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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 florescence 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 subclinical 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.