J. Dairy Sci. 100:1–11
https://doi.org/10.3168/jds.2016-12020

Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol

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ABSTRACT

This study evaluated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) for the identification of bovine-associated coagulase-negative staphylococci (CNS), a heterogeneous group of different species. Additionally, we aimed to expand the MALDI-ToF MS database with new reference spectra as required to fill the gaps within the existing commercial spectral library. A total of 258 isolates of CNS were used in the study, covering 16 different CNS species. The majority of the isolates were previously identified by *rpo*B gene sequencing (n = 219), and the remainder were identified by sequencing of 16S rRNA, *hsp*60, or both *rpo*B and *hsp*60. The genotypic identification was considered the gold standard identification. All MALDI-ToF MS identifications were carried out using the direct transfer method. In a preliminary evaluation (n = 32 isolates; 2 of each species) with the existing commercial database, MALDI-ToF MS showed a typeability of 81% (26/32) and an accuracy of 96% (25/26). In the main evaluation (n = 226 isolates), MALDI-ToF MS with the existing commercial Biotyper (Bruker Daltonics Inc., Billerica, MA) database achieved a typeability of 92.0% (208/226) and an accuracy of 99.5% (207/208). Based on the assessment of the existing commercial database and prior knowledge of the species, a total of 13 custom reference spectra, covering 8 species, were created and added to the commercial database. Using the custom reference spectra expanded database, isolates were identified by MALDI-ToF MS with 100% typeability and 100% accuracy. Whereas the MALDI-ToF MS manufacturer's cutoff for species-level identification is 2.000, the reduction of the species level cutpoint to ≥1.700 improved the species-level identification rates (from 64 to 92% for the existing commercial database) when classifying CNS isolates. Overall, MALDI-ToF MS using the direct transfer method was shown to be a highly reliable tool for the identification of bovine-associated CNS.

Key words: bovine coagulase-negative staphylococci, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, species identification

INTRODUCTION

Mastitis remains an important disease of dairy cattle and causes substantial economic loss (Halasa et al., 2007; Ruegg, 2012). Pathogen-specific diagnosis is important for the management of bovine mastitis because modes of transmission and infection, as well as treatments, differ between different mastitis-causing organisms (Ruegg, 2012; Royster and Wagner, 2015). In many countries, as the prevalence of major mastitis pathogens such as *Streptococcus agalactiae* and *Staphylococcus aureus* has declined, a shift toward a greater detection of CNS has been observed (Makovec and Ruegg, 2003; Pitkälä et al., 2004; Piepers et al., 2007; Ruegg, 2012). According to a national-level study of the epidemiology of IMI in Canada conducted in 2007 and 2008, CNS was the most common bacterial group isolated among clinically normal quarters (recovered in 5.4% of quarters) and were approximately twice as common as the next most frequent organism (*S. aureus*, recovered in 2.4% of samples; Reyher et al., 2011).

The classification of CNS represents a group of species instead of a single pathogen species, as is common for most other mastitis etiologic diagnoses. Most diagnostic laboratories group these staphylococci together because of inaccuracy or cost of more explicit species

Received September 20, 2016.
Accepted October 25, 2016.
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determination (Zadoks and Watts, 2009; Ajitkumar et al., 2013). However, CNS species have vast differences in pathogenicity, ecology, and epidemiology, differences that arguably would rival those between other species of mastitis pathogens (Sampimon et al., 2011; Supré et al., 2011; Vanderhaeghen et al., 2014).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-ToF MS**) is a rapid, robust, and cost-effective technology that has revolutionized routine microbial diagnostics over the past half-decade (Kliem and Sauer, 2012). This technology relies on the generation of unique protein profiles (mass spectrum) captured from small amounts of bacterial colony material followed by software-assisted searching of a database containing known microbial spectra. The performance of MALDI-ToF MS systems is thus dependent upon the species coverage of the reference database. With regard to CNS, the commercially available Bruker MALDI Biotyper system employs a database that contains 32 different species of CNS (Biotyper reference database version 4.0.0.1 with 5,627 entries; Bruker Daltonics Inc., Billerica, MA). Included in this collection are *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus simulans*, and *Staphylococcus xylosus*, which are reported to be the most prevalent species recovered in bovine milk in studies from around the world (Vanderhaeghen et al., 2015). In a small study of 108 bovine CNS isolates speciated by PCR-RFLP, 95% were correctly identified by MALDI-ToF MS (Tomazi et al., 2014). A limitation of that study was an uneven distribution of species such that of the 108 isolates of 11 species of CNS, 74.0% (n = 80) were *S. chromogenes*. Moreover, the reference methodology (*groEL* PCR-RFLP) has only been validated against biochemical testing, which has been shown to suffer from a low accuracy (Vanderhaeghen et al., 2015). Whereas the results of this previous study show promise, further studies of the evaluation of MALDI-ToF MS for bovine-associated CNS using a greater number of isolates originating from bovine-associated habitats such as milk and better representation of species are required. The results of MALDI-ToF MS validation research will be valuable to the increasing number of diagnostic laboratories that are adopting MALDI-ToF MS for microbial identification. The main objectives of this study were therefore to evaluate MALDI-ToF MS for the identification of bovine-associated CNS and to expand the MALDI-ToF MS database with new reference spectra as required to fill the gaps within the existing commercially available spectral library.

Table 1. Collection of 16 species of CNS analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Species[1]	Number	Percentage of total
Staphylococcus capitis	3	1.2
Staphylococcus chromogenes	75	29.1
Staphylococcus cohnii	15	5.8
Staphylococcus devriesei	4	1.6
Staphylococcus epidermidis	34	13.2
Staphylococcus equorum	23	8.9
Staphylococcus fleurettii	5	1.9
Staphylococcus gallinarum	9	3.5
Staphylococcus haemolyticus	26	10.1
Staphylococcus hyicus	4	1.6
Staphylococcus saprophyticus	3	1.2
Staphylococcus sciuri	11	4.3
Staphylococcus simulans	11	4.3
Staphylococcus succinus	4	1.6
Staphylococcus warneri	7	2.7
Staphylococcus xylosus	24	9.3
Total	258	100

[1]Determined by *rpo*B (219/258), *rpo*B and *hsp*60 (16/258), *hsp*60 (12/258), or 16*S* (11/258) gene sequencing.

MATERIALS AND METHODS

Bacterial Isolates and Culture

One hundred and one isolates belonging to the Mastitis Pathogen Culture Collection (**MPCC**) of the Canadian Bovine Mastitis and Milk Quality Research Network (Ajitkumar et al., 2013) and 157 isolates sourced from Ghent University, Belgium (Supré et al., 2009), were used in the study, covering 16 different CNS species (Table 1). Isolates were mainly recovered from milk samples (n = 241; 93%), with the remainder isolated from teat apex swabs. The majority of the isolates were previously identified by *rpo*B gene sequencing with ≥94.0% sequence homology to the GenBank database (n = 238); the remainder were identified by sequencing of 16S rRNA (with ≥98.7% homology; n = 12) or *hsp*60 (with ≥97% homology; n = 8; Mellmann et al., 2006; Supré et al., 2009; Ajitkumar et al., 2013).

Stock isolates, which were stored at −80°C, were thawed at room temperature for 15 min. Columbia agar plates containing 5% sheep blood were streaked with a 1-μL sterile disposable loop to obtain isolated colonies. Plates were incubated for 18 to 24 h at 35°C. The following day, cultures were examined for growth and purity. Single colonies were sub-cultured onto blood agars and incubated for 18 to 24 h at 35°C.

MALDI-ToF Mass Spectrometry

Sample Preparation. All isolate classifications were carried out using the direct transfer method (MALDI Biotyper 3.1 User Manual, Bruker Daltonics Inc.). Briefly, a single-use, 15-cm sterile wooden applicator stick was used to lift material from a well-isolated bacterial colony followed by smearing a thin film of