Veterinary Microbiology 173 (2014) 147–151

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Identification of *Corynebacterium* spp. isolated from bovine intramammary infections by matrix-assisted laser desorption ionization-time of flight mass spectrometry

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ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form 16 June 2014
Accepted 28 June 2014

Keywords:
Subclinical mastitis
Corynebacterium spp.
Mass spectrometry

ABSTRACT

Corynebacterium species (spp.) are among the most frequently isolated pathogens associated with subclinical mastitis in dairy cows. However, simple, fast, and reliable methods for the identification of species of the genus *Corynebacterium* are not currently available. This study aimed to evaluate the usefulness of matrix-assisted laser desorption ionization/mass spectrometry (MALDI-TOF MS) for identifying *Corynebacterium* spp. isolated from the mammary glands of dairy cows. *Corynebacterium* spp. were isolated from milk samples via microbiological culture ($n = 180$) and were analyzed by MALDI-TOF MS and 16S rRNA gene sequencing. Using MALDI-TOF MS methodology, 161 *Corynebacterium* spp. isolates (89.4%) were correctly identified at the species level, whereas 12 isolates (6.7%) were identified at the genus level. Most isolates that were identified at the species level with 16 S rRNA gene sequencing were identified as *Corynebacterium bovis* ($n = 156$; 86.7%) were also identified as *C. bovis* with MALDI-TOF MS. Five *Corynebacterium* spp. isolates (2.8%) were not correctly identified at the species level with MALDI-TOF MS and 2 isolates (1.1%) were considered unidentified because despite having MALDI-TOF MS scores >2, only the genus level was correctly identified. Therefore, MALDI-TOF MS could serve as an alternative method for species-level diagnoses of bovine intramammary infections caused by *Corynebacterium* spp.

1. Introduction

Bovine mastitis is the most frequent and costly disease in the dairy industry and affects both the quality and yield of milk (Halasa et al., 2007). *Corynebacterium* spp. have been identified as common agents of subclinical intramammary infections (IMI) and are thought to account for 10–25% of the reported cases of this infection (Haltia et al., 2006; Schukken et al., 2009). These mastitis pathogens are generally associated with moderate increases in the somatic cell counts (SCC) from the affected dairy cows (Schukken et al., 2009).

Despite the high percentage of mastitis infections caused by *Corynebacterium* spp., these organisms cannot be accurately identified at the species level using conventional bacteriological techniques. Accordingly, knowledge regarding the number of species is limited, and the role of *Corynebacterium* in bovine mastitis is poorly understood.

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http://dx.doi.org/10.1016/j.vetmic.2014.06.028
0378-1135/

Moreover, conventional tests to identify *Corynebacterium* spp. in bovine milk have a reported misidentification rate of nearly 30% (Coyle and Lipsky, 1990; Watts et al., 2000) and, in most cases, isolate identification is used to distinguish the isolates from other more pathogenic microorganisms such as *Nocardia* spp. (Hogan et al., 1999). Hence, simple and rapid methods could assist in the identification of *Corynebacterium* spp. isolated from milk samples and allow a better understanding of their role in mastitis.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) can be used to rapidly detect and characterize bacterial strains; this method can detect a large range of biomolecules within complex mixtures at a high speed and sensitivity level (Maier et al., 2006; Welker, 2011). Our group has previously reported the use of MALDI-TOF MS to identify bacteria isolated from subclinical bovine mastitis samples (Barreiro et al., 2010; Braga et al., 2013); however these studies did not focus on the identification of mastitis-causing *Corynebacterium* spp. In bacterial characterization, the major use of MS is the direct acquisition of a unique protein biomarker profile for each microorganism from an intact sample (Welker, 2011).

Corynebacterium spp. isolated from clinical human samples have been identified by MS (Theel et al., 2012) but the identification of mastitis-causing *Corynebacterium* spp. has not yet been reported. Therefore, this study aimed to evaluate the ability of MALDI-TOF MS to identify *Corynebacterium* spp. isolated from the milk samples of dairy cows with IMI.

2. Materials and methods

2.1. Sample collection and Corynebacterium spp. isolation

Mammary quarter milk samples ($n = 1140$) were collected from 285 lactating cows that were distributed among 21 dairy herds (Oliver et al., 2004). A total of 180 isolates were presumptively identified as *Corynebacterium* spp. via microbiological culture. Samples with more than two morphologically distinct bacterial isolates ($n = 16$) were considered contaminated and were excluded from further analysis.

To isolate *Corynebacterium* spp. for microbiological culture-based identification, a 0.01-mL aliquot of milk was spread onto a quadrant of a blood agar plate that contained 5% defibrinated bovine blood. The plates were aerobically incubated at 37 °C ± 1 °C for up to 72 h and were evaluated for bacterial growth after 24, 48, and 72 h to evaluate the bacteria colonies according to the morphology (shape, size, number, and color), hemolytic ability (presence and type), and possible contamination (Oliver et al., 2004). After growth on blood agar, a single small, circular colony (approximately 1 mm in diameter) with a white–gray or yellowish color and a slightly raised, dry and/or flaky, and nonhemolytic appearance was streaked on a plate of trypticase soy agar (TSA, Becton, Dickinson and Company, Sparks, MD, USA) that had been supplemented with 1% Tween 80 (Sigma, St. Louis, MO, USA); this plate was then aerobically incubated at 37 °C ± 1 °C for 72 h. One loopful of a single colony was inoculated in 1 mL of trypticase soy broth (TSB, Becton, Dickinson and Company, Sparks, MD, USA) and aerobically incubated at 37 °C ± 1 °C for 72 h, followed by centrifugation at 10,000 × *g* for 10 min. The bacterial pellet was washed with 1 mL of autoclaved Milli-Q water and centrifuged again under the same conditions, after which 1 mL of TSB containing 10% glycerol was added, followed by homogenization. The contents were transferred to a sterile 1.5-mL microcentrifuge tube and cryopreserved at −20 °C. All isolates were cryopreserved and resuspended to ensure identification before being subjected to *Corynebacterium* spp. identification via 16S rRNA gene sequencing and MALDI-TOF MS.

2.2. MALDI-TOF MS sample preparation

Sample preparation for MALDI-TOF MS was performed as previously described (Barreiro et al., 2010). Briefly, a few (2–4) *Corynebacterium* spp. colonies from a fresh and pure overnight culture that had been grown on TSA supplemented with 1% Tween 80 at 37 °C under aerobic conditions were suspended in 300 μL of distilled water, to which 900 μL of absolute ethanol was added.

Subsequently, the microcentrifuge tubes containing the bacterial isolates in ethanol solution were centrifuged at 13,000 × *g* for 2 min, after which the supernatants were removed by careful pouring from the microcentrifuge tubes. After centrifugation, the remaining liquid was carefully removed with a pipette. The bacterial pellets were air dried at room temperature for 5–10 min. A 70% formic acid solution was added to lyse the cells and release the intracellular proteins, particularly the ribosomal proteins that produce the diagnostic ions required for MALDI-TOF MS fingerprinting (Ryzhov and Fenselau, 2001). The amount of formic acid solution added to each sample was proportional to the size of the pellet (usually 20–40 μL), and the microcentrifuge tubes were shaken vigorously to completely dissolve the pellets. Subsequently, an equal volume of 100% acetonitrile was added to each sample to yield a bacterial extract in a 1:1 solution of 70% formic acid and acetonitrile. A final centrifugation step was performed to separate the bacterial cell debris from the supernatant, as the latter contained the intracellular proteins used for bacterial identification.

The MALDI-TOF MS analyses were performed in a Bruker Autoflex (Bruker Daltonik, Bremen, Germany) Smart Beam III mass spectrometry unit that was operated in the linear mode and equipped with a 337-nm nitrogen laser. To prepare the MALDI target plate, 1 μL of each bacterial extract was placed onto a 384-spot target plate (polished stainless steel) and dried at room temperature. The dried sample spot was then overlaid with 1 μL of a matrix solution comprising α-cyano-4-hydroxy-cinnamic acid diluted in a solution of 50% acetonitrile and 2.5% trifluoroacetic acid.

The Bruker Bacterial Test Standard (BTS) was used for the mass calibration and instrument parameter optimization. Mass spectra data were collected within the range of 2000–20,000 *m/z*, using the FlexControl 3.3 software package. Three thousand laser shots were combined to generate each spectrum; the Biotyper 3.0 software package was used to analyze the results at the default settings.

The Biotyper Real Time Classification 3.0 software package (Bruker Daltonik, Bremen, Germany) was used to

process the raw spectra acquired by the Autoflex. The data were analyzed according to the built-in main spectra projection feature of the Biotyper software; this feature is a proprietary algorithm for spectral pattern matching that produces logarithmic scores from 0 to 3. Using the standard parameters of the pattern-matching algorithm, the peak lists were compared with each entry in the Biotyper database, which contained 3995 references (March, 2012). The identification score cut-off values were applied to each measurement as follows: a score of $\geq$2.000 indicated a species-level identification, a score of 1.700–1.999 indicated a genus-level identification, and a score of $\leq$1.699 indicated that no reliable identification could be performed. Isolates that presented identification disagreement between both methods were named misidentified (MI). On the other hand, isolates with identification agreement at genus level but not at species level by both identification methods were classified as unidentified (UI). All the isolates classified as MI and UI were tested three times.

2.3. DNA extraction and PCR

Prior to DNA extraction, all *Corynebacterium* spp. isolates were previously cultured overnight on TSA supplemented with 1% Tween 80 at 37 °C under aerobic conditions to evaluate the purity of the colonies. Subsequently, a single *Corynebacterium* spp. colony was suspended in 90 μL of autoclaved Milli-Q water and the resulting solution was centrifuged at 10,000 × *g* for 10 min. Next, 100 μL of a lysozyme buffer solution and 4 μL of lysozyme (10 mg/mL; Merck, Whitehouse Station, NJ, USA) were added to the bacterial pellet and the suspension was incubated at room temperature for 15 min. Subsequently, 100 μL of a resin solution (10 mg/mL, Chelex® 100 resin; Bio-Rad Laboratories, Richmond, CA, USA) was added to the suspensions, which were then heated in a thermocycler at 99 °C for 10 min (Vaneechoutte et al., 1995). The samples were centrifuged at 16,000 × *g* for 10 min, after which 5 μL of the supernatant were aliquoted for a conventional polymerase chain reaction (PCR) analysis. All PCR reactions were adjusted to a total volume of 50 μL and comprised the 5-μL supernatant aliquot as well as 45 μL of a PCR mixture (16 μL of distilled water, 25 μL of 2× Master Mix® (Promega, Madison, WI, USA), and 2 μL each of the forward and reverse primers).

All *Corynebacterium* spp. isolates were submitted to PCR amplification as described by Huxley et al. (2004) with the primer pair F-5′GCGAACGGGTGAGTAACACG3′ and R-5′TCTGCGATTACTAGCGACTCCG3′. Isolates that were not amplified by this amplification method were submitted to a second PCR analysis as described by Watts et al. (2000) with primers F-5′AGAGTTTGATCCTGGCTCAG3′ and R-5′AAGGAGGTGATCCAGCCGCA3′. This second amplification protocol was used to amplify other species than *C. bovis*. All PCR reactions targeted the 16S rRNA gene. The cycling conditions were set as described in Huxley et al. (2004) and Watts et al. (2000). The reactions were performed in a Veriti® Thermal Cycler (Life Technologies™, Foster City, CA, USA).

The resulting PCR products were analyzed via electrophoresis on a 1% (wt/vol) agarose gel prepared with Tris/borate/EDTA (TBE) buffer. A 4-μL aliquot of a molecular marker containing a 100-base pairs (bp) DNA Ladder® (Life Technologies™, Foster City, CA, USA) was added to the first well of each gel. The negative (PCR mix + primers) and positive controls (*C. bovis*) were added to the remaining wells of each gel.

After a 30-min electrophoretic run (5 min at 80 V and 25 min at 100 V), the 1250 bp DNA bands were visualized by ultraviolet transillumination. The bands of interest were isolated and purified with a commercially available gel purification kit (GE Health Care illustra™ GFXTM, Buckinghamshire, UK) according to the manufacturer's instructions.

2.4. Sequencing reactions and precipitation protocols

The isolates were genotypically identified by sequencing the 16S rRNA analytes as described previously by Watts et al. (2000). The purified PCR products were sequenced unidirectionally with the reverse primer. Each sequencing reaction was performed with 1 μL of primer R-5′AAGGAGGTGATCCAGCCGCA3′ (5 pmoL/μL), 1.5 μL of 5× buffer, 2 μL of purified DNA (20 ng/μL), 4.5 μL of nuclease-free water and 1 μL of BigDye® from the Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The PCR cycling conditions were the same as those described in a previous section.

The precipitation procedure included two ethanol washes (Sigma, St. Louis, MO, USA). The first wash was accomplished with 21 μL of absolute ethanol, the solution was held at room temperature for 5 min and subsequently centrifuged at 3000 × *g* for 30 min at 15 °C. The second wash was accomplished with 35 μL of 75% ethanol, and the solution was centrifuged at 1650 × *g* for 15 min at 15 °C. The ethanol was discarded by inverting the plates, which were then heated to 95 °C for 8 min followed by the addition of 10 μL of formamide Hi-DiTM (Life Technologies™, Foster City, CA, USA). The sequencing reaction products were analyzed via automatic sequencer capillaries on the ABI 3500 Genetic Analyzer® (Applied Biosystems, Foster City, CA, USA).

All sequences obtained from the 16S rRNA gene sequences were confirmed in the GenBank Library Reference online data. Isolates were regarded as identified at the species level when their similarities to reference sequences were $\geq$98% (Watts et al., 2000).

3. Results and discussion

The MALDI-TOF MS data were analyzed using the Biotyper software, which is a database that contains conserved bacterial protein profiles. In our study, MALDI-TOF MS correctly identified 161 (89.4%) *Corynebacterium* spp. isolates at the species level (Table A1). Additionally, MALDI-TOF MS correctly identified 12 *Corynebacterium* spp. isolates (6.7%) at the genus level, resulting in a total identification at both species and genus level of 173 isolates (96.1%). This rate is higher than 88.5% reported by Theel et al. (2012) with a tube extraction of ribosomal proteins. In our study, tube extraction was used specifically to increase the identification scores as described previously by Alatoom et al. (2012).

Nine isolates (5%) that were identified by 16S rRNA gene sequencing as *C. bovis* ($n = 6$), *Corynebacterium auriscanis* ($n = 2$), and *Corynebacterium efficiens* ($n = 1$) were correctly identified at the species level by MALDI-TOF MS; however, the scores ranged from 1.7 to 2. Three isolates (1.7%) that were identified by 16S rRNA gene sequencing as *Corynebacterium* sp., *C. bovis* and *Corynebacterium amycolatum* were identified by MALDI-TOF MS as *C. auriscanis*, *Corynebacterium pseudotuberculosis* and *Corynebacterium* sp., respectively. However, these isolates were identified by MALDI-TOF MS with scores ranging from 1.7 to 2, indicating identification only at the genus level (Table A1).

Five isolates (2.8%) were not correctly identified at the species level by MALDI-TOF MS but were identified by 16S rRNA gene sequencing as *C. bovis* ($n = 4$) and *Corynebacterium* spp.; however, when these five isolates were analyzed by MALDI-TOF MS, the identification scores ranged from 1.7 to 2 and were identified as *Arthrobacter globiformis* ($n = 1$), *Bacillus clausii* ($n = 1$), *Curtobacterium luteum* ($n = 1$) and *Propionibacterium acnes* ($n = 2$). Therefore, the MALDI-TOF MS results for these five isolates were classified as misidentified (MI). A possible explanation for these MI results could be based on a previous phylogenetic study of these non-*Corynebacterium* species, which were reported to have chemotaxonomic similarities to *Corynebacterium* spp. (Pascual et al., 1995). Additionally, the MALDI-TOF MS results for two isolates (1.1%) were considered to be unidentified (UI) because despite having a MALDI-TOF scores >2, only the genus levels were correctly identified (Table A1). These identification failures likely reflect the lack of reference spectra for these microorganisms in the Biotyper 3.0 database (Maier et al., 2006). All of the isolates classified as MI and UI were tested three times according to the MALDI-TOF MS method. Among the *Corynebacterium* spp. isolates that were identified by 16S rRNA gene sequencing at the species level, 156 (86.7%) were identified as *C. bovis*, the species with the highest frequency of identification, followed by *Corynebacterium casei* ($n = 1$) and *Corynebacterium xerosis* ($n = 1$). The current study yielded a higher frequency of *C. bovis* relative to other *Corynebacterium* spp. in the milk samples from cows with intramammary infection, a finding that was similar to those reported for previous studies (Huxley et al., 2004; Watts et al., 2000).

However, four isolates were only identified at the genus level by 16S rRNA gene sequencing. These isolates were successfully identified by MALDI-TOF MS as *C. xerosis* ($n = 2$) and *C. bovis* ($n = 1$) with scores ≥2.0 and as *C. auriscanis* ($n = 1$) with scores of 1.7–2 (Table A1).

In our study, the identification cutoff scores used for *Corynebacterium* spp. identification by MALDI-TOF MS were ≥2.0 (species level) and ≥1.7 (genus level); these were similar to the cutoff scores described in other studies (Alatoom et al., 2012; Konrad et al., 2010; Vila et al., 2012). In contrast, Theel et al. (2012) suggested the use of lower cutoff score values such as ≥1.5 (genus level) and ≥1.7 (species level).

The MALDI-TOF MS analysis described in the present study might serve as an alternative method for *Corynebacterium* spp. identification and might help to elucidate the role played by these species in dairy cow intramammary infections.

Acknowledgments

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil (FAPESP) for research funding (2011/14284-5) and to José Garcia Moreno Franchini and Lucinéia Mestieri for technical assistance.

Appendix A. Appendix

Table A1

Table A1
Corynebacterium spp. isolates identified by 16S rRNA gene sequencing versus MALDI-TOF MS.

16S rRNA gene sequencing		MALDI-TOF MS			
Corynebacterium species	*n*	Correctly identified at species level (scores of >2)		MI[1]	UI[2]
		n	Suggested species		
Corynebacterium aquilae	2	0	*Corynebacterium* sp.	0	2
Corynebacterium bovis	156	156	*Corynebacterium bovis*	0	0
Corynebacterium bovis	1	0	*Arthrobacter globiformis*	1	0
Corynebacterium bovis	1	0	*Bacillus clausii*	1	0
Corynebacterium bovis	1	0	*Curtobacterium luteum*	1	0
Corynebacterium bovis	1	0	*Propionibacterium acnes*	1	0
Corynebacterium casei	1	1	*Corynebacterium casei*	0	0
Corynebacterium xerosis	1	1	*Corynebacterium xerosis*	0	0
Corynebacterium sp.	2	2	*Corynebacterium xerosis*	0	0
Corynebacterium sp.	1	1	*Corynebacterium bovis*	0	0
Corynebacterium sp.	1	0	*Paenibacillus lactis*	1	0
Subtotal	168	161		5	2
		Correctly identified at genus level (scores of 1.7–2)			
Corynebacterium amycolatum	1	1	*Corynebacterium* sp.	0	0
Corynebacterium auriscanis	2	2	*Corynebacterium auriscanis*	0	0
Corynebacterium bovis	6	6	*Corynebacterium bovis*	0	0
Corynebacterium bovis	1	1	*Corynebacterium pseudotuberculosis*	0	0
Corynebacterium efficiens	1	1	*Corynebacterium efficiens*	0	0
Corynebacterium sp.	1	1	*Corynebacterium auriscanis*	0	0
Subtotal	12	12		0	0
Total	180	173		5	2

[1] MI is the misidentified.
[2] UI is the unidentified.

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