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Direct identification of bovine mastitis pathogens by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in pre-incubated milk

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ABSTRACT

The present study aimed to compare two MALDI-TOF identification methods [(a) direct sample identification after pre-incubation; or (b) use of bacteria isolated on pre-culture)] to standard, traditional bench microbiology. A total of 120 quarter milk samples from 40 Holstein lactating cows were screened based on culture-positive results obtained by microbiological culture (reference method) with the following numbers of quarters positive per cow: 4 cows with 1, 8 cows with 2, 12 cows with 3 and 16 cows with 4 infected quarters per cow. For direct identification method, quarter milk samples ($n = 120$) were skimmed by centrifugation (10,000 × *g*/10 min) and pre-incubated at 37 °C for 12 h. After pre-incubation, quarter milk samples were submitted to total bacterial count by flow cytometry and for a preparation protocol for bacterial ribosomal protein extraction followed by MALDI-TOF MS analysis. The direct MALDI-TOF MS identification method compared to microbiological culture correctly identified isolates of coagulase-negative *Staphylococci* (27.2%), *Streptococcus agalactiae* (21.8%), *Staphylococcus aureus* (14.2%), and *Streptococcus uberis* (5.2%). The pre-incubation protocol of milk samples, associated to the direct identification method by MALDI-TOF MS, did not increase the identification at species level (score >2.0) of pathogens causing subclinical mastitis in comparison to the method without previous incubation.

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Introduction

Conventional microbiological culture of milk samples is based on biochemical tests for identification of microorganisms causing subclinical mastitis. Different bacterial characteristics are evaluated for identification of a single species, such as growth conditions, colony morphology, growth characteristics in selective medium, carbohydrate fermenting capacity, metabolic and antigenic characteristics, and antibiotic susceptibility.[1] The procedures are time consuming, and it may take 2–7 days for the complete diagnosis of the causative pathogen of the intramammary infection (IMI).

The mass spectrometry (MS) technique using matrix assisted laser desorption/ionization source (MALDI) and a time-of-flight type mass analyzer (TOF) can be used for rapid identification of bacteria and yeast from colonies previously cultured on solid medium. Such methodology provides a rapid identification of mastitis-causing bacteria by means of the extraction of ribosomal proteins from bacterial colonies cultured on blood agar[2] or using a direct transfer protocol.[3] However, the MALDI-TOF MS method has been used for direct identification of bacteria from human blood samples as a rapid diagnostic tool in hospital laboratories, since it does not depend on previous bacterial isolation using microbiological culture.[4,5] The direct classification of Gram-positive and Gram-negative bacteria at the genus level showed a 100% positive predictive value (PPV) when spectra consisting of joint ribosomal proteins ("fingerprints") were acquired from blood samples.[4,6]

Urinary tract pathogens have been also successfully directly identified by MALDI-TOF MS using urine samples with bacterial counts >10^5 cfu/mL. According to Ferreira et al.,[7] the direct identification of microorganisms by MALDI-TOF MS in urine samples showed 91.8% agreement at the species level and 92.7% at the genus level, when compared to microbiological culture identification. These results suggested that MALDI-TOF MS allowed the identification of Gram-negative bacteria directly from urine samples in a short period of time when samples contained elevated bacterial counts.[7] The MALDI-TOF MS method was also applied to clinical samples of cerebrospinal fluid and correctly classified the pathogens when bacterial counts in samples were between 10^4 and 10^6 cfu/mL.[8]

The scores used for the direct (nonculture based) identification MALDI-TOF method (direct-MALDI-TOF) in blood samples were lower than those of isolates obtained from bacterial cultures obtained from hemoculture, indicating that these scores can be influenced by the bacterial count. Previous studies utilized serial dilutions and obtained excellent identification spectra when the bacterial count in the blood sample was ≥10^6 cfu/mL, though the direct classification of microorganisms at the species level (75.8%) by MALDI-TOF MS was done considering scores ≥1.7.[9]

The total bacterial count is a critical factor for the direct identification of pathogens that cause mastitis by MALDI-TOF MS.[3] According to Moussaoui et al.,[10] the pre-incubation of blood samples enabled higher precision in the direct identification of bacteria using the MALDI-TOF MS method. Recently, our research group has evaluated the detection limit of MALDI-TOF MS for direct identification, without previous microbiological culture, of bovine mastitis-causing bacteria from milk samples.[3] Therefore, we suggested that the non-culture-based protocol could be applied in diagnostic laboratories by subjecting all milk samples to direct MALDI-TOF, and those without a positive identification could be submitted to pre-incubation protocol, being identified by MALDI-TOF MS combined with standard bacteriology. However, the effect of pre-incubation of quarter milk samples from cows affected with subclinical mastitis has not been evaluated using direct identification of bacteria by the MALDI-TOF method. Thus, the objective of the present study was to compare two MALDI-TOF identification methods [(a) direct sample identification after pre-incubation protocol; or (b) use of bacteria isolated on pre-culture] to standard, traditional bench microbiology.

Material and methods

Ethics approval was obtained through the Ethical Committee on the Use of Animals of the School of Veterinary Medicine and Animal Science (University of São Paulo, Brazil, protocol number 3002/2013) before the commencement of the study.

Sample collection and bacterial identification

Composite milk samples were collected from all cows on 2 commercial dairies located in the Midwest region of São Paulo State, Brazil. Based upon these results, milk samples were aseptically collected from all quarters of previously culture-positive cows. Microbiological culture (reference method) was performed using procedures consistent with National Mastitis Council guidelines. Briefly, 10 μL of milk per sample (quarter) were inoculated on blood agar plates with 5% defibrinated bovine blood. Inverted plates were incubated aerobically at 37 °C for 48 h and observed every 24 h for colony characteristics (shape, size, number, and color), hemolytic ability (presence and type). Gram stain, potassium hydroxide test (KOH) and catalase tests were performed to determine the morphology and differentiation between genera. Specific microbiology procedures such as coagulase, CAMP, esculin, bile esculin and pyr test were performed as described by Oliver et al.[11] A total of 120 quarter milk samples from 40 cows were positive, with the following numbers of quarters positive per cow: 4 cows with 1, 8 cows with 2, 12 cows with 3 and 16 cows with 4 infected quarters per cow. All quarter milk samples were also submitted to the nonculture based identification MALDI-TOF method (direct sample identification-MALDI-TOF).

Direct sample identification of mastitis-causing pathogen by MALDI-TOF MS

For direct sample identification of mastitis causing pathogens, fat was removed (skimmed) from 1 mL of milk samples by centrifugation (10,000 × *g*/10 min, followed by removal of the superior layer of fat. After skimming, milk samples were agitated in a vortex for 30 s (Kasvi basic K45 2820, Paraná, Brazil) and submitted to pre-incubation at 37 °C for 12 h in a water bath with agitation (Solab, SL 155/10, São Paulo, Brazil). After the incubation period, one aliquot of each milk sample (40 mL)

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