Short communication: Identification of *Corynebacterium bovis* by MALDI-mass spectrometry

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ABSTRACT

Corynebacterium bovis is a mastitis-causing micro-organism responsible for economic losses related to decrease in milk production. The aim of the study was identify *Corynebacterium* spp. strains recovered from milk samples of subclinical mastitis by using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Samples were collected during a 10-mo mastitis-monitoring program in a high-production dairy farm. In this study, 80 strains were analyzed; from these 54 (67.5%) were identified at species level as *Corynebacterium bovis*, 24 (31.2%) isolates were identified at the genus level as *Corynebacterium* spp., and only 1 (1.35%) isolated had unreliable identification. Results demonstrated that MALDI-MS could be an important technique for the identification of *Corynebacterium* spp. in milk.

Key words: mastitis, *Corynebacterium bovis*, proteomic characterization

Short Communication

Mastitis is an important cause of economic losses in dairy farms. This disease has great relevance to public health mainly by direct transmission of food-borne pathogens or due to the ingestion of their produced toxins and enterotoxins (de Freitas Guimarães et al., 2013; Langoni, 2013).

The most frequent etiological agents of bovine mastitis are *Staphylococcus aureus*, CNS, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus*

uberis, *Escherichia coli*, and *Corynebacterium bovis*. The latter is the most common among *Corynebacterium* spp. involved in bovine IMI (Watts et al., 2001). Whereas many authors consider *C. bovis* either a normal inhabitant of the teat canal or a minor pathogen of bovine mastitis (Pankey et al., 1985; Ngatia et al., 1991; Bexiga et al., 2011), other studies described this organism as an important causative agent of mastitis cases (Costa et al., 1985; Haltia et al., 2006; Schukken et al., 2009; Langoni et al., 2016) with significant deteriorating effects on milk production (Domingues et al., 1998; Gonçalves et al., 2016).

Studying mammary glands naturally infected by *C. bovis*, Victória et al. (2005) observed median SCC equal to 262×10^3 and 806×10^3 cells/mL in milk samples negative and positive to *C. bovis*, respectively, with some samples as high as 1×10^6 cells/mL. Currently available techniques used for *Corynebacterium* spp. identification are laborious biochemical schemes or molecular diagnosis, requiring the use of many reagents and specific primers (besides previous standardization), specialized laboratories, and professional expertise (Bexiga et al., 2011).

The matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) technique permits rapid, precise, and species-specific diagnosis for identification of mastitis pathogens (Gonçalves et al., 2014; Tomazi et al., 2014). Considering the relevance of *C. bovis* as an etiological agent on bovine mastitis, the objective of the present study was to identify *Corynebacterium* spp. strains recovered from milk samples of subclinical mastitis by MALDI-MS.

The criterion adopted for selection was the isolation of *Corynebacterium* spp. during a 10-mo, monthly sample strategy of all lactating cows in a large high-production dairy farm. A total of 244 Holstein cows

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were evaluated. The entire herd of lactating cows was screened using a strip cup and the California mastitis test (Schalm and Noorlander, 1957). Mammary quarters positive by strip cup test or California mastitis test score 1 to 3 were aseptically sampled and milk samples were stored in refrigerated isothermal boxes for transport to the laboratory for bacteriological analysis (NMC, 1999).

In the laboratory, 10 µL of each sample was streaked both on 8% sheep blood agar and on MacConkey agar (Oxoid Ltd., Basingstoke, UK) plates. The inoculated plates were incubated, aerobically, at 37°C during 3 d, with growth observation every 24 h (NMC, 1999). *Corynebacterium* spp.-suspected colonies were morphologically characterized by Gram stain, phenotypically classified according to biochemical tests suggested by Krieg and Holt (1994), and then inoculated in brain and heart infusion for proteomic analysis

Pleomorphic gram-positive isolates with characteristics of *Corynebacterium* spp. were cryopreserved (1:1) by adding 500 µL of Tween 80 (Vetec, Rio de Janeiro, Brazil) enriched brain and heart infusion containing microorganisms in 500 µL of 40% sterile glycerol in sterile 1.5-mL cryogenic tubes, kept frozen at -20°C until characterization was performed by MALDI-MS (Barreiro et al., 2010; Gonçalves et al., 2014; Tomazi et al., 2014).

Samples were thawed and individually plated by streaking on 8% sheep blood agar and then incubated under 37°C for 48 h. For MALDI-MS, colonies were transferred to sterile microtubes filled with 300 µL of deionized water, homogenized, and 900 µL of ethanol (HPLC) was added for bacterial inactivation.

Microtubes were centrifuged at 1,500 × g for 3 min at room temperature and the supernatant was discarded inverting the tubes. After another centrifugation, the remaining ethanol was removed. For bacterial lysis, after drying for 30 min at room temperature, a 70% formic acid solution was mixed at a sufficient volume to cover the pellet (approximately 30 µL). The content was homogenized in a shaker for 2 min until the whole pellet was completely undone. We then added 30 µL of 100% acetonitrile, homogenized the mixture in a tube shaker, and centrifuged at 14,000 × g for 3 min

at room temperature to separate proteins from bacterial cells, specifically the ribosomal proteins (Fenselau and Demirev, 2001) from which MALDI-MS spectrums were obtained and used to identify the agent.

One microliter of this bacterial extract was placed onto a 384-well plate (Bruker Daltonics, Billerica, MA) and dried at room temperature. The dried bacterial extract was overlaid with 1.0 µL of matrix solution composed of α-cyano-4-hydroxy-cinnamic acid (CHCA) diluted in 50% acetonitrile and 2.5% trifluoroacetic acid.

The MALDI-MS analysis was performed in an Autoflex III (Bruker Daltonics, Bremen, Germany) MALDI time-of-flight mass spectrometer operated in the linear mode and equipped with a 337-nm smart beam laser using FlexControl 3.3 software (Bruker Daltonics). The bacterial test standard (Bruker Daltonics) was used for the mass calibration. The mass spectra were collected within the mass range of *m/z* 2,000 to 20,000. The spectra were analyzed with the database library MALDI Biotyper 3.0 software (Bruker Daltonics). The algorithm used by MALDI Biotyper confronts the spectra of the unknown sample with pattern samples existing in a database (Lartigue et al., 2009).

Out of 1,813 milk samples submitted to microbiological culture, *Corynebacterium* spp. strains were isolated in 600 (33%) at the beginning. Considering the 244 studied cows, 50 (20.5%) were positive for *Corynebacterium* spp. in one or more teats; from these teats, the same pathogen was recurrently isolated at least once again in 23 (46%), which were included in the study.

In relation to the persistence of this pathogen causing subclinical infection during the lactation, it was observed that in 43.48% (10/23) of these animals *Corynebacterium* spp. was recovered only twice, within a mean time equal to 111 d of subclinical infection. In 26.1% (6/23), there were 3 isolations, with an average of 190 d of subclinical infection; 30.43% (7/23) showed 4 or more isolations within a mean time of 197 d of infection.

According to the MALDI-MS technique, 54 (67.5%) *Corynebacterium* spp. isolated were identified as *Corynebacterium bovis* (Table 1), reinforcing its involvement in mastitis epidemiology of the considered herd. This

Table 1. Identity of 80 isolates of *Corynebacterium* spp. according to the pattern *Corynebacterium bovis*, according to matrix-assisted laser desorption/time-of-flight mass spectrometry (MALDI-MS) technique

Number of samples	Percentage	Identity with <i>Corynebacterium bovis</i> pattern
54	67.50	High identity with pattern sample
25	31.25	Probable identity with pattern sample
1	1.25	Unidentified species
80 (total)	100	

supported the conclusions of Costa et al. (1985) and Haltia et al. (2006), who found this pathogen in subclinical and clinical mastitis cases.

The genus *Corynebacterium* includes over 85 species isolated from animals or humans beings (Funke and Bernard, 2011). Species identification of *Corynebacterium* is challenging, and clinical laboratories need better strategies for identification. Alatoom et al. (2012) studied 92 *Corynebacterium* spp. clinical isolates from human cases and concluded that MALDI is a useful approach for identification of this genus species; however, *C. bovis* was not included among these isolates. Gonçalves et al. (2014) analyzed 180 isolates presumptively classified as *Corynebacterium* spp. and verified that MALDI correctly identified 161 (89.4%) of the *Corynebacterium* spp. isolates at species level.

According to the results of the present study, *C. bovis* were identified in 67.5% of all *Corynebacterium* spp. isolates; *C. bovis* was positively identified in 31.25% isolates. This demonstrates that MALDI-MS could be an important tool for the identification of these pathogens and confirms the importance of this pathogen as subclinical mastitis agent.

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