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Applications of flow cytometry to mycoplasmology

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1. ABSTRACT

Flow cytometry has become a valuable tool in different fields of microbiology, such as clinical microbiology, aquatic and environmental microbiology, food microbiology, and biotechnology. It combines direct and rapid assays to determine numbers, biochemical and physiological characteristics of individual cells, revealing the heterogeneity present in a population. This review focuses on the applications of flow cytometry to the field of mycoplasmology. It tries to give a scope of the important breakthroughs which occurred in this field in the last decades, and in the advantages of introducing flow cytometry in research and routine diagnostic procedures of mycoplasmas.

2. INTRODUCTION

Flow cytometry is a sensitive, specific and rapid technique which can be used to analyse a large number of cellular parameters at the same time (1). In recent years, researchers in different fields have understood and recognised the scope offered by flow cytometry in studies of microbial cell populations on a single cell level, instead of in bulk by biochemical levels (2, 3).

Advances in the applications of flow cytometry to mammalian cells facilitated the emergence of simpler, cheaper and more sensitive equipment, which was essential for the spread of this technology in microbiology laboratories (2). In this context flow cytometry has been successfully applied to several fields of microbiology. As some examples, flow cytometry has been used to study drug and antibiotic susceptibility, discrimination and identification of microbial cells by using light scattering and fluorocromes, antibodies, or fluorescent oligonucleotides, assessment of bacterial viability and analysis of its physiological state. All of these issues are well covered in the following reviews, which document the use of flow cytometry to general microbiology (2, 4, 5, 6, 7, 8, 9) clinical microbiology (10), aquatic and environmental microbiology (11, 12, 13, 14), food microbiology (15), and biotechnology (3, 16).

Although the number of publications on flow cytometry applied to different microorganisms has increased, as well as specific courses and conferences, it seemed that the mycoplasmology field has been left aside, since only in the review published by Fouchet *et al.* (2)

have mycoplasmas been mentioned. Although publications on studies of mycoplasmas by flow cytometry have not increased on an equal basis with the rest of the microbiology fields, it is certain that in the last decade some effort has been made to improve this issue.

The aim of this review is to highlight the important breakthroughs that have occurred in the field of mycoplasmology and flow cytometry, and in the advantages of introducing this technique in research and routine diagnostic procedures of mycoplasmas.

3. FLOW CYTOMETRY APPLIED TO MYCOPLASMAS

3.1. Interaction of mycoplasmas with other systems

Considerable advances have been made toward a better understanding of mycoplasma pathogenesis. Most impressive are the findings concerning the interaction of mycoplasmas with the immune system, macrophage activation, cytokine induction, mycoplasma cell components acting as superantigens, and autoimmune manifestations. The demonstration of the ability of mycoplasmas to enter host cells and the possibility that several human mycoplasmas act as accessory factors in the activation of AIDS played a role in intensifying research on mycoplasma pathogenesis, bringing more researchers into the circle of those interested in this group of organisms (17).

For this reasons, the most usual application of flow cytometry to mycoplasmology has been the study of the interaction of mycoplasmas with eukaryotic cells and the immune system, in order to elucidate the pathogenic mechanisms of mycoplasma infections, rather than the direct application of flow cytometry to the study of mycoplasma cells themselves.

In this context, flow cytometry has been applied to study the response of the immune system to vaccines (18) and experimental infections (19, 20) with different species of mycoplasmas.

In particular, some studies, based on confocal microscopy and flow cytometry of fluorochrome-labeled mycoplasmas, revealed that *M. penetrans*, *M. pneumoniae*, and *M. genitalium* entered the intracellular spaces and were located throughout the cytoplasm and perinuclear regions of cultured human cells (21).

Cheek *et al.* (22) reported the *in vitro* detection of *M. fermentans* binding to B-lymphocytes in fresh peripheral blood using a polyclonal antibody and flow cytometry. At the same time, Shibata and Watanabe (23) observed that *M. fermentans* enhances concanavalin A-induced apoptosis of mouse splenic T cells, and (24) that spiralin, a mycoplasmal membrane lipoprotein, induced T-cell-independent blastogenesis of murine B-cells, and secretion of proinflamatory cytokines. Furthermore, it has been shown by flow cytometric analysis that a soluble recombinant fusion protein (FP29), representing the abundant P29 surface lipoprotein of *M. fermentans*, binds human HeLa cells and inhibits *M. fermentans* binding to these cells (25).

Ferronha *et al.* (26) reported that *M. mycoides* subsp. *mycoides* Small Colony-type did not affect neutrophil integrity and not even impaired their ability to be stimulated. More recently, Vanden Bush et al (27) by using flow cytometry, reported that *M. bovis* induced apoptotic death of bovine lymphocytes and that the death was not dependent upon the addition of apoptotic inducers as shown with other mycoplasmas.

Furthermore, flow cytometry has been used to study the interaction of *M. arthritidis* superantigen with human T-cells (28, 29) and whole blood cultures (30). It was demonstrated that activation of the immune system with *M. arthritidis* superantigen *in vivo* did not increase joint inflammation or cartilage degradation (31). Moreover, it was reported that mycoplasma-infected cells secrete HIV-1 at a higher rate, have a slight increase in cell surface expression of lipoproteins, and are less sensitive to immunotoxins than uninfected cells (32).

3.2. Detection of *Mycoplasma* contamination in cell cultures

Mycoplasma contamination is known to be a major problem in the cultivation of continuous cell lines. These contaminations have a multitude of effects on cultured cell lines that can potentially influence the results of experiments and pollute bioactive substances used in human medicine (33). Mycoplasma can influence any parameter and activity of the cell line, such as cell growth rate, cell morphology, cell response to stimulation and intracellular signalling (34, 35, 36). Therefore, mycoplasma infected cell cultures cannot be used for experimental or diagnostic purposes.

It has been reported that about 15–35% of all cell lines are infected with a limited number of mycoplasma species of predominantly human, swine or bovine origin. Most of the cultures are infected with *Mycoplasma fermentants*, *M. hyorhinis*, *M. orale*, *M. arginini*, *Acholeplasma laidlawii* and *M. hominis* (35). Infection of cell cultures by mycoplasma is persistent, difficult to be detected and to be cured, in part because mycoplasma contamination is not obvious, either macroscopically or microscopically, especially at the early phase of infections (37).

The specific, sensitive, and reliable detection of mycoplasma contamination is an important part of mycoplasma control and should be an established method in every cell culture laboratory (38). Mycoplasma contamination can be examined by numerous techniques such as classical microbiological culture assay on agar plates, DNA staining, RNA hybridization, PCR assays, ELISA assays (37). However, performing these assays, although some of them take only a few hours, is still time-consuming and represents a delay in many experiments.

Taking into consideration these statements, there is still the need to develop methods which can be

performed in parallel with the designed experiments and do not require extra time, in which flow cytometry can have an important role.

In 1990, Monsigny *et al.* (39) described the synthesis of a new benzoxazinone derivative suitable to detect early infection of cultured cells with mycoplasmas. This molecule was coupled to kanamycin A, leading to a cationic fluorescent probe which fluoresces at 600 nm upon excitation at 490 nm. This fluorescent probe was shown to heavily label the glycocalyx of all the mycoplasma strains tested, which are found to be associated with contaminated cultured cells and allowed a rapid detection of mycoplasma contamination by flow cytometry.

More recently, another rapid, simple, inexpensive and reliable flow cytometry method was described for the routine testing of mycoplasma contamination of a mouse macrophage-like cell line J774.2. This flow cytometry method was based on the observations that mycoplasma contamination had a marked effect on expression of CD14 and CD80 molecules (37). Importantly, these phenotypic changes can be detected already in the earliest phases of mycoplasma contamination, and are thus ideal when cell line assays are followed using direct immunofluorescence staining with a panel of specific monoclonal antibodies (MHC class II, CD11a, CD11b, CD14, CD16 + 32, CD21 + 35, CD45, CD71, CD80, CD86, F4/80, MOMA-2) and flow cytometric analysis (37), which is a currently used approach in experiments with culture cell lines.

3.3. Detection and differentiation of *Mycoplasma* species with antibodies

Mycoplasma infections and their management and control have considerable economic impact on animal production. One of the reasons for developing methods for *Mycoplasma* identification based on flow cytometry is to reduce the diagnostic time required, since accurate and rapid diagnosis of *Mycoplasma* infections would help in eradication, isolation, and treatment of the organisms and would be of great benefit to the industry and to researchers.

Mycoplasma isolates from chicken are usually identified by a fluorescent antibody technique that requires 7 to 10 days from sampling to the completion of identification (40). Previously, May et al. (41) were able to detect and distinguish *M. gallisepticum* and *M. symoviae* in broth cultures with specific FITC-labelled polyclonal antibodies by flow cytometry. Afterwards, the aim was to use the same approach to accurately diagnose infected birds (40). The flow cytometric procedure that used the same antibody-FITC conjugate reduced the diagnostic time required to only 3 or 4 days in comparision with the traditional fluorescence antibody technique (40). However, when samples collected from infected animals were immediately analysed by flow cytometry in the absence of multiplication in culture, the results were ambiguous. In this case, a wide range of fluorescent intensities was observed with no clear peak of fluorescence and no definitive diagnosis was possible (40). Only when cultures had changed colour, indicating organism multiplication in culture, was it possible to make a clear diagnosis by flow cytometry (39). Afterwards, May *et al.* (42) have been able to distinguish between two strains of *M. gallisepticum* using monoclonal antibodies and flow cytometry.

3.4. Studies on *Mycoplasma* antigen variability

The discovery that the minute mycoplasmas possess an impressive capability of maintaining a surface architecture that is antigenically and functionally versatile has placed the mycoplasmas in the “elite” group of bacterial pathogens and parasites distinguished by remarkable antigenic variability. This variability plays an important role in the adaptation of these fragile micro-organisms and helps them to survive on various mucosal tissues and also to evade the immune system (17, 43, 44, 45, 46, 47, 48).

The classical techniques used to detect variable expression of antigens are based on immunological reactions involving the transfer of colonies onto nitrocellulose filters and staining with monoclonal antibodies. This permits a qualitative evaluation of the variability that is materialised by portions of colonies that selectively express, or do not express, a specific epitope. However, it does not permit a quantitative analysis (47). The main advantage of flow cytometry is that it allows an easy and quantitative evaluation of the expression at the cell level by comparing multi-parameters, like fluorescence, size, and complexity.

Flow cytometry was used to study the epitope diversity of F strain *M. gallisepticum* with a specific FITC-labelled monoclonal antibody (MAb 6F10), after inoculation of leghorn hens (49). The authors suggested that the microenvironment of the colonization site in the hen induced epitope diversity in this strain, as evidenced by the loss in the expression of the MAb 6F19-defined epitope. This fact was further confirmed by the observations that after isolation of the organism from hens and propagation for several in vitro passages resulted in the re-expression of the epitope defined by the MAb.

Furthermore, the expression of *M. mycoides* subsp. *mycoides* Small Colony-Type surface antigen with a monoclonal antibody (117/5) was studied, and it was observed that the maximum expression of the epitope occurred at a late stage of culture (day 13), when mycoplasmas were no longer multiplying (47). The authors suggested that late cultures should be used for ELISA antigen production. The possible outcomes of flow cytometry are numerous (47): It offers an easy way to determine the accessibility of the epitopes at the surface of mycoplasmas; it should permit quantification of the expression of surface epitopes depending on the conditions and particularly depending on the medium composition, therefore enabling an improvement of the antigen yield; it will ensure a quantitative analysis of the antigens which are produced for diagnostic assays and facilitate its quality control; and it should also permit to monitor the quality of the antigens which are used for trials with inactivated antigens as it may be used to control a possible degradation of the antigen during the purification process.

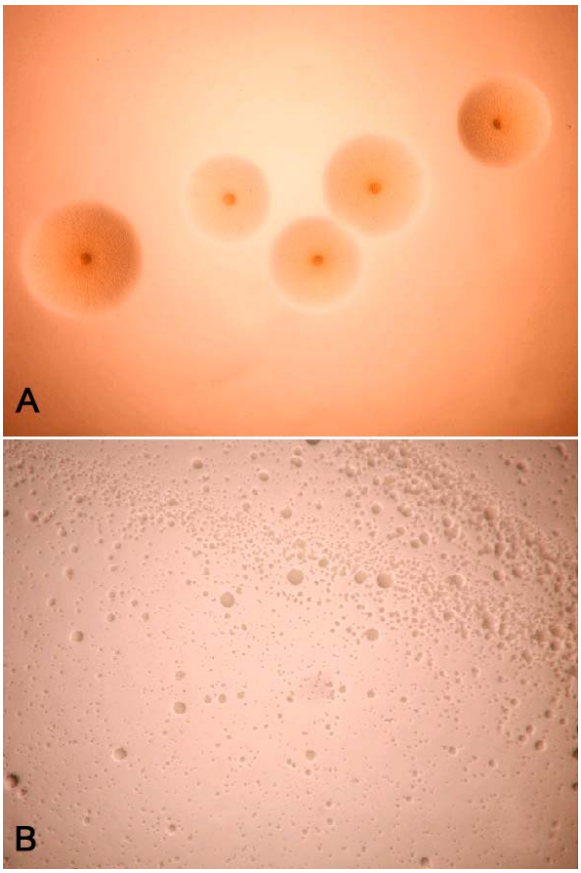

Figure 1. Mycoplasma colonies with the typical "fried-egg" morphology (A), and *M. hyopneumoniae* colonies which are characterised by the lack of the central area (B). Amplification: 40x.

3.5. Quantification of mycoplasmas in broth medium

Even though several techniques have been proposed to assess the number of cultured *Mollicutes* cells (50, 51, 52, 53, 54, 55) accurate determination of *Mycoplasma* growth still remains a difficult task (54).

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 under-estimated 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 under-estimation of cell numbers (52, 54).

There is 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 (55). Traditional count methods like plate count method [Colony-Forming Units (CFU)] and Colour-Changing Units (CCU), although routinely used in all laboratories for estimation of mycoplasma cell number in broth medium, are tedious and time-consuming, since they can take days or even weeks, depending on the mycoplasma species. This is too late when the knowledge of the exact number of mycoplasma cells in a culture is required, if decisions about a process are to be made, like in vaccine production, industrial fermentation, animal or cell experimental inoculations, or diagnostic assays like determination of antibiotic sensitivity, metabolism inhibition test, growth inhibition test.

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 (9). 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 (3, 16).

A recent study of *M. hyopneumoniae* growth in broth medium (56) has shown that analysis of these cells just by using light scattering was not enough to discriminate them from medium components and background noise. For this reason, fluorochromes Syto 9 and/or Propidium Iodide (PI) were used to stain nucleic acids of *M. hyopneumoniae* cells before flow cytometry analysis. Since *M. hyopneumoniae* strains exhibit a reduced ability to form colonies (Figure 1), the traditional CCU method was used to validate the flow cytometry method. It was demonstrated that a good relationship existed between growth estimations performed using the semi-quantitative CCU and flow cytometry method in the different phases of the growth curve (logarithmic, stationary and senescence phases). Labelling with Syto 9 alone was sufficient to differentiate *M. hyopneumoniae* cells with different amounts of nucleic acids, in the stationary and senescence phase of the *M. hyopneumoniae* growth curve (Figure 2), whereas, PI labelling did not detect cell death in the end phase of *M. hyopneumoniae* growth. It was suggested that cells with reduced fluorescence during the senescent phase could be attributed to the presence of nonviable or viable but not culturable subpopulations of cells with lower nucleic acid content. The major outcome of this study is that flow cytometry method allowed results in 20-30 min, while at least 15 days were necessary to obtain results with the traditional semi-quantitative CCU.

It was demonstrated that flow cytometry is a very useful, practical and fast technique to study the growth rates of *M. hyopneumoniae* in broth medium (56). These findings suggest that flow cytometry could be a good alternative to replace other time-consuming techniques that are currently used to enumerate mycoplasma in broth medium, such as plate count (CFU) and CCU method. Flow cytometry was found to be specially important if applied to slow-growing mycoplamas and/or mycoplasmas with a reduced ability to form colonies, like *M. hyopneumoniae*.

4. TECHNICAL CONSIDERATIONS

Flow cytometry of microorganisms presents several challenges that are not often encountered in measurements of mammalian cells. Bacteria are typically

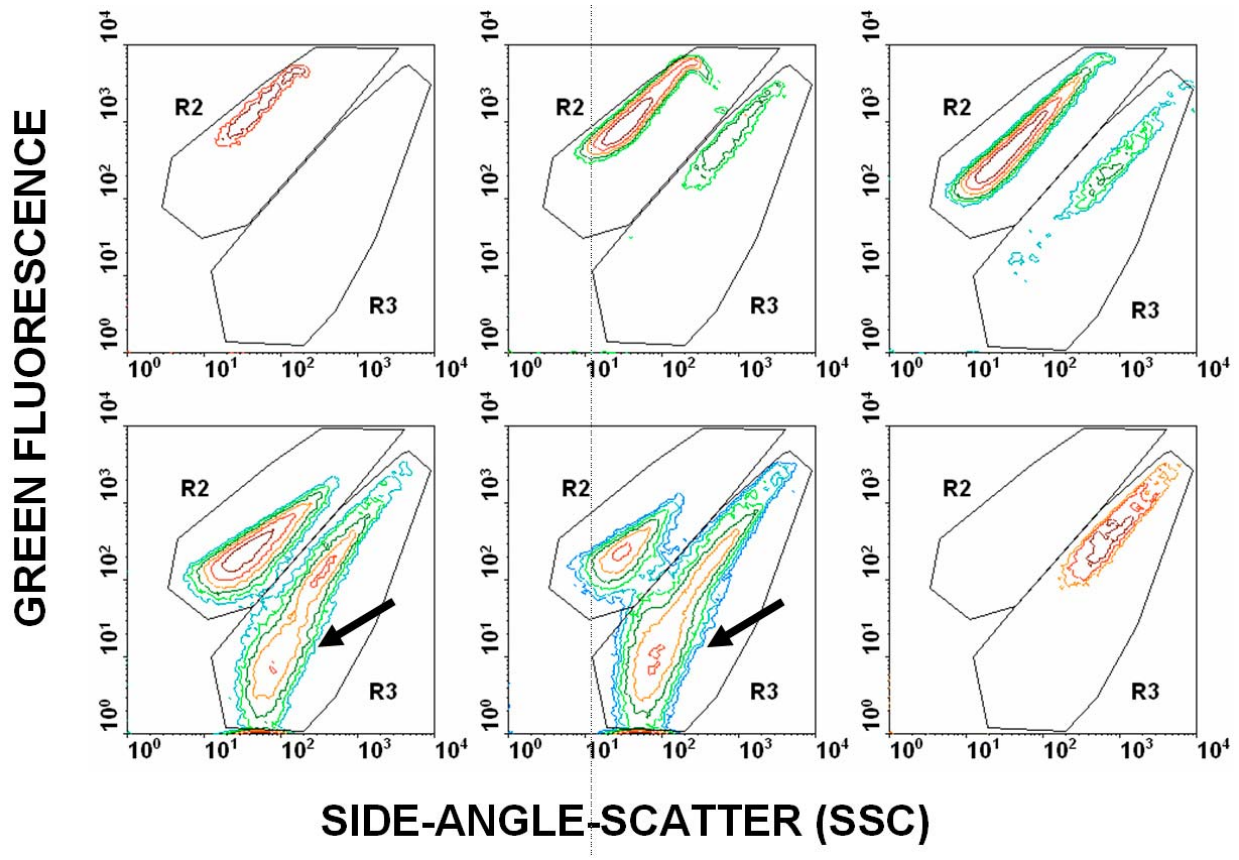

Figure 2. Dual parameter contour plot of green fluorescence (FL1) versus Side-Angle-Scatter (SSC) of *M. hyopneumoniae* cells (Type Strain). Cells were stained with Syto 9. From left to right and from top to bottom: samples colleted at 0, 24, 48, 72, 96 h and broth medium (control). R2 corresponds to *M. hyopneumoniae* cell and R3 to non-specific signals derived from the medium components. The arrows denote the appearance of a new population characterized by lower intensity of green fluorescence.

some three orders of magnitude smaller that mammalian cells with regard to cell volume as well as DNA and protein content (57). As regards mycoplasma, these differences are larger, resulting in the major problem for analysing mycoplasma by flow cytometry being undoubtedly its reduced size. Differentiation of mycoplasma from background noise or debris in the flow cytometer may be difficult if only light scatter parameters are used. For these reasons, mycoplasma detection and discrimination can best be achieved by using nucleic acids specific stains or labelled specific antibodies in combination with intrinsic light scatter measurements. In this case, the use of very bright fluorochromes like the SYTO and Sybr Green families can improve the sensitivity of the method (56, 58).

Due to mycoplasma's small size, instrumentation strategy is of great importance, since instruments have different ranges of application, depending on their quantitative and qualitative sensitivities. The quantitative limits, accuracy, speed of analysis, type of samples which can be analysed and light sources of these instruments are important selection criteria (6).

There are two types of flow cytometer, depending on the illumination source: those with a laser light source, and those with an arc lamp source. Each has its own advantages and disadvantages, but the main differences lie in their fields of application. Arc lamp cytometers are frequently used in microbiological applications due to their better scatter resolution and versatility. In contrast, laser flow cytometers have wider applications in immunology and hematology because they excite fluorochromes associated with cells (10). However, studies comparing the two types of cytometers have concluded that the selection of one rather than the other depends mainly on the range of wavelenghts required for the excitation of the selected florescent stains (10).

The other aspect that might make a difference when analysing small microorganisms like mycoplasmas is the type of flow cell of the flow cytometer. The flow cell consists of a conical nozzle that either produces a jet in air or leads into a rectangular tube (closed flow chamber or "cuvette"). The "jet in air" flow cell is preferable for fast and reliable sorting of cells, but it may increase the background level of the light scattering detectors, set a lower limit to the flow velocity, which in turn limits the increase in sensitivity that can be obtained by reducing flow velocity, and are more susceptible to disturbance from impurities in the orifice than are closed flow cells.

Futhermore, they are not compatible with immersion opticals, and because the orifice is usually smaller than the crossed section of closed flow cells, it is more prone to clogging, especially when running cells with a tendency to aggregate (57). The main disadvantages of the "jet in air" flow cell described here are avoided with closed flow cells ("cuvette"). On the other hand, a "jet on open surface" type (employed by a microscope-based flow cytometer) exhibits fewer interfaces than closed flow cytometers, and therefore gives rise to less stray light and a correspondingly lower background (59, 60).

In conclusion, flow cytometry offers the possibility to perform rapid and sensitive analysis on mycoplasma individual cells. Its ability to give real-time results is extremely important for the detection and quantification of mycoplasmas, especially of the slow growing ones. However, flow cytometry has some limitations, and the choice of the appropriate instrumentation together with very sensitive stains is important for the success of the application of interest. Furthermore, flow cytometry is not adapted to the detection of rare events since it is difficult to detect less than 100 bacteria per ml (6). In this context, other techniques such as Real Time PCR and reverse transcriptase Real Time PCR (61) which are characterised by a higher sensitivity, lower instrumentation costs and require less qualified personnel may be an alternative to flow cytometry. However, PCR-based methods do not allow single-cell analysis or the possibility to physically sort them as flow cytometry does.

5. PERSPECTIVES

The ability of flow cytometry to give information on cell size, macromolecular content, and cellular functions has given rise to new methods in microbial analysis. By comparison with the often lengthy and tedious classical methods of microbial analysis, flow cytometry analysis offers numerous possibilities for cell enumeration, determination of the physiological state of the cells, and rapid identification of microorganisms. Moreover, it can quantify the heterogeneities of microbial cell populations, which cannot be measured by classical biochemical methods (2).

Although flow cytometry has become a valuable tool in microbiology, it seems that mycoplasmology and flow cytometry are still in its beginnings. Nevertheless, mycoplasmology would have a lot to gain with the introduction of this technique, since flow cytometry would add sensitivity, specificity and rapidity in routine diagnostic and research procedures.

Mycoplasmas are the smallest and simplest self-replicating bacteria known, which are difficult to grow in artificial media, especially on primary isolation. In fact, the consensus is that only a small fraction of mycoplasmas existing in nature have been cultivated so far (17, 62). Taking into count these considerations, flow cytometry together with molecular probes, could have a great benefit in the detection and identification of uncultured mycoplasmas, similar to other uncultured bacteria (63, 64, 65).

It is very likely that in the future flow cytometry will become an essential tool for modern mycoplasmology, just as it has become indispensable in other fields, such as haematology, blood transfusion, transplantation and immunology (66) and more recently for different fields of microbiology.

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