APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1986, p. 689–695
0099-2240/86/100689-07$02.00/0

Vol. 52, No. 4

Method for Flow Cytometric Detection of *Listeria monocytogenes* in Milk

CATHERINE W. DONNELLY[1]* AND GREGORY J. BAIGENT[2]

Department of Animal Science, College of Agriculture,[1] and Department of Medical Microbiology, College of Medicine,[2] The University of Vermont, Burlington, Vermont 05405

Received 21 April 1986/Accepted 8 July 1986

This report describes a method for the detection of *Listeria monocytogenes* in raw milk by flow cytometric analysis of fluorescently labeled bacterial populations. The use of immunofluorescence in combination with measures of DNA content by propidium iodide labeling and size by light scattering enabled specific identification of *L. monocytogenes* from *Streptococcus faecalis*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Staphylococcus epidermidis*, and *Staphylococcus hyicus*. Additional specific resolution of *L. monocytogenes* populations was achieved through selective enrichment of raw milk in *Listeria* enrichment broth. These procedures should permit the rapid screening of milk and other food samples for *L. monocytogenes* and eliminate many of the short-comings associated with conventional fluorescent-antibody procedures.

Listeria monocytogenes is a gram-positive, nonsporeforming, aerobic to facultatively anaerobic, rod-shaped bacterium which exhibits pathogenicity towards humans and other animals. Although not generally recognized as a foodborne pathogen, three recent outbreaks of listeriosis may indicate that this organism is becoming more prevalent as an agent of foodborne disease. The first documented outbreak of foodborne listeriosis occurred in Nova Scotia, Canada, in 1981. Cole slaw was implicated as the vehicle of infection. Case studies determined that cabbage used to prepare cole slaw had been grown in soil fertilized with sheep manure. Several sheep, which were sources of manure, had died of listeriosis prior to this outbreak (19). In a second outbreak which occurred between June and August 1983, 49 patients in Massachusetts were hospitalized with septicemia or meningitis caused by *L. monocytogenes*. The source of infection was traced to whole or 2% pasteurized milk. Fourteen deaths resulted from this outbreak (5). A third outbreak of listeriosis occurred in Los Angeles and Orange Counties, California, between January and June 1985. The infective vehicle linked to 29 deaths in this outbreak was Mexican-style cheese (11).

To monitor the incidence of *L. monocytogenes* in milk and food products and ultimately prevent future outbreaks of listeriosis from occurring, methods must be developed whereby *L. monocytogenes* can be rapidly identified. Current methods for the detection of *L. monocytogenes* are tedious, and often samples must be subjected to several months of cold enrichment prior to detection of *Listeria* spp. (7, 15, 18). No official methods exist for the isolation of *L. monocytogenes* from food products.

Khan et al. (14) described a fluorescent-antibody technique for the rapid identification of *L. monocytogenes* in milk and meat. However, because of nonspecific cross-reactivity of *Listeria* antisera with streptococci and staphylococci, contaminating organisms could be mistakenly identified as *Listeria* spp. To increase the specificity of fluorescent-antibody procedures for the rapid identification of *L. monocytogenes*, we investigated the feasibility of automating fluorescent-antibody techniques through the use of flow cytometry (FCM). FCM permits the rapid characterization of a cell population based on a number of parameters such as morphology, nucleic acid content, and surface antigenicity. Cells are treated with appropriate stains and passed rapidly in suspension on a cell-by-cell basis through a laser beam. By analyzing the interactions of each cell (light scatter, fluorescence) with the beam, a representation of the distribution of the desired parameter within the population is acquired. Microbiological applications of FCM have included the characterization of oral bacteria and etiologic agents of oral infections (1), the differentiation of bacterial species by nuclear base pair ratio measurements (22), the characterization of the intracellular accumulation of poly-β-hydroxybutyrate in *Alcaligenes eutrophus* (20), and the detection of *Legionella* spp. in cooling tower water (21). This paper describes our efforts to automate fluorescent-antibody techniques through FCM as a means to rapidly screen enriched raw milk samples for the presence of *L. monocytogenes*.

MATERIALS AND METHODS

Cultures. Strains of *L. monocytogenes* used in this study included serotypes most frequently isolated from raw milk (5). These strains were *L. monocytogenes* ATCC 19111 (serotype 1), ATCC 19114 (serotype 4a), ATCC 19115 (serotype 4b), ATCC 19116 (serotype 4c), ATCC 19117 (serotype 4d), and ATCC 19118 (serotype 4e), all from the American Type Culture Collection, Rockville, Md; and F5027 (serotype 1a) and F5069 (serotype 4b), both raw milk isolates, which were obtained from Robert Weaver, Centers for Disease Control, Atlanta, Ga. Other organisms used in this study were *Staphylococcus aureus* ATTC 25923, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus faecalis* ATCC 19433. These cultures were obtained as dehydrated disks from Difco Laboratories (Detroit, Mich.). *Streptococcus uberis*, *Streptococcus agalactiae*, *Staphylococcus hyicus* subsp. *hyicus* and *Staphylococcus hyicus* subsp. *chromogene* were isolated from quarter milk samples by J. S. Hogan and J. W. Pankey, University of Vermont, Burlington. All cultures were maintained at 4°C on tryptose-phosphate agar (Difco) slants.

Bacterial enumeration. Viable cell counts of *L. monocytogenes* and raw milk microflora were determined with tryptose-phosphate agar and plate count agar (Difco), respec-

* Corresponding author.

tively (3). Bacterial suspensions were diluted in Butterfield phosphate buffer and pour plated in duplicate. Tryptose-phosphate agar plates were incubated at 37°C for 48 h, while plate count agar plates were incubated at 32°C for 48 h.

Cell labeling procedures. *L. monocytogenes* was grown in tryptose-phosphate broth (Difco) for 16 h at 37°C prior to FCM analysis. At this phase, cultures were consistently present at levels ranging from 5.0×10^8 to 1.3×10^9 CFU/ml. A 2.5-ml aliquot of culture was transferred to sterile polypropylene culture tubes (12 by 75 mm) (Fisher Scientific Co., Medford, Mass.). Cells were sonicated at 20% power for 15 sc (Branson Sonic Power, Danbury, Conn.) to disrupt cell chains. Sonicated cells were centrifuged at 5,000 × *g* for 5 min at room temperature, using a Savant HSC 10,000 centrifuge (Savant Instruments, Farmingdale, N.Y.), and fixed in 1 ml of 95% ethanol for 15 min at room temperature. Cells were again centrifuged as described above and washed in 2.5 ml of 0.22-μm filtered phosphate-buffered saline (PBS; pH 7.2). The washed cells were suspended in 0.5 ml of a 1-mg/ml solution of RNase A (Sigma Chemical Co., St. Louis, Mo.) and incubated at 37°C for 15 min. Cells were again washed with PBS and then immunofluorescently labeled.

Two protocols were used to immunofluorescently label cells. For direct labeling, cells were suspended in 100 μl of fluorescein isothiocyanate (FITC)-labeled antilisteria polyvalent antisera types 1 and 4 (Difco) for 45 min at room temperature, washed in PBS, and suspended in 2.5 ml of PBS. Negative controls were prepared by incubating cells with FA rabbit globulin (Difco) instead of *Listeria* antisera.

For indirect labeling, cells were incubated for 45 min at room temperature with 100 μl of *Listeria* O antiserum type 1, *Listeria* O antiserum type 4, or polyvalent *Listeria* O antiserum (Difco). Cells were washed in PBS and suspended in 100 μl of FITC-labeled goat anti-rabbit immunoglobulins (immunoglobulins G, A, and M, 1:40 dilution; CalBiochem-Behring, La Jolla, Calif.). Cells were incubated for 15 min at room temperature, washed in PBS, and suspended in 2.5 ml of PBS. Negative controls for indirectly labeled cells were stained only with FITC-labeled goat anti-rabbit immunoglobulins.

To label cellular DNA, following immunofluorescent staining, 1 ml of propidium iodide (PI; 100 μg/ml in 1% trisodium citrate; Sigma) was added to cell suspensions in 2.5 ml of PBS. Cells were stored in PI at 4°C prior to analysis.

FCM. The instrument used in these studies was an Ortho 50-H/H Dual Laser Cytofluorograf equipped with an Ortho 2150 data handling system (Ortho Diagnostics, Westwood, Mass.). The argon laser (Coherent 90-5; Coherent, Inc., Palo Alto, Calif.) emitting 0.5 W of 488-nm light was used for characterization of all parameters. In experiments where DNA and surface immunofluorescence were studied simultaneously, red (PI) and green (FITC) emissions were divided by using a 560-nm dichroic filter (Omega Optics, Brattleboro, Vt.). Red fluorescence was then passed through a 625-nm Schott filter and green fluorescence was passed through a 530/30 barrier pass filter (Omega).

Prior to bacterial analysis and characterization, the instrument was calibrated by using 2.0-μm Fluoresbrite fluorescent beads (Polysciences, Warrington, Pa.) and standardized with a suspension of *Listeria* bacteria.

RESULTS

Bacterial gating and characterization. Initial phases of this study were devoted to the investigation of parameters which could serve to set gating regions permitting the differentiation of *Listeria* from non-*Listeria* contaminants. Preliminary trials combined forward angle light scatter and immunofluorescence as bacterial characterization and gating parameters. Although immunofluorescence was useful as a gating parameter, due to the small size of the organisms used in this study, forward angle light scatter was not capable of reliably resolving bacterial cells from noncellular material and electronic noise. A 90° scatter was a slightly more useful gating parameter, but when pure populations of *L. monocytogenes* were analyzed by this parameter, two distinct regions could be discerned on scatter diagrams (Fig. 1). Adjustment of flow rate from 100 to 2,000 cells per s did not change the ratio of cells appearing in each region. This would be expected if these regions were due to alignment of cells in the flow stream. Flow systems such as the 50-H/H orient particles by achieving a condition of laminar flow within the flow cell. Bacteria contained within a sample are confined to a narrow stream of sample fluid surrounded by a stream of sheath fluid. Under these conditions, particles are aligned with their long axis parallel to the direction of flow. This tendency is reduced as particle size decreases. Since the limit of the Ortho 50-H/H system to align cells is 5 μm this would explain why adjustment of flow rate of this system did not influence alignment of *L. monocytogenes*, whose size is 0.5 by 1.0 to 2.0 μm (7). This was confirmed when resorting each region of the scatter diagram (Fig. 1). Analysis of the sorted populations gave histograms identical to the unsorted population (data not shown). Due to the appearance of dual regions of cell populations on scatter diagrams, it was concluded that 90° scatter would not be useful as a gating parameter. In addition, pure cultures of *Listeria* spp. and other organisms examined gave very broad, overlapping forward angle light scatter distributions. A further problem arose from the inability to resolve single cells from doublets and clumps by using light scatter alone. Nonspecific trapping of antibody in negative controls resulted in unacceptably high levels of reactivity during early experiments (data not shown).

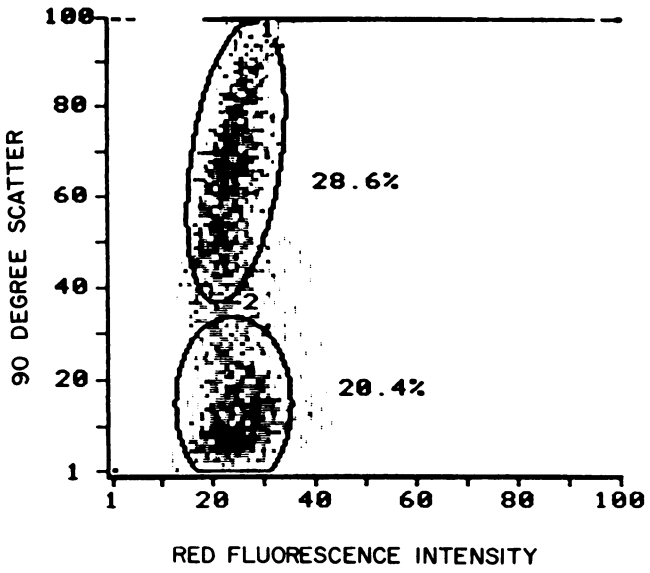

FIG. 1. Cytogram pattern of *L. monocytogenes* serotype 1 (ATCC 19111). Cells were grown in tryptose-phosphate broth for 16 h prior to use, fixed in 95% EtOH for 15 min, and labeled with PI prior to analysis.

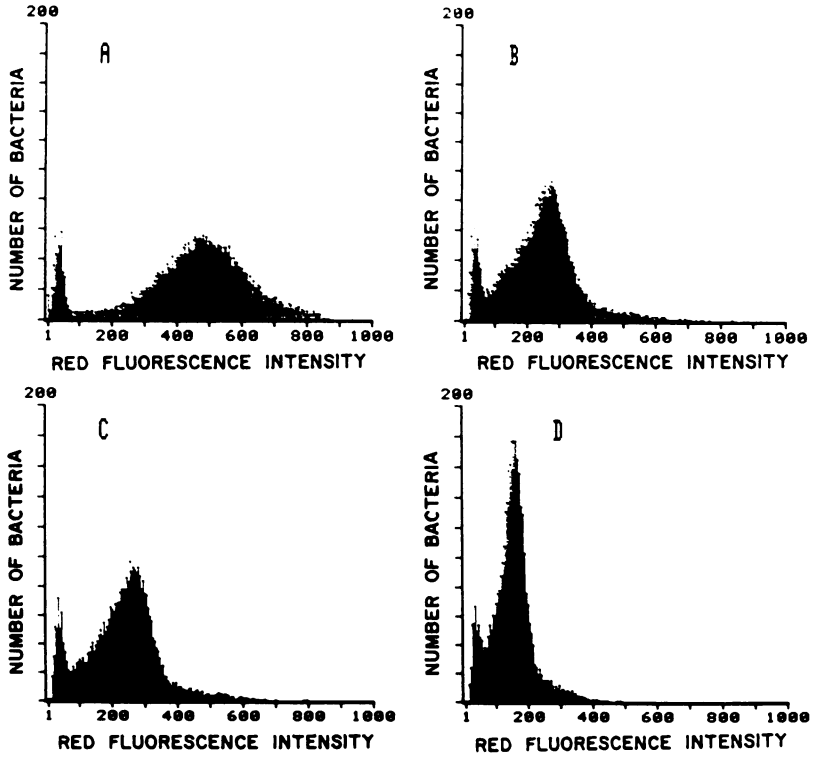

FIG. 2. Effect of RNase treatment on *L. monocytogenes* F5069. Parentheses indicate the mean ($\bar{x}$) and standard error of the population distribution: (A) no RNase, 15 min, 37°C ($\bar{x}$ = 482 ± 135.9); (B) 1 mg of RNase per ml, 15 min, 37°C ($\bar{x}$ = 259 ± 72.6); (C) 1 mg of RNase per ml, 30 min, 37°C ($\bar{x}$ = 253.3 ± 69.2); (D) 1 mg of RNase per ml, 15 min, 37°C, excess PI removed ($\bar{x}$ = 147 ± 36.3).

Due to these limitations and due to the requirement for a gating parameter which would enable the exclusion of contaminating organisms from analysis, the potential of cellular DNA measurement as a gating parameter was investigated. PI was used as the DNA stain because it can be measured in combination with FITC following excitation by a common light source. PI intercalates into all double-stranded nucleic acid molecules; therefore, cells must be treated with RNase prior to staining with PI to make this stain DNA specific. The effect of RNase treatment on DNA (red) fluorescence signals appears in Fig. 2. Cells without RNase treatment (Fig. 2A) displayed broad distributions and large standard errors of values around the peak mean. RNase treatment for 15 min (Fig. 2B) reduced the width of the DNA distribution and sharpened the DNA fluorescence signal. Since no differences were observed between 15 (Fig. 2B) and 30 (Fig. 2C) min of incubation with RNase, the shorter incubation period was chosen. To remove excess PI (Fig. 2D), cells were centrifuged and suspended in PBS immediately prior to sorting. This procedure sharpened the DNA fluorescence signal and sharply reduced the standard error of mean values of the DNA peaks. This was confirmed by statistical analysis of these values (Fig. 2).

Modifying the fluorescent labeling procedure by mildly sonicating the cells improved resolution of *Listeria* spp. Sonication settings and times were selected which maximized disruption of cell chains without decreasing cellular viability. Sonication was found to result in decreased background fluorescence and improved detection of individual cells when using DNA-associated fluorescence as a gating parameter.

Direct and indirect labeling methods were used to develop cell surface immunofluorescence. Although acceptable results were obtained by using direct methods, more specificity could be achieved when indirect labeling methods were used. With indirect labeling, primary antibody titer greatly influenced degree of positive fluoresence. For *L. monocytogenes* ATCC 19111 (serotype 1) (Fig. 3), a 1:2 dilution of primary antibody resulted in 52% of the population exhibiting positive fluoresence, while 1:20 and 1:40 dilutions resulted in 98 and 99% positive fluorescence, respectively. Similar results were observed with serotype 4b strains of *L. monocytogenes* (data not shown). At high primary antibody concentrations, precipitation of cells from the suspension

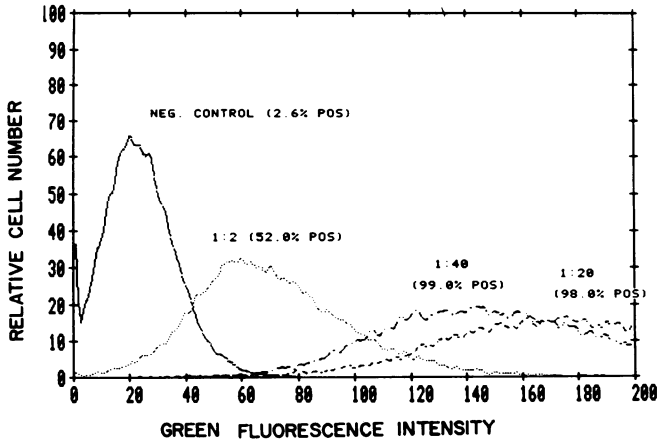

FIG. 3. Effect of antiserum dilution on immunofluorescence of *L. monocytogenes* serotype 1 (ATCC 19111). Cells were indirectly labeled (secondary antibody concentration, 1:40).

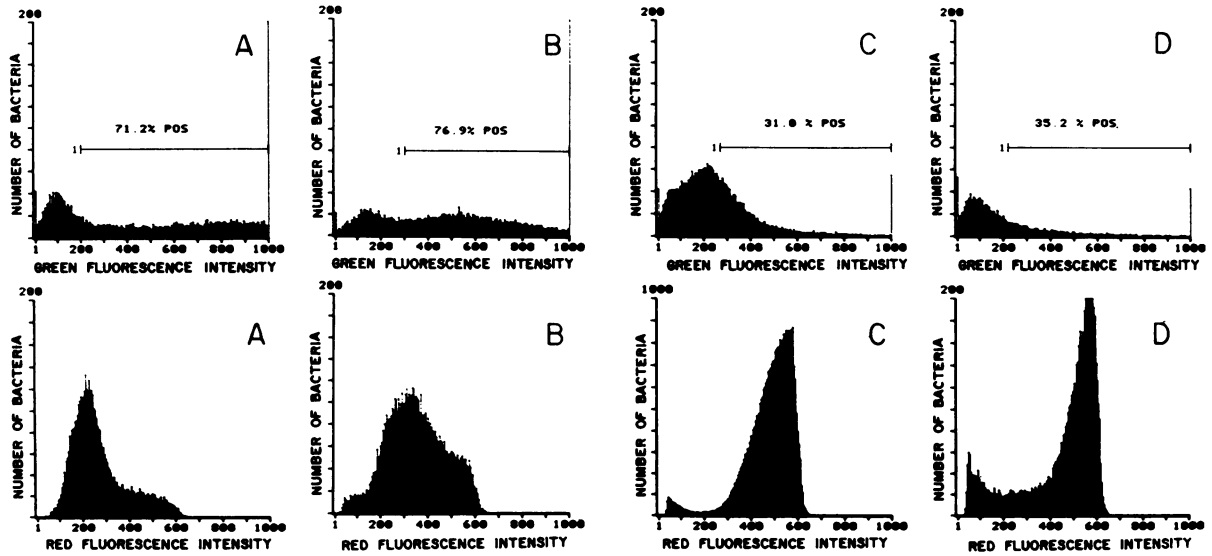

FIG. 4. Immunofluorescence (green) and DNA-associated fluorescence (red) profiles of (A). *L. monocytogenes* 5027 (serotype 1), (B) *Staphylococcus aureus*, (C) *Streptococcus faecalis*, and (D) *Streptococcus agalactiae*.

was frequently observed. For analysis of *L. monocytogenes* by FCM, it was necessary to determine the titers of each new lot of primary antibody suspension and routinely use the concentration which provided maximum fluorescence.

Detection of *L. monocytogenes* in milk by FCM. The goal of this study was to apply FCM to the detection of *L. monocytogenes* in raw milk. Khan et al. (14) developed a method for the immunofluorescent identification of *L. monocytogenes* in milk through fluorescence microscopy. These authors observed cross-reactivity of *Listeria* antisera with streptococci and staphylococci. Commonly isolated raw milk contaminants were therefore labeled with *Listeria* antisera to determine the extent of nonspecific reactivity of these cells. Figure 4 compares DNA-associated fluorescence (red) and immunofluorescence (green) profiles of *L. monocytogenes* with those of common raw milk bacteria. Based on DNA fluorescence profiles alone, *Streptococcus faecalis* (Fig. 4C) and *Streptococcus agalactiae* (Fig. 4D) could be differentiated from *L. monocytogenes* (Fig. 4A) and showed only slight cross-reactivity with *Listeria* antiserum. Similar results were observed for *Staphylococcus hyicus* subsp. *hyicus*, *Streptococcus uberis*, *Staphylococcus hyicus* subsp. *chromogene*, and *Staphylococcus epidermidis* (data not shown). *Staphylococcus aureus* proved to be a problem, however, with DNA distributions falling in the region of *Listeria* DNA distributions and cells exhibiting high degrees of cross-reactivity with *Listeria* antibodies (Fig. 4B).

To prevent *Staphylococcus aureus* and other contaminating microflora from obscuring the detection of *L. monocytogenes* in milk samples, several selective enrichment media were tested to inhibit growth of raw milk contaminants and select for *L. monocytogenes*. The most successful medium for this application was a modification of enrichment medium III, described by Rodriguez et al. (18). This medium was designated *Listeria* enrichment broth (LEB) and is composed of the following: proteose peptone (Difco), 5.0 g; tryptone (Difco), 5.0 g; Lab-Lemco powder (Oxoid Ltd., Columbia, Md.), 5.0 g; yeast extract, 5.0 g; sodium chloride, 20.0 g; disodium phosphate-2-hydrate, 12.0 g; potassium phosphate monobasic, 1.35 g; esculin (Sigma), 1.0 g; naladixic acid (Sigma), 40 mg; and acriflavin HCl (Sigma), 12 mg. All components except esculin, naladixic acid, and acriflavin HCl were combined with 980 ml of distilled water and autoclaved at 121°C for 15 min. A 20-ml portion of a 5% esculin solution, 2 ml of a 2% naladixic acid solution (in 0.1 N NaOH), and 1 ml of a 1.2% acriflavin HCl solution were added to the cooled medium. Esculin and acriflavin HCl were sterilized by autoclaving at 121°C for 15 min. Naladixic acid was filler sterilized by passing the solution through a 0.22-μm filter (Gelman Sciences, Inc., Ann Arbor, Mich.). LEB contains naladixic acid, which inhibits the growth of gram-negative organisms, and acriflavin hydrochloride, which inhibits many gram-positive bacteria. Standardized suspensions of a variety of gram-positive bacteria were used to inoculate LEB. Of these test organisms, only *L. monocytogenes* strains were capable of growth within 24 h at 37°C (Table 1). Prior growth of pure populations of *L. monocytogenes* in LEB did not result in altered DNA profiles or immunofluorescent signals when compared with cells grown in tryptose-phosphate broth.

TABLE 1. Growth of *L. monocytogenes* and common raw milk contaminants in LEB[a]

Bacterial strain	Growth
L. monocytogenes 19111	+
L. monocytogenes F5027	+
L. monocytogenes 19114	+
L. monocytogenes 19115	+
L. monocytogenes 19116	+
L. monocytogenes 19117	+
L. monocytogenes 19118	+
L. monocytogenes F5069	+
Staphylococcus aureus 25923	−
Staphylococcus epidermidis 12228	−
Staphylococcus hyicus subsp. *hyicus*	−
Staphylococcus hyicus subsp. *chromogene*	−
Streptococcus agalactiae	−
Streptococcus uberis	−
Streptococcus faecalis 19433	−

[a] Initial cell levels were approximately 5×10^2 CFU/ml. LEB was incubated at 37°C for 24 h and scored as + (positive visual turbidity) or − (negative visual turbidity).

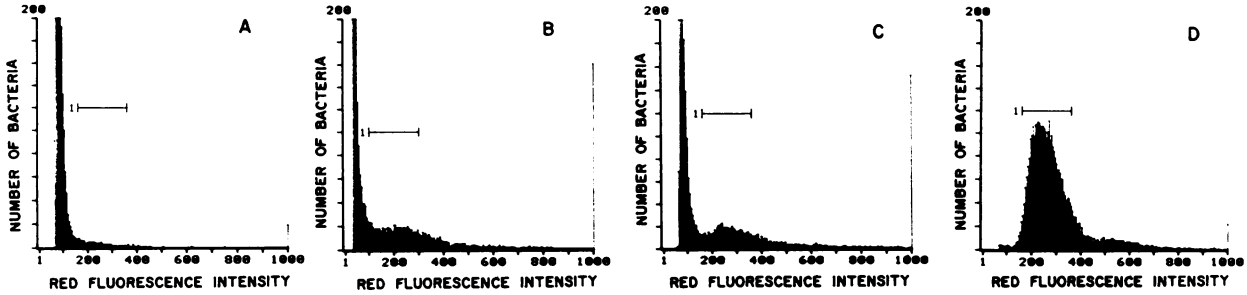

FIG. 5. DNA-associated fluorescence (red) profiles of *L. monocytogenes* F5027. Histograms depict the analyzed cell population after growth for 24 h in LEB. (A) Initial cell population, 0; after 24 h in LEB, 0. (B) Initial cell population, 8.5×10^0 CFU/ml; after 24 h in LEB, 1.7×10^6 CFU/ml. (C) Initial cell population, 6.0×10^1 CFU/ml; after 24 h in LEB, 8.8×10^6 CFU/ml. (D) Initial cell population, 7.8×10^2 CFU/ml; after 24 h in LEB, 7.6×10^7 CFU/ml.

Because population levels of *L. monocytogenes* in raw milk are variable, and often low-level contamination is reported (4), it was necessary to determine the lower limits of detection of *L. monocytogenes* in milk by FCM analysis. To determine detectable limits under ideal conditions, a stationary-phase culture (5.0×10^8 CFU/ml) of *L. monocytogenes* serotype 1 (ATCC 19111) grown in tryptose-phosphate broth was serially diluted to extinction in PBS. Diluted cell suspensions were transferred to 10 ml of sterile whole milk. Viable counts of *L. monocytogenes* in whole milk were determined prior to enrichment. Aliquots (1 ml) of whole milk containing known concentrations of *L. monocytogenes* were transferred to 100 ml of LEB and enriched for 24 h at 37°C. Cells from LEB were fluorescently labeled and analyzed by FCM. Figure 5 depicts DNA (red) fluorescence histograms generated by varying population levels of *L. monocytogenes*. Milk initially containing 8.5×10^0 *L. monocytogenes* per ml grew to a population of 1.7×10^6 *L. monocytogenes* per ml in LEB within 24 h at 37°C. Stained preparations of cells at this level yielded a discernible DNA peak which could be resolved from unbound stain and background noise (Fig. 5B). At final achievable population levels of $<10^6$ *L. monocytogenes* per ml of LEB, resolution of an *L. monocytogenes* DNA peak was not possible. As populations of *L. monocytogenes* in LEB increased, a more definable *L. monocytogenes* DNA peak was apparent (Fig. 5D).

Upon analysis of raw milk for *L. monocytogenes*, numerous raw milk samples contained unidentified microorganisms which grew in LEB. Because contaminating organisms capable of growth in LEB could obscure DNA-associated fluorescence signals due specifically to *L. monocytogenes*, the previous experiment was repeated by inoculating raw milk with low populations of *L. monocytogenes*. Following enrichment in LEB and fluorescent labeling, samples were analyzed by FCM. During analysis of mixed bacterial populations containing *L. monocytogenes*, DNA-associated red fluorescence was selected as the triggering and primary gating parameter. It consistently separated signals due to *Listeria* spp. from those due to debris and unbound stain. The primary gating region was set to include cells falling within two standard deviations of the peak mean of an *L. monocytogenes* standard culture used for instrument calibration prior to FCM analysis. While initial experiments determined that 90° scatter and forward angle light scatter were not useful for the characterization of pure populations of *L. monocytogenes*, scatter analysis of mixed cell suspensions proved to be useful to gate out non-*Listeria* isolates which were capable of growth in LEB. The scatter window was set, using an *L. monocytogenes* standard culture, to include 95% of cells falling within this region. Figure 6 depicts a DNA and scatter histogram of raw milk microflora in which no *L. monocytogenes* are present (Fig. 6A and B) and in which levels of 5.0×10^0 *L. monocytogenes* per ml of raw milk were initially present (Fig. 6C and D). Region 1 of Fig. 6A and C represents the DNA gating region, while region 1 of Fig. 6B and D represents the scatter gating region. In the presence of high levels of contaminating microorganisms, resolution of a DNA fluorescence peak generated by low numbers of *L. monocytogenes* was not possible (Fig. 6C). However, scatter analysis of this DNA-gated population clearly identified a subpopulation attributable to *L. monocytogenes* (Fig. 6D). During routine analysis of milk samples for *L. monocytogenes*, only cells falling into both region 1 on DNA fluorescence histograms and region 1 of scatter histograms were analyzed for immunofluorescence. The use of scatter and DNA fluorescence profiles in addition to immunofluorescence more clearly defined a bacterial population as *L. monocytogenes* and eliminated many of the problems of cross-reactivity associated with traditional fluorescent-antibody microscopic techniques for identification of *L. monocytogenes*.

DISCUSSION

In this report, a technique for the detection of *L. monocytogenes* in raw milk samples using FCM has been described. *L. monocytogenes* is extremely difficult to isolate from infected tissues, milk, silage, and feces by traditional cultural procedures because of overgrowth by competing microorganisms (7). Numerous attempts have been made to devise cultural procedures whereby *L. monocytogenes* has selective growth advantages over competing microflora. Since it is a gram-positive rod, potassium tellurite (8, 13), nitrofuran compounds such as guanofuracin (5-nitro-2-furfurylidene aminoguanidine hydrochloride) (7), furacin, and furadantin (17), chloramphenicol (7), salt (7), naladixic acid (2), amphotericin B (16), and polymixin B (9) have been incorporated as selective agents into plating media. However, with each of these reports have surfaced conflicting data concerning the efficacy of the selective component or isolation procedure. The most reliable method for isolating pure cultures of *L. monocytogenes* remains cold enrichment (15). Samples from infected tissues, milk, silage, or other sources are mixed with a suitable dilutent and stored at 4°C. Storage at 4°C provides *L. monocytogenes* with a competitive advantage over contaminating microflora or other inhibitors which may be associated with the sample. However, at

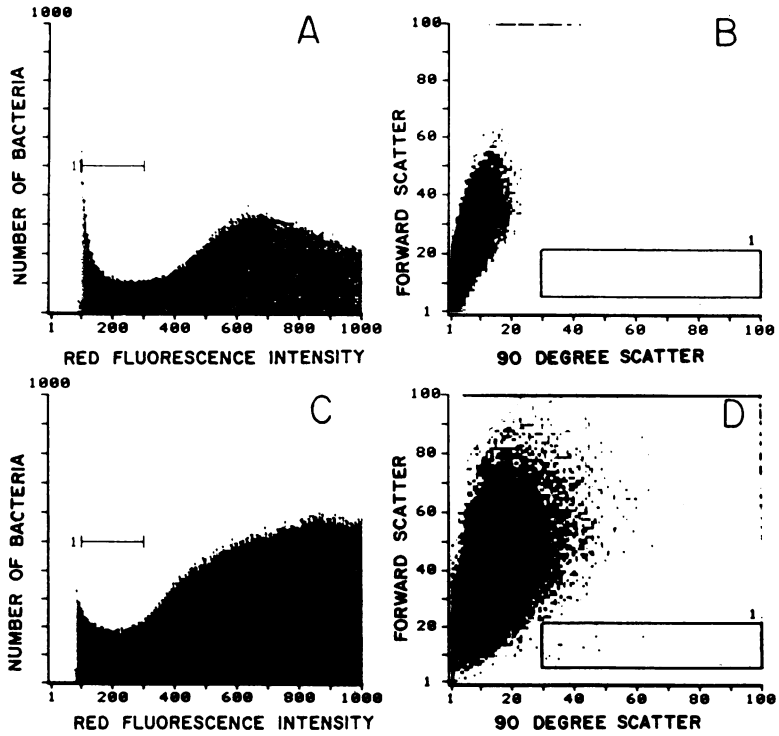

FIG. 6. DNA-associated fluorescence (red) and scatter histograms of raw milk microflora enriched in LEB. Region 1 of each histogram depicts an *L. monocytogenes* standard gating region. (A) Red fluorescence histogram depicting raw milk in which no *L. monocytogenes* are present. (B) Scatter analysis of raw milk without *L. monocytogenes*. (C) Red fluorescence histogram of *L. monocytogenes* (region 1) contained within a population of raw milk microflora. (D) Scatter analysis of raw milk containing *L. monocytogenes* (region 1).

4°C isolation of *L. monocytogenes* may take as long as 3 months. Any methodology for the isolation and identification of *L. monocytogenes*, if it is to be used successfully as a screening technique, must provide more rapid results.

Fluorescent-antibody techniques exist for the rapid identification of *L. monocytogenes* in milk and meat (14). A major limitation of these methods, however, is that antibodies against *L. monocytogenes* exhibit cross-reactivity with members of the genera *Streptococcus* and *Staphylococcus* (6, 10, 14). Visual resolution of these cell types from *Listeria* spp. is difficult, and thus fluorescence microscopy is not a reliable method for the screening of milk samples for the presumptive presence of *L. monocytogenes*. An alternative to the use of fluorescence microscopy is FCM. Fluorescence-activated cell sorters enable the morphological characterization and fluorescence detection of individual cells suspended in solution as they travel through a laser beam (1). Cells possessing specific antigenicity and morphology can be separated, quantitated, and summarized as distributions which represent the analyzed cell population. FCM has recently been used for a variety of microbiological applications (1, 20–22) and appears to have potential for providing a method to rapidly detect the presence of *L. monocytogenes* in raw milk samples.

Since detection of *L. monocytogenes* present in mixed populations is dependent on immunofluorescence, it is advantageous to treat samples in a manner which will maximize fluorescence. We found that the degree of positive fluorescence exhibited by populations of *L. monocytogenes* was dependent on primary antibody titer. At high antibody concentrations, cells precipitated from solution and formed clumps and aggregates, which resulted in decreased fluorescence on histograms. Also, when working with microorganisms which have the tendency to form chains or clumps, nonspecific trapping of antibody in these clumps may yield false fluorescence signals or obscure detection of organisms. To circumvent this problem, mild sonication of cells was found to be a useful procedure.

Compared with fluorescence microscopy, the use of FCM for bacterial detection and characterization of microbial populations has numerous advantages. Thousands of cells can be analyzed in seconds, in comparison to the time-consuming and subjective nature of visual microscopic analysis. Furthermore, fluorescent parameters in addition to immunofluorescence can be utilized to gather more information regarding a microbial population. In our procedure, in addition to immunofluorescence, PI was used to label cellular DNA. The fluorescent signal emitted by PI is related to the DNA content of a given bacterium (12). Since related bacteria possess similar DNA contents, the use of PI enables the separation of microbial populations based on DNA content. PI is also useful in excluding cell debris, which would be devoid of nucleic acids, from analysis.

The use of DNA-associated red fluorescence as a primary gating parameter allowed further characterization of the gated cell population by light scatter. Both forward angle and 90° scatter can be used to characterize cell populations and establish gating regions (20). Forward angle light scatter, primarily a combination of diffraction and refraction, relates the size information of a cell. A 90° scatter, which is light scattered by a cell at a right angle to the beam, is primarily a reflection phenomenon and is thought to be related to the internal structure of the cell. Numerous authors studying the microbiological applications of flow cytometry have con-

cluded that light scatter is not a useful parameter for differentiating between cocci and rods (1). The small size of most bacterial cells approaches the lower limits of resolution by FCM and the signals generated by light scatter are often difficult to resolve from background noise. In our study, the 90° light scatter distribution of a pure population of *Listeria* fell into two distinct regions due to alignment of cells in the flow stream. While this parameter alone was not useful for characterization of pure populations of *Listeria* spp., the use of forward versus 90° scatter was extremely useful for gating out non-*Listeria* contaminants based on size and morphology. This parameter enabled further resolution of *Listeria* spp. in mixed bacterial populations.

In conclusion, we have shown that FCM can rapidly identify the presence of *L. monocytogenes* intentionally added to raw milk samples. In addition, we have successfully used this method to detect naturally occuring *L. monocytogenes* in raw milk (C. W. Donnelly, E. H. Briggs, and G. J. Baigent, Abstr. 73rd Annu. Meet. Int. Assoc. Milk Food Environ. Sanitarians, abstr. no. 14, p. 5, 1986). Presumably, this technique could also be used to analyze a wide variety of other food as well as clinical specimens. Use of this method assumes familiarity with basic FCM principles, and, once mastered, assays can be run easily on a routine basis. At present, this method is dependent on commercially available polyclonal antibodies. However, with the rapid developments being made in monoclonal antibody research, highly specific antibodies may soon be available against a wide variety of bacterial species. These developments will vastly increase the utility of FCM as a tool for microbiological analysis. In addition to detection of *L. monocytogenes* in milk and other food products, it is expected that FCM analysis could be useful in a clinical laboratory for the rapid detection of *L. monocytogenes* in patient blood or spinal fluid and thus enable rapid diagnosis and treatment of patients with listeriosis.

ACKNOWLEDGMENTS

This work was supported by U.S. Department of Agriculture grant 58-6125-4-16 and by State and Hatch Funds allocated to the Vermont Agricultural Experiment Station.

LITERATURE CITED

1. **Barnett, J. M., M. A. Cuchens, and W. Buchanan.** 1984. Automated immunofluorescent speciation of oral bacteria using flow cytometry. J. Dent. Res. **63**:1040–1042.
2. **Beerens, H., and M. M. Tahon-Castel.** 1966. Milieu á l'acide n'aladixique pour l'isolement des streptocoques, *D. pneumoniae*, *Listeria*, *Erysipelothrix*. Ann. Inst. Pasteur (Paris) **111**:90–43.
3. **Clark, W. S., A. R. Brazis, J. L. Fowler, C. K. Johns, and F. E. Nelson.** 1978. Standard plate count method, p. 77. *In* E. H. Marth (ed.), Standard methods for the examination of dairy products, 14th ed. American Public Health Association, Inc., Washington, D.C.
4. **Donker-Voet, J.** 1962. My view on the epidemiology of *Listeria* infections, p. 113–139. *In* M. L. Gray (ed.), Second symposium on listeric infection. Montana State College, Bozeman.
5. **Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reingold.** 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. **312**:404–407.
6. **Forsgren, A., and U. Forsum.** 1970. Role of protein A in nonspecific immunofluorescence of *Staphylococcus aureus*. Infect. Immun. **2**:387–391.
7. **Gray, M. L., and A. H. Killinger.** 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. **30**:309–382.
8. **Gray, M. L., M. J. Stafseth, and F. Thorp, Jr.** 1950. The use of sodium azide, potassium tellurite, and acetic acid in a selective medium for the isolation of *Listeria monocytogenes*. J. Bacteriol. **59**:443–444.
9. **Groenstoel, H., and E. Aspoey.** 1977. A new selective medium for the isolation of *Listeria monocytogenes*. Nord. Veterinaermed. **29**:451–466.
10. **Hopfer, R. L., R. Pinzon, M. Wenglar, and K. V. I. Rolston.** 1985. Enzyme release of antigen from *Streptococcus faecalis* and *Listeria monocytogenes* cross-reactive with Lancefield group G typing reagents. J. Clin. Microbiol. **22**:677–679.
11. **James, S. M., S. L. Fannin, B. A. Agree, B. Hall, E. Parker, J. Vogt, G. Run, J. Williams, L. Lieb, C. Salminen, T. Prendergast, S. B. Werner, and J. Chin.** 1985. Listeriosis outbreak associated with Mexican-style cheese—California. Morbid. Mortal. Weekly Rep. **34**:357–359.
12. **Kerker, M., M. A. Van Dilla, A. Brunsting, J. P. Kratohvil, P. Hsu, D. S. Wang, J. W. Gray, and R. G. Langlois.** 1982. Is the central dogma of flow cytometry true: that fluorescence intensity is proportional to cellular dye content? Cytometry **3**:71–78.
13. **Khan, M. A., A. Seaman, and M. Woodbine.** 1973. Differential media in the isolation of *Listeria monocytogenes*. Zentralbl. Infektionskr. Hyg. Abt. 1 Orig. Bakteriol. Parasitenkd. Reihe A **224**:362–365.
14. **Khan, M. A., A. Seaman, and M. Woodbine.** 1977. Immunofluorescent identification of *Listeria monocytogenes*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **239**:62–69.
15. **Krieg, N. R.** 1981. Enrichment and isolation, p. 112–142. *In* P. Gerhardt, R. G. E. Murray, R. N Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
16. **Mavrothalassitis, P.** 1977. A method for the rapid isolation of *Listeria monocytogenes* from infected material. J. Appl. Bacteriol. **43**:47–52.
17. **McBride, M. E., and K. F. Girard.** 1960. A selective method for the isolation of *Listeria monocytogenes* from mixed bacterial populations. J. Lab. Clin. Med. **55**:153–157.
18. **Rodriquez, L. D., G. S. Fernandez, J. Fernandez, F. Garayzabel, and E. R. Ferri.** 1984. New methodology for the isolation of *Listeria* microorganisms from heavily contaminated environments. Appl. Environ. Microbiol. **47**:1188–1190.
19. **Schlech, W. F., P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome.** 1983. Epidemic listeriosis—evidence for transmission by food. N. Engl. J. Med. **308**:203–206.
20. **Srienc, F., B. Arnold, and J. E. Bailey.** 1984. Characterization of intracellular accumulation of poly-B-hydroxybutyrate (PHB) in individual cells of *Alcaligenes eutrophus* H16 by flow cytometry. Biotechnol. Bioeng. **26**:982–987.
21. **Tyndall, R. L., R. E. Hand, Jr., R. C. Mann, C. Evans, and R. Jernigan.** 1985. Application of flow cytometry to detection and characterization of *Legionella* spp. Appl. Environ. Microbiol. **49**:852–857.
22. **Van Dilla, M. A., R. G. Langlois, D. Pinkel, D. Yajko, and W. K. Hadley.** 1983. Bacterial characterization by flow cytometry. Science **220**:620–621.