Detection of Mycoplasmas in Goat Milk by Flow Cytometry

Patricia Assunção, Hazel M. Davey, Ruben S. Rosales, Nuno T. Antunes, Christian de la Fe, Ana S. Ramirez, Carlos M. Ruiz de Galarreta, Jose B. Poveda

Abstract
The detection of mycoplasma in milk can be performed by either culture techniques or polymerase chain reaction (PCR) based methods. Although PCR can reduce the average diagnostic time to 5 h in comparison with the several days for the isolation of the agent, there is still a need to develop methods, which could give earlier results. For this purpose, we tested the ability of flow cytometry (FC) to detect mycoplasmas in milk samples. Milk samples inoculated with four different mycoplasmas, Mycoplasma agalactiae, Mycoplasma putrefaciens, Mycoplasma capricolum subsp. Capricolum, or Mycoplasma mycoides subsp. mycoides large-colony type, known to cause contagious agalactia in goats, were stained with the DNA stain SYBR Green I and analyzed by FC. Three goat milk samples, from which mycoplasmas have been isolated in broth medium were also analyzed. All mycoplasmas were easily distinguished from debris of milk samples, but it was not possible to distinguish between the different mycoplasma species. In our conditions, the detection limit of the technique was of the order of 10^3–10^4 cells ml⁻¹.

Furthermore, mycoplasmas were also distinguished from Staphylococcus aureus. FC together with SYBR Green I was able to distinguish between mycoplasma cells and debris present in milk samples and gave results in 20–30 min. This is an important first step in developing a robust, routine flow cytometric method for the detection of mycoplasmas in milk samples.

Key terms
mycoplasma; flow cytometry; SYBR Green I; contagious agalactiae; milk

CONTAGIOUS agalactia of small ruminants is a disease that is notifiable to the World Organization for Animal Health (OIE, old B list) and is responsible for causing severe economic losses in goat- and sheep-farms. It has a worldwide distribution, being endemic in the Mediterranean area and in certain African and Asian regions (1–3). The main causative agent of the disease in sheep and goats is Mycoplasma agalactiae, however, contagious agalactia can also be caused by Mycoplasma putrefaciens, Mycoplasma capricolum subsp. Capricolum, or Mycoplasma mycoides subsp. mycoides large-colony type (1). Contagious agalactia causes a variety of clinical syndromes like mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion (1,3). Taken together, this disease has been estimated to cause annual losses in excess of $30 million in European countries around the Mediterranean (4) and consequently methods for confirming suspected infections are required.

Early detection of infection, or the potential for infection, gives the opportunity to control further spread of the disease with vaccines or antibiotics (1).

The detection of mycoplasmas in goat milk can be performed by culture techniques or by polymerase chain reaction (PCR) based methods. Culture techniques are the most common but have as a major disadvantage the long time required to obtain results (several days), making them time consuming and labour demanding. In addition, problems can occur due to growth of contaminants that out-compete the more slowly-growing mycoplasmas. PCR based methods offer a substantial
advantage in that they reduce the average diagnostic time to about 5 h (6). Positive results from PCR assays enable a full investigation to take place, however negative results should not be considered definitive.

Flow cytometry (FC) is a sensitive technique, which avoids the need for culturing and can be both qualitative and quantitative (7). FC has been used to detect, enumerate and differentiate several bacteria with a combination of fluorescent stains, antibodies, or oligonucleotide probes (7–9). The detection, enumeration, and determination of viability of mycoplasmas in laboratory culture have been demonstrated (10,11), however, we are not aware of any efforts to detect, enumerate, or differentiate mycoplasmas in milk samples by FC.

The aim of the present study was thus to develop a method based on FC in order to detect total mycoplasmas in goat milk samples.

Materials and Methods

Strains and Culture Conditions

The reference strains of M. mycoides subsp. mycoides large-colony type (LC), M. agalactiae (Ma), M. putrefaciens (Mp), and M. capricolum subsp. capricolum (Mcc) were obtained from the National Collection of Type Cultures (NCTC, United Kingdom). Furthermore, a field strain of Staphylococcus aureus isolated from a clinical case (goat milk) submitted to the Department of Epidemiology and Preventive Medicine of the Las Palmas de Gran Canaria University (Spain) was also used to show that mycoplasmas could be differentiated from larger bacteria associated with similar infections. Mycoplasmas were propagated in PH broth medium under aerobic conditions (12) and S. aureus was propagated in Blood Agar Medium (Oxoid), both at 37°C for 24 h.

Milk Samples

Mycoplasmas [10^6–10^8 Colony Forming Units (CFU) ml^{-1}] and S. aureus (10^7 CFU ml^{-1}) were inoculated separately or together into goat milk (collected from healthy animals from Las Palmas Veterinary Faculty experimental farm, Spain), or an infant milk formula (Nestlé, Switzerland), which was reconstituted in sterile water following the instructions of the manufacturer. The infant milk formula was used in order to optimise the method. In addition, three goat milk samples that had been remitted to our lab for routine diagnostics, from which Mycoplasma spp. had been detected using culture-based techniques, were also analyzed by FC. The number of CFU was determined as described elsewhere (13).

Fluorescence Labeling and Flow Cytometric Analysis

Milk samples (100 µl) were diluted to 1 ml with sterile-filtered saline solution (0.85% NaCl) and stained (15 min at room temperature in the dark) with the cell-permeant DNA-fluorochrome Sybr green-I (SYBR, Amresco Inc., Ohio) used at a final concentration 1:5,000 (vol/vol) of the commercial stock solution. Neither the molecular weight nor the chemical formula are provided by the manufacturer, however it is reported to be [2-N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinoxilinium] (14). Before FC analysis, to avoid coincidence, the samples were further diluted between 4 and 4,000 times in a 0.85% NaCl solution, in order to maintain the flow-rate below 2,000 events s^{-1}.

Sample analysis was performed in a Coulter Epics XL-MCL flow cytometer (Coulter, Miami, FL) equipped with an air-cooled 488 nm argon-ion laser (15 mW output). Each cell was characterised by three optical parameters: Side-Angle-Scatter (SSC), Forward angle scatter (FSC) and green fluorescence for SYBR (525 ± 20 nm, FL1 detector) and data were acquired on a four-decade logarithmic scale. Green fluorescence from SYBR was collected combining a 550 dichroic long filter and a 525 band pass filter.

Optical alignment was based on an optimised signal from 10 µm fluorescent beads (Flow-check, Beckman-Coulter Inc., Fullerton, CA). For absolute counts we used the Coulter Fix Volume System analysis and the discriminator was set on green fluorescence (FL1). The number of cells counted was then converted to cells ml^{-1}.

Data Analysis

Data were analyzed with the SYSTEM II software (Coulter, Miami, FL) and the WinMDI software version 2.8 (Joseph Trotter, The Scripps Research Institute La Jolla, CA). SPSS (Statistical Package for Social Science) version 12.0 was used for the statistic analysis; data were analyzed by least squares linear regression.

Results

In this study, we investigated the potential of FC in combination with a fluorescent dye (SYBR) to rapidly detect and enumerate mycoplasmas in goat milk samples.

In these experiments, we inoculated the different species of mycoplasmas in the infant milk and in goat milk samples, before staining them with SYBR and performing flow cytometric analysis. Samples of each of the pure cultures of mycoplasmas were also analyzed by FC. Results show that mycoplasma cells stained with the nucleic acid-specific SYBR dye were easily distinguished from the debris that was present in the culture medium or the milk samples. Infant milk and mycoplasma cultures demonstrated less background noise than goat milk samples when analyzed by FC, however, even the higher background present in the goat milk did not interfere with the detection of mycoplasmas. Analysis of clinical samples known to contain mycoplasmas gave similar results (Fig. 1).

To assess the sensitivity of the FC method we performed additional experiments using tenfold dilutions of the different mycoplasma cultures in milk samples. Data derived from flow cytometric analysis using dual parameter contour plots of FL1 vs. SCC demonstrated that the detection limit for mycoplasma cells stained with SYBR was in the order of 10^3–10^4 cells ml^{-1}. A good correlation (r^2 = 0.96) was obtained between FC and plate count method.
Furthermore, in order to validate the FC method, a field strain of *S. aureus* was inoculated into milk samples to determine whether the method was capable of distinguishing mycoplasmas from other bacteria. As shown by the data in Figure 2, there is clear evidence that under these conditions mycoplasmas could be easily distinguished from *S. aureus* by the optical parameters FSC together with SSC and green fluorescence (SYBR) together with FSC or SSC. Using the flow cytometric method described results were obtained in <30 min, since, after a 20 min incubation with SYBR, the flow cytometric analysis takes approximately 1 min.

**DISCUSSION**

Techniques based on flow cytometric principles are routinely applied in the dairy industry for measuring the total bacterial count within a few minutes (7,8). However, so far, there aren’t any reports of flow cytometric methods for the detection of mycoplasmas in milk samples. This may be due to the fact that mycoplasmas are the smallest self-replicating microorganisms known (15), and this may have discouraged researchers from analysing these microorganisms using flow cytometric techniques. The small size and correspondingly lower concentrations of cellular constituents of mycoplasmas results in smaller optical signals that are more difficult to resolve from the background noise than those obtained from mammalian cells or even from larger microorganisms.

In this study, we demonstrate by flow cytometric analysis, that the four species of mycoplasmas (LC, Ma, Mcc, and Mp), known to cause contagious agalactia in goats, can be easily resolved against the background debris present in both infant formula and goat milk without any previous treatment of the samples. Furthermore, we show that the method is capable of distinguishing between mycoplasmas and *S. aureus*. In previous studies related to the detection of bacteria, researchers had to perform a pre-treatment of the milk samples before being analyzed by FC (7,8,16–20). Gunasekera et al. (7) reported that before *Escherichia coli* and *S. aureus* could be detected in milk samples, a prior treatment with protease K or savinase for UHT milk samples and of savinase plus Triton X-100 for raw milk samples was necessary. Initially, we investigated these protocols, but we found that these treatments also caused loss of the mycoplasma cells, thus preventing their detection by FC. Nonetheless, as shown above we have found that by using SYBR mycoplasmas can be readily detected without recourse to time-consuming pre-treatments and thus the FC-based assay is not only quicker than culture-based methods but also more rapid than PCR-based techniques.

*S. aureus* is the most frequently isolated pathogen known to be involved in mammary infections of goats (21–25). Consequently, to be a robust method, any protocol for identifying mycoplasma infection of milk must be capable of distinguishing between *Mycoplasma* spp. and *S. aureus*. As shown by the results in infant milk, the differentiation between mycoplasmas and *S. aureus* is possible due to the differences in size (FSC), complexity (SSC), and fluorescence due to SYBR staining (FL1). In the future it may be possible to improve the methods described here by the use of specific probes such as fluorescently labelled antibodies or oligonucleotides to effective diagnosis at the generic or species level. However, suitable probes that are both reliable and brightly fluorescent are not yet available commercially for this purpose. In any case, it is likely that an initial screen of the type described here would be a useful screening step prior to the use of more costly and time consuming specific assays.

In the analyses reported here, data were collected for 1 min and this corresponds to 20 µl of sample. This approach resulted in a detection limit of the order of $10^{3}$–$10^{4}$ cells ml$^{-1}$. The detection limit could be improved by analyzing a larger sample volume however it is unlikely that concentrations below 100 bacteria ml$^{-1}$ could be detected since FC is not adapted to the detection of rare events (26). Nevertheless, Cai et al. (27) found that when using real-time PCR to detect *Mycoplasma bovis* in clinical manifestations of bovine mastitis, the *M. bovis* culture-positive milk samples were estimated to contain between $5 \times 10^{5}$ and $7.7 \times 10^{8}$ cells ml$^{-1}$. These values are in the range of detection of mycoplasmas in milk samples by the FC method thus indicating that the sensitivity of the method is appropriate for diagnostic purposes.

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**Figure 1.** Dual parameter dot plot of green fluorescence (FL1) versus side angle scatter (SSC) of goat milk samples of three clinical cases from which *Mycoplasma* spp. was detected by cultured based techniques. SSC and FL1 data were acquired in a four-decade logarithmic scale. *Mycoplasma* spp. (M), Debris (D). FC *Mycoplasma* spp. cell counts: $1.1 \times 10^{7}$ counts ml$^{-1}$ (A); $8.1 \times 10^{7}$ counts ml$^{-1}$ (B); $9.6 \times 10^{8}$ counts ml$^{-1}$ (C).
Furthermore, it is expected that a slight underestimation of total counts will be obtained by the flow cytometric analysis method since mycoplasma clumps will be enumerated as one unit in FC analysis. This phenomenon also occurs with the traditional plate count method that is used for this purpose and with which we compared our flow cytometric results.

It is important to note that the flow cytometric method presented here does not distinguish between Mycoplasma species. This is not considered problematic from a veterinary viewpoint as knowledge of the species of mycoplasma involved in the infection process is not important for the establishment of adequate treatment and control measures.

**Figure 2.** Differentiation of cultures of mycoplasmas (a mix of the four species at a concentration of $10^7$ CFU ml$^{-1}$) from *S. aureus* ($10^7$ CFU ml$^{-1}$) in infant milk by different optical parameters (FSC, SSC, and FL1). FSC, SSC, and FL1 data were acquired in a four-decade logarithmic scale. Infant milk alone (A), mycoplasmas (B), *S. aureus* (C), mycoplasmas and *S. aureus* (D).
In conclusion, this study represents to our knowledge, the first step in developing a routine FC based method for the detection of total mycoplasmas in milk samples. We have demonstrated the ability of FC to discriminate mycoplasmas from other bacteria and from debris in milk. The main advantage of the flow cytometric approach, when compared with culture-based or PCR-based methods, is the increased speed with which results are available. The flow cytometric approach allows results to be obtained in 20–30 min. Since the microbiological content of raw milk affects quality, shelf life and safety of milk and dairy products (28), our development of a method for the detection of bacteria in milk that does not require time-consuming pre-treatments may have wider applicability in the dairy industry and for monitoring the general health status of livestock. Furthermore, the need for methods of screening herds for mycoplasma infection is likely to increase as a consequence of a perceived bioterrorism threat. Contagious bovine pleuropneumonia, a highly infectious disease of cattle and buffalo which is caused by \textit{M. mycoides} subsp. \textit{mycoides} small-colony type has been identified as a potential agroterrorism threat (29) and it is likely that the flow cytometric method would be appropriate for detection of this pathogen.

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**LITERATURE CITED**


