator of the fraction of growing cells (19, 20).

Regarding Mh growth curve, a better correlation was obtained between FC and CCU method, with respect to that reported previously (11). This is due to the fact that we had set the discriminator of the flow cytometer to green fluorescence. In the previous work, flow cytometry anakysis of cells with the discriminator set on FSC (Forward-Angle-Scatter) which is known to be related with particle size (17). This can have limited the mycoplama detection by FC due to the mycoplasma's small size (2).

Traditional count methods like CFU and CCU, although routinely used in laboratories for estimation of mycoplasma cell number in broth medium, are tedious and time-consuming. In our study, plate count (CFU) method took from 24 to 72h, depending on the mycoplasmas species, and CCU method took 15 days. This is too long when knowledge of the exact number of mycoplasma cells in a culture is required and decisions about a process are to be made, such as during vaccine production, industrial fermentation, animal or cell experimental inoculations, or diagnostic assays like antibiotic sensitivity, metabolism and growth inhibition. In contrast, FC together with SYBR, gave results in just 20-30 min, which is extremely useful, especially for enumerating the mycoplasma species such as *M. hyopneumoniae* where the traditional methods take too long,.

SYBR is a low cost dye that can be obtained from different commercial laboratories, whereas, SYTO dyes, used in previous experiments (11) can only be obtained from Molecular Probes (AA Leiden, the Netherlands) and at a higher cost. SYBR is a very sensitive fluorochrome that binds specifically to nucleic acid and has an extremely high affinity to bind to DNA (19). SYBR has been successfully used to enumerate both bacteria (20, 23, 24, 25) and viruses (26, 27, 28).

It can be concluded that flow cytometry is a very powerful technique to discriminate and enumerate mycoplasma cells in broth medium in "real time", which can replace the traditional time-consuming plate count (CFU) and CCU methods. We believe that it can be applied to any species of mycoplasma.

6. ACKNOWLEDGMENTS

This work was supported by Ministerio de Educación y Ciencia (AGL 2003-07526) and by Gobierno de Canarias (IDT-LP-04/016). We thank Dr. Anthony Ellis (UK) for revising the English language of the manuscript and Esther Díaz and Maria de los Reyes Suarez for its technical assistance.

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Key Words: Flow Cytometry, Mycoplasma, Sybr green-I, enumeration, CFU

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