Gram-typing of mastitis bacteria in milk samples using flow cytometry

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ABSTRACT

Fast identification of pathogenic bacteria in milk samples from cows with clinical mastitis is central to proper treatment. In Denmark, time to bacterial diagnosis is typically 24 to 48 h when using traditional culturing methods. The PCR technique provides a faster and highly sensitive identification of bacterial pathogens, although shipment of samples to diagnostic laboratories delays treatment decisions. Due to the lack of fast on-site tests that can identify the causative pathogens, antibiotic treatments are often initiated before bacterial identification. The present study describes a flow cytometry-based method, which can detect and distinguish gram-negative and gram-positive bacteria in mastitis milk samples. The differentiation was based on bacterial fluorescence intensities upon labeling with biotin-conjugated wheat germ agglutinin and acridine orange. Initially 19 in-house bacterial cultures (4 gram-negative and 15 gram-positive strains) were analyzed, and biotin-conjugated wheat germ agglutinin and acridine orange fluorescence intensities were determined for gram-negative and gram-positive bacteria, respectively. Fluorescence cut-off values were established based on receiver operating characteristic curves for the 19 bacterial cultures. The method was then tested on 53 selected mastitis cases obtained from the department biobank (milk samples from 6 gram-negative and 47 gram-positive mastitis cases). Gram-negative bacteria in milk samples were detected with a sensitivity of 1 and a specificity of 0.74, when classification was based on the previously established cut-off values. However, when receiver operating characteristic curves were constructed for the 53 mastitis cases, results indicate that a sensitivity and specificity of 1 could be reached if cut-off values were reduced. This flow cytometry-based technique could potentially provide dairy farmers and attending veterinarians with on-site information on bacterial gram-type and prevent ineffective antimicrobial

treatment in mastitis cases caused by gram-negative bacteria.

Key words: gram-type, bacterial pathogen, bovine mastitis, flow cytometry

INTRODUCTION

Mastitis caused by bacterial pathogens is of major economic importance because it decreases milk yield and milk quality, increases the risk of early culling, and adds on to the daily work load (Seegers et al., 2003). Furthermore, mastitis compromises animal welfare (Rasmussen et al., 2011; Fogsgaard et al., 2012). An early identification of the pathogenic bacteria is essential to treatment (Pinzón-Sánchez and Ruegg, 2011). In Denmark, milk samples from cows with clinical or sub-clinical mastitis (high SCC) are commonly grown on blood agars for 24 to 48 h and bacterial pathogens are identified by the attending veterinarian based on colony morphology and taxonomic tests [i.e., KOH string test, oxidase, catalase, and Christie, Atkins, Munch-Petersen (CAMP) reactions]. At present, the use of a commercial PCR kit for detection of mastitis pathogens (Koskinen et al., 2009) is increasing in some Northern European countries such as Finland, Denmark, and Germany, the method being a highly sensitive technique for bacterial detection. The technique does not require overnight incubation and, consequently, bacterial identification times are also considerably reduced (6 h; Koskinen et al., 2009; Shome et al., 2011). However, at present, the PCR technique is not economically accessible for on-site solutions and, consequently, shipment of samples to diagnostic laboratories prolongs bacterial identification times. Other concerns regarding the PCR technique are the identification of numerous bacteria in each milk sample, including bacteria that may not originate from the udder, the inability to distinguish between live and dead bacteria, and the inability to obtain antibiograms.

In Denmark, the treatment of clinical mastitis is, therefore, often initiated before final bacterial diagnosis, and consequently ineffective antibiotic treatment of gram-negative bacterial infections occurs regularly. The development of a fast on-site solution that can determine bacterial gram-type with minimal preincu-

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bation could potentially reduce the ineffective use of antibiotics in gram-negative mastitis cases.

The original Gram staining procedure was first described by Christian Gram and his colleague Carl Friedlander back in the 1880s (Madigan et al., 2009). At present, the KOH string test is a simpler and less labor-intensive test for gram typing of bacteria, gram-positive bacteria being identified with 100% sensitivity and 99% specificity (Arthi et al., 2003).

Flow cytometry is a commonly used technique for quantifying bacteria in bulk milk (Gunasekera et al., 2003; Holm and Jespersen, 2003), milk powder (Flint et al., 2007), and dairy starters (Bunthof and Abee, 2002), and it is presently used for quantification of bacteria in several commercial instruments such as the BactoScan (Foss Electric A/S; Hillerød, Denmark), BactoCount (Bentley Instruments Inc., Chaska, MN), and BactiFlow ALS (AES Chemunex Inc., Cranbury, NJ). In addition, flow cytometry-based methods have also been used for gram-typing bacteria in bulk milk when labeling with DNA-associated fluorochromes and fluorochrome-conjugated lectins (Gunasekera et al., 2003; Holm and Jespersen, 2003). The same principle could potentially be used for gram-typing of mastitis-associated bacteria in individual cow or quarter milk samples. However, the flow cytometry-based method has not been further developed for mastitis diagnostics because of difficulties in analyzing nonhomogeneous mastitis milk samples, the background noise caused by high SCC, a relatively poor detection limit, and misclassification due to contaminating bacteria.

In the present study, a new adapted procedure for identification and gram-typing of bacteria in mastitis milk samples is described. Diluted and preincubated mastitis milk samples were labeled with a combination of biotin-conjugated wheat germ agglutinin (WGA) and acridine orange (AO) and their fluorescence intensities were measured by flow cytometry. The hypotheses were that bacterial populations in mastitis milk with high SCC could be detected and that gram-negative and gram-positive bacterial populations could be distinguished using flow cytometry.

MATERIALS AND METHODS

In-House Bacterial Cultures

Nineteen different bacterial strains/isolates were evaluated in the study (Table 1). The bacteria included 15 commercial reference strains and 4 bovine field isolates from other Danish laboratories; among these, 15 were gram-positive and 4 were gram-negative bacterial strains. The bacteria listed are referred to as in-house bacteria. The in-house bacteria were used to establish

the method presented and to determine the receiver operating characteristic (ROC) curves and cut-off values as described in the data analysis section.

Bits of frozen bacterial culture (-20°C) were scraped onto blood agars (Eurofins Steins Laboratory A/S, Holstebro, Denmark) and BD CHROMagar Orientation Mediums (BD Diagnostic Systems, Heidelberg, Germany). Both agars were incubated at 37°C overnight and were evaluated for viable monocultures the following day. One colony from blood agars of each bacterial monoculture was transferred to 3 mL of brain heart infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) and incubated at 37°C overnight before fluorochrome labeling and flow analysis were performed (Figure 1).

Mastitis Milk Samples

In total, 53 quarter milk samples from cows with mastitis were selected from the Department of Animal Science (Aarhus University, Tjele, Denmark) biobank and included in the study. Quarter milk samples were taken out aseptically, as initial jets of milk were discarded and teat openings were wiped with 70% ethanol before sample collection. The SCC was determined on fresh milk in the barn using a DCC cell counter (DeLaval A/S, Vejle, Denmark). Milk samples were frozen immediately after collection (-20°C). Samples were transported from dairy farms to the laboratory, where they were stored at -20°C upon arrival. Milk samples were incubated on blood agars (Eurofins Steins Laboratory A/S) and on BD CHROMagar Orientation Mediums (BD Diagnostic Systems) at 37°C . The agars were read after 24 and 48 h. Pathogens were identified by classical bacteriologic procedures (i.e., colony morphology, KOH string test, oxidase, catalase, coagulase, and CAMP reactions). *Staphylococcus aureus* and *Streptococcus dysgalactiae* mastitis cases were confirmed using Slidex Staph-Kit and Slidex Strepto Plus, respectively; both kits were from BioMérieux (Marcy L'Étoile, France). Type B streptococci with a positive CAMP test were sent to a diagnostic laboratory (Eurofins Steins Laboratory A/S) according to the national guidelines. The milk samples were then included in the biobank at the Department of Animal Science (Aarhus University). Prior to flow analysis, the milk samples were thawed and 50 μL was transferred to 5 mL of BHI broth (Merck KGaA) for incubation at 37°C overnight (Figure 1). The following day, the samples of the incubated milk in BHI broth were transferred to blood agars (Eurofins Steins Laboratory A/S) and BD CHROMagar Orientation media (BD Diagnostic Systems) and incubated at 37°C overnight. Classical bacteriological procedures were repeated to confirm bacterial viability and the previous bacteriological identification.

Figure 1. (A) Timeline (48 to 72 h) for the in-house bacterial cultures, which describes bacterial incubation on agar plates, followed by preincubation of a single colony in brain heart infusion (BHI) broth before bacterial labeling and flow analysis; (B) timeline (24 h) for the frozen mastitis milk samples showing the dilution and preincubation of milk in BHI broth before bacterial labeling and flow analysis. For reference, a sample of the preincubated milk in BHI broth was transferred to agar plates and the bacterial pathogen was determined based on colony morphology and taxonomic tests 24 to 48 h after flow analysis was performed.

Preparation of Fluorochromes and Labeling of Bacteria

Biotin-conjugated WGA (Sigma-Aldrich, St. Louis, MO) was dissolved in 1 mL of PBS; the stock solu-

tion (1 mg/mL) was divided into aliquots and frozen at -20°C . To produce the amount of biotin-conjugated APC streptavidin-WGA complex to stain bacteria present in 1 sample, 0.5 μL of biotin-conjugated WGA (stock solution) was mixed with 0.312 μL of APC strep-

Table 1. In-house bacterial cultures used to evaluate the flow cytometry-based method including 15 commercial strains and 4 bovine field isolates from cows with mastitis

No.	Bacterial culture	Reference strain	Transmission ¹	Gram-type
1	<i>Staphylococcus aureus</i>	Bovine field, A25, phage type 29/52, ribo-type 1 ²	Cont	Positive
2	<i>Staph. aureus</i>	CCUG 4151 ³	Cont	Positive
3	<i>Staph. aureus</i>	SLV-350 ⁴	Cont	Positive
4	<i>Staphylococcus epidermis</i> (CNS)	CCUG 46178 ³	Envir	Positive
5	<i>Staphylococcus simulans</i> (CNS)	CCUG 46177 ³	Envir	Positive
6	<i>Staphylococcus saprophyticus</i> (CNS)	SLV-013 ⁴	Envir	Positive
7	<i>Streptococcus agalactiae</i>	CCUG 4208T (ATCC13813) ³	Cont	Positive
8	<i>Streptococcus dysgalactiae</i>	CCUG 27301T ³	Envir	Positive
9	<i>Strep. dysgalactiae</i>	Bovine field, D8 ⁵	Envir	Positive
10	<i>Streptococcus uberis</i>	CCUG 37983 (ATCC9927) ³	Envir	Positive
11	<i>Strep. uberis</i>	CCUG 17930, type 1, Lancefield gr E ³	Envir	Positive
12	<i>Strep. uberis</i>	Bovine field, U103 ⁵	Envir	Positive
13	<i>Enterococcus casseliflavus</i>	CCUG 34231, Lancefield gr D ³	Envir	Positive
14	<i>Enterococcus faecalis</i>	CCUG 19916T (ATCC 19433), Lancefield gr D ³	Envir	Positive
15	<i>Enterococcus faecium</i>	ATCC 35667 ⁴	Envir	Positive
16	<i>Escherichia coli</i>	Bovine field, k2bh2 ⁶	Envir	Negative
17	<i>Klebsiella pneumoniae</i>	CCUG 225T (ATCC 13883) ³	Envir	Negative
18	<i>Serratia marcescens</i>	CCUG 6 ³	Envir	Negative
19	<i>Pseudomonas aeruginosa</i>	CCUG 17619 (ATCC 27853) ³	Envir	Negative

¹Cont = contagious pathogen; Envir = environmental pathogen.

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tavidin (BD Pharmingen, Franklin Lakes, NJ) and 4.2 μ L of sterile 0.9% saline, and the conjugated complex was then left in the dark for 30 min. 3-*N*,3-*N*,6-*N*,6-*N*-Tetramethylacridine-3,6-diamin (AO; Invitrogen, Nærum, Denmark) was used in a stock solution of 10 mg/mL of water.

Samples with in-house bacterial cultures incubated overnight in BHI broth were diluted with saline to an expected bacterial concentration of 10^6 bacteria/mL. The diluted samples (500 μ L) were initially labeled with 5 μ L of biotin-conjugated allophycocyanin (APC) streptavidin-WGA complex and left in the dark for 10 min. Then, samples were labeled with 1 μ L of AO stock solution and left in the dark for 5 min, and immediately thereafter flow analyses were performed. Bacterial pathogens in mastitis milk samples preincubated overnight in BHI broth (500 μ L) were labeled according to the protocol for the in-house bacterial cultures described above.

Flow Analysis

Flow analyses of the in-house bacterial cultures and milk samples were conducted on a BD FACS-Canto Flow Cytometer (BD Biosciences, San Jose, CA) equipped with a blue laser (488 nm) using forward scatter (FSC) and side scatter (SSC) signals and green (525-nm) fluorescent signals, and with a red laser (635 nm) using red (661-nm) fluorescent signals. Voltage settings for FSC, SSC, fluorescein isothiocyanate (FITC), and APC channels were 250, 375, 400, and 600, respectively. The detectors were all set to logarithmic amplification. The SSC threshold was set at 200, whereas no threshold was set for FSC; however, all events with an FSC below 400 were eliminated by gating (Figure 2). The flow analyses were performed without compensation. Day-to-day alignment was performed using BD FACS 7-color setup beads (BD Biosciences) and gram-positive and gram-negative controls. Flow analyses were performed using a low flow rate and analyses were stopped after 3 min or when total events in the Q1-1 and Q2-1 gates reached 5,000 (Figure 2). Based on flow analyses from 7 sterile milk samples with SCC <100,000 cells/mL, the gate Q1-1 was set to eliminate unspecific binding (artifacts) from milk components observed in gate Q2-1 (Figure 2). During the flow analysis of milk samples, the in-house bacterial cultures *Staph. aureus* (no. 1) and *Escherichia coli* (no. 16) were included as gram-positive and gram-negative controls, respectively (Figure 2). The day-to-day coefficients of variation for *Staph. aureus* were 1.6% (AO FITC) and 1.1% (WGA APC), and for *E. coli* they were 3.7% (AO FITC-H) and 2.8% (WGA APC). The gates shown in Figure 2 were applied in all of the flow analyses performed.

Data Analysis

In-house bacterial cultures were plotted with respect to AO FITC mean fluorescence intensity (MFI) and WGA APC MFI to evaluate the distribution pattern for gram-negative and gram-positive bacteria, respectively. A linear model was fitted to the AO FITC MFI and WGA APC MFI variables, with gram-type included as a fixed effect. The MFI values were log transformed before statistical analysis and results are expressed as medians with corresponding 95% confidence intervals. Median MFI values in mastitis milk samples were also determined for gram-negative and gram-positive cases and for the individual pathogens.

The sensitivity and specificity for the present Gram-staining method were calculated from the results of the 53 mastitis cases based on AO FITC and WGA APC MFI cut-off values determined for the in-house bacterial cultures. The golden standard for bacterial gram-type used in the calculation of reported sensitivities and specificities was the reported gram-type for all in-house bacterial cultures and gram-type results obtained with the KOH string test for all bacterial pathogens in mastitis cases. Cut-off values were identified for in-house bacterial cultures AO FITC and WGA APC MFI based on individual ROC curves. In addition, ROC curves with an added smooth function were constructed for AO FITC and WGA APC MFI in mastitis cases.

For all the statistical analyses performed, significance was stated at the 5% level. Data were analyzed using the software R (R Development Core Team, 2012; <http://www.R-project.org>).

RESULTS

In-House Bacterial Cultures

In Figure 3, the AO FITC MFI for the 19 in-house bacterial cultures was plotted against the WGA APC MFI. The median AO FITC was 6,066 (3,779; 9,735) MFI and 16,267 (12,742; 20,767) MFI for gram-negative and gram-positive bacteria cultures, respectively, and differed significantly ($P = 0.002$). The median WGA APC was 289 (87; 964) MFI for gram-negative bacteria cultures, which was significantly lower than the 1,402 (753; 2,611) MFI for the gram-positive bacteria cultures ($P = 0.036$). The cut-off values with corresponding sensitivity and specificity are presented on the ROC curves shown in Figure 4.

Mastitis Milk Samples

In total, 53 mastitis milk samples from cows with clinical mastitis were included in the study, the mean

Figure 2. (A) Gram-positive in-house bacterial culture (no. 1; *Staphylococcus aureus*); (B) gram-negative in-house bacterial culture (no. 16; *Escherichia coli*); (C) sterile milk sample with low SCC; (D) mixed sample. The gate Q1-1 was set to include gram-negative and gram-positive controls and Q2-1 to eliminate artifacts from unspecific staining of milk components. Fluorescein isothiocyanate (FITC-H) mean fluorescence intensity (MFI) for labeling with acridine orange (AO) and allophycocyanin (APC-H) MFI for labeling with wheat germ agglutinin (WGA) are shown.

SCC was $2.2 \times 10^6/\text{mL}$ (SD $1.8 \times 10^6/\text{mL}$). The samples included 6 gram-negative bacterial pathogens [i.e., *E. coli* ($n = 6$)], and 47 gram-positive bacterial pathogens [i.e., CNS ($n = 17$), *Staph. aureus* ($n = 7$), *Streptococcus agalactiae* ($n = 1$), *Strep. dysgalactiae* ($n = 6$), *Streptococcus uberis* ($n = 6$), and *Enterococcus faecalis* ($n = 10$)]. The AO FITC MFI and WGA APC MFI were significantly different in gram-negative compared with gram-positive bacterial pathogens, with $P = 0.002$ and $P < 0.001$, respectively (Figure 5). In addition, significant differences among the individual bacterial pathogens were observed. The AO FITC MFI was significantly lower in *E. coli* compared with CNS, *Staph. aureus*, *Strep. dysgalactiae*, and *E. faecalis*, and the WGA APC MFI was significantly lower in *E. coli*

compared with CNS, *Staph. aureus*, *Strep. dysgalactiae*, *Strep. uberis*, and *E. faecalis* (Figure 5).

When using the cut-off values for both AO FITC MFI and WGA APC MFI determined for the in-house bacterial cultures, the sensitivity was 1 and the specificity was 0.74 for detection of gram-negative bacteria in milk samples, and 77% of the pathogens were gram-typed correctly; 3/17 CNS, 1/7 *Staph. aureus*, 1/1 *Strep. agalactiae*, 2/6 *Strep. uberis*, and 5/10 *E. faecalis* were classified incorrectly. However, based on the evaluation of ROC curves for the 53 mastitis cases, the identified cut-off values for the in-house bacterial cultures were most likely overestimated (Figure 4). Using the cut-off values determined from the ROC curves based on the milk samples {AO FITC 9,037 MFI [sensitivity = 1 (1;

Figure 3. In-house bacterial cultures are plotted with respect to fluorescein isothiocyanate (FITC) and allophycocyanin (APC) mean fluorescence intensity (MFI). The identification number refers to the individual bacterial cultures listed in Table 1. 1–3 = *Staphylococcus aureus*; 4–6 = CNS; 7 = *Streptococcus agalactiae*; 8–9 = *Streptococcus dysgalactiae*; 10–12 = *Streptococcus uberis*; 13–15 = *Enterococcus* spp.; 16 = *Escherichia coli*; 17 = *Klebsiella pneumoniae*; 18 = *Serratia marcescens*; 19 = *Pseudomonas aeruginosa*. Gram-negative bacteria cultures are symbolized by open squares (□) and gram-positive bacteria cultures by filled squares (■). AO = acridine orange; WGA = wheat germ agglutinin.

1), specificity = 0.72 (0.6; 0.85)] and WGA APC 303 MFI [sensitivity = 1 (1; 1), specificity = 0.94 (0.85; 1)]}, both sensitivity and specificity would be 1 in the present study.

DISCUSSION

In the present study, a flow cytometry-based method was set up to detect and distinguish gram-negative and gram-positive mastitis-associated bacteria in milk samples from cows with clinical mastitis. Bacterial detection was achieved through preincubation and by diluting milk samples in connection with the preincubation and labeling procedures before flow analysis. This approach made it possible to separate bacteria from somatic cells and cell debris in the milk when labeling with AO. Gram-type was determined based on bacterial fluorescence intensity upon labeling with AO and biotin-conjugated APC streptavidin-WGA complexes. Staining intensity was affected by cell wall composition of the bacteria; generally, gram-negative bacteria that had a thin peptidoglycan layer surrounded by an outer lipopolysaccharide layer stained less intensively.

The present results indicate that it may be possible to detect and determine the gram-type of pathogenic bacteria in mastitis milk samples based on bacterial AO FITC and WGA APC fluorescence intensity. Using this fluorescent staining procedure, we confirmed that gram-negative bacteria stained less intensively with the WGA complex compared with gram-positive bacteria, as has previously been reported (Sizemore et al., 1990; Holm and Jespersen, 2003). Wheat germ agglutinin binds to N-acetylglucosamine in the peptidoglycan layer, which is prevented in gram-negative bacteria, as WGA cannot penetrate the outer lipopolysaccharide layer. In addition, gram-negative bacteria also took up less AO. Previously, it has been shown that gram-negative bacteria stained with AO decolorized differently when subjected to ethyl alcohol or acetone (Fazii et al., 2002), indicating that the cell wall of gram-negative bacteria was less capable of retaining AO.

In a prevalence study performed in Finland, the most common bacterial pathogens isolated from quarter milk samples were CNS, *Corynebacterium bovis*, *Staph. aureus*, *Strep. uberis*, *Enterococcus* spp., coliforms, *Strep. dysgalactiae*, and *Strep. agalactiae*, in the order

Figure 4. (A) and (B): receiver operating characteristic (ROC) curves constructed for acridine orange (AO) and wheat germ agglutinin (WGA), respectively, based on the 19 in-house bacterial cultures. (C) and (D): ROC curves (smooth function applied) for AO and WGA, respectively, based on the 53 mastitis milk samples. In mastitis milk samples, the AO fluorescein isothiocyanate (FITC) cut-off value of 9,037 mean fluorescence intensity (MFI) had a sensitivity [median (CI)] of 1 (1; 1) and a specificity [median (CI)] of 0.72 (0.6; 0.85), and the WGA allophycocyanin (APC) cut-off value of 303 MFI had a sensitivity of 1 (1; 1) and a specificity of 0.94 (0.85; 1) for detection of gram-negative mastitis cases. By using the 2 cut-off values in combination, a sensitivity (Se) and specificity (Sp) of 1 were obtainable.

Figure 5. (A) The median acridine orange (AO) fluorescein isothiocyanate (FITC) and wheat germ agglutinin (WGA) allophycocyanin (APC) mean fluorescence intensity (MFI) for the 6 gram-negative and the 47 gram-positive bacterial pathogens in mastitis milk samples; (B) the median AO FITC MFI and (C) the median WGA APC MFI for each bacterial pathogen in the mastitis milk samples. Significant differences in MFI among bacterial pathogens are indicated by the different letters (a-e). *Staph.* = *Staphylococcus*; *Strep.* = *Streptococcus*; *E. faecalis* = *Enterococcus faecalis*; *E. coli* = *Escherichia coli*.

mentioned (Pitkälä et al., 2004). In the present study, the in-house bacterial cultures and mastitis cases were selected to represent the most common bacterial udder infections, although the study lacks results for *C. bovis*, as it is considered a minor mastitis pathogen (Pitkälä et al., 2004). *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Pseudomonas aeruginosa* were included to represent different gram-negative strains.

The gram-negative bacterium *P. aeruginosa* was observed to stain intensively with the biotin-conjugated APC streptavidin-WGA complex, which has also been reported by Sizemore et al. (1990). In addition, *P. aeruginosa* also stained intensely with AO. Consequently, *P. aeruginosa* increased the AO FITC and WGA APC cut-off values determined for the in-house bacterial cultures (no. 19; Figure 2). Mastitis cases did not include infections caused by *P. aeruginosa* and, therefore, specificities were higher at lower cut-off values for AO FITC and WGA APC MFI, when looking at the ROC curves for the milk samples.

In mastitis milk samples, gram-negative *E. coli* differed significantly with respect to AO FITC and WGA APC MFI compared with the gram-positive *Staphylococcus* spp., *Strep. dysgalactiae*, and *E. faecalis*. In addition, *E. coli* differed compared with *Strep. uberis* with respect to WGA APC MFI. However, the single gram-positive *Strep. agalactiae* mastitis case included in the study stained similar to *E. coli*, which was not observed with the in-house *Strep. agalactiae* strain (no. 7; Figure 3), which stained as was expected for gram-positive bacteria. Most gram-positive bacteria have carbohydrate polymers incorporated into their cell walls and these carbohydrate polymers are highly diverse and often species or strain specific (Weidenmaier and Peschel, 2008). Holm and Jespersen (2003) argue that lowering pH affects the binding of WGA for some gram-positive bacteria, as it affects the rigid structure of the glycopolymer teichoic acid, thereby increasing access to the WGA binding site. However, in the present study we did not lower pH to enforce WGA binding. Misclassification of *Strep. agalactiae* with the present method is a cause for concern, as it is a contagious pathogen of major economic importance and, furthermore, an ongoing Danish surveillance program exists for this pathogen (Mweu et al., 2012). As *Strep. agalactiae* multiplies in chain formation, the bacterium can be separated from gram-negative bacteria based on its FSC and SSC position during flow analysis (S. N. Langerhuus, unpublished data). Further analysis with additional *Strep. agalactiae* mastitis cases or isolates must be carried out to verify if this atypical gram classification involves other strains of *Strep. agalactiae*.

The present flow cytometry-based method was envisioned as a fast on-site solution to be used on fresh milk samples obtained from cows with clinical mastitis, potentially as a cow-site analysis performed by the farmer in the barn. In our study, analyses were conducted on frozen milk samples, which may delay initial bacterial growth and the number of viable bacteria for some species (Schukken et al., 1989; Sánchez et al., 2003); consequently, we used 24-h preincubations. Therefore, it is relevant to analyze fresh mastitis milk samples using shorter preincubation times and repeated measurements to determine how early bacteria can be detected and a gram-type diagnosis made. Moreover, as the bacterial concentrations in milk vary depending on bacterial species and time of sampling in relation to disease kinetics (Schukken et al., 2011), preincubation times are expected to vary from 1 to 2 to 48 h.

In accordance with our observations (S. N. Langerhuus, unpublished data), the bacterial detection limit with flow cytometry has been reported to be $\leq 10^4$ bacteria/mL (Gunasekera et al., 2000; Gruden et al., 2004). Bacterial concentrations in quarter milk samples from cows with clinical mastitis have been reported to reach 1×10^6 to 1×10^8 bacteria/mL (Jeffrey and Wilson, 1987; Leigh et al., 2010; Schukken et al., 2011). However, concentrations are often much lower, as cows are frequently sampled after the bacterial growth has peaked in the mammary gland (Dinsmore et al., 1992).

With the present method, milk samples were diluted 100-fold to circumvent the noise from high SCC. Subsequently, the preincubation step increased bacterial numbers, resulting in an even lower bacteria:SCC noise ratio. Moreover, the use of preincubation of milk samples before the standard culture technique has been documented to increase the probability of diagnosing some mastitis bacteria (Dinsmore et al., 1992; Sol et al., 2002; Artursson et al., 2010). For instance, a short preincubation time will increase the likelihood of identifying *E. coli* in mastitic milk samples (Dinsmore et al., 1992), whereas a long preincubation time before culturing will increase the percentage of cows diagnosed with mastitis caused by *Staph. aureus* (Artursson et al., 2010). Nevertheless, viability is a criterion for bacterial growth during preincubation. Thus, if a milk sample contains as little as 1 viable bacterium that multiplies during preincubation, it can be detected and gram-typed. Extended preincubation times may, however, be complicated by growth and additional noise from contaminant bacteria (Dinsmore et al., 1992).

Specificity of the present method may improve by incubating milk samples in selective growth mediums favoring gram-negative or gram-positive bacterial

growth, and valuable information could be gained by introducing media that may differentiate *Streptococcus* spp. from *Staphylococcus* spp. Furthermore, introducing a medium with penicillin or other types of antibiotics along with a nonselective medium such as BHI may give additional information on antibiotic resistance among pathogens.

The present method cannot compete with the traditional culturing techniques or PCR technology with respect to bacterial identification. In contrast to the PCR method, this combined preincubation and fluorescent staining method has the potential to differentiate between living and dead bacteria and to include an antibiogram.

CONCLUSIONS

Bacteria in diluted and preincubated mastitis milk samples can be detected and gram-typed by AO and WGA fluorochrome labeling using flow cytometry. In the future, this method could potentially provide the dairy farmer or the attending veterinarian with on-site information on bacterial detection and gram-type in cases of clinical mastitis. At present, flow cytometry is accessible in minor portable units (Gruden et al., 2004), and with this technology the method could potentially be further developed for cow-side or online automatized solutions.

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