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Species level identification of coagulase negative *Staphylococcus* spp. from buffalo using matrix-assisted laser desorption ionization–time of flight mass spectrometry and *cydB* real-time quantitative PCR

Lucas J.L. Pizauro[a,1], Camila C. de Almeida[b,1], Glenn A. Soltes[c,1], Durda Slavic[d], Oswaldo D. Rossi-Junior[a], Fernando. A. de Ávila[b], Luiz. F. Zafalon[e], Janet I. MacInnes[c,*]

[a] Department of Veterinary Preventive Medicine and Animal Reproduction, São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, Jaboticabal, Brazil
[b] Department of Veterinary Pathology, São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, Jaboticabal, Brazil
[c] University of Guelph, Department of Pathobiology, 50 Stone Rd. East, Guelph, Ontario, N1G 2W1, Canada
[d] Animal Health Laboratory, University of Guelph, Post Office 3612, Guelph, Ontario, N1H 6R8, Canada
[e] Brazilian Agricultural Research Corporation (EMBRAPA), Embrapa Southeast Livestock, Washington Luiz road, Km 234, 13560-970, Sao Carlos, Sao Paulo, Brazil

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ABSTRACT

Incorrect identification of *Staphylococcus* spp. can have serious clinical and zoonotic repercussions. Accordingly, the aim of this study was to determine if matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and/or *cydB* real- time quantitative PCR (qPCR) could be used to accurately identify coagulase negative *Staphylococcus* spp. (CoNS) obtained from buffalo milk and milking environment samples. Seventy-five of 84 CoNS isolates could be identified to the species level (score value > 1.99) using MALDI-TOF MS. However, as determined by cytochrome d ubiquinol oxidase subunit II (*cydB*) qPCR and by 16S RNA and *cydB* gene sequencing, 10 *S. agnetis* strains were wrongly identified as *S. hyicus* by MALDI-TOF MS. In addition, 9 isolates identified by MALDI-TOF only to the genus level (score values between 1.70 and 1.99) could be identified to species by *cydB* qPCR. Our findings suggest that MALDI-TOF MS is a reliable method for rapid identification of *S. chromogenes* and *S. epidermidis* (species of interest both in human and veterinary medicine) and may be able to correctly identify other *Staphylococcus* spp. However, at present not all *Staphylococcus* spp. found in buffalo milk can be accurately identified by MALDI-TOF MS and for these organisms, the *cydB* qPCR developed in the current study may provide a reliable alternative method for rapid identification of CoNS species.

1. Introduction

Buffalo milk and milk products are of growing importance in the dairy industry worldwide (Camargo et al., 2015). Recent studies have suggested that CoNS can be significant mastitis causing-agents in buffalo (El-Jakee et al., 2013). For example, in studies of Chavoshi and Husaini (2012), coagulase negative staphylococci (CoNS) were the most frequently isolated pathogens in buffalo subclinical mastitis in Iran. In Italy, 66% subclinical mastitis in buffalos were caused by CoNS (Moroni et al., 2006). *Staphylococcus* spp. can cause a variety of pathological effects on the mammary gland. For example, coagulase negative *Staphylococcus* species (CoNS) can induce a mild inflammatory reaction and a modest increase in milk somatic cell counts (SCC) in dairy cattle (Schukken et al., 2009). Thought to act mainly as minor mastitis pathogens, CoNS are also reported to block udder infection by major mastitis pathogens (Piepers et al., 2013). *Staphylococcus* spp. can be transmitted by food products including milk and affect healthy people. Like *Staphylococcus aureus,* some CoNS species secrete enterotoxins that are responsible for food poisoning (Nunes et al., 2016).

Identification of CoNS can be a very difficult and time consuming task. Failure to identify staphylococcal species can have serious clinical and zoonotic repercussions (Martins and Cunha, 2007). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) can differentiate microorganisms based on their protein profiles. Increasingly, MALDI-TOF MS is being used in human medicine and more recently, in veterinary medicine. MALDI-TOF MS is reported

* Corresponding author.
E-mail address: macinnes@uoguelph.ca (J.I. MacInnes).
[1] These authors contributed equally to this work.

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to correctly identify 95% of the CoNS species and is considered a rapid and reliable method of identification (Randall et al., 2015).

Compared with classical microbiological methods, molecular techniques such as qPCR may offer greater specificity for the identification of microorganisms (Britten, 2012). However, to date, no standard methodology has been widely accepted for the identification of the causative agents of mastitis (Tomazi et al., 2014). An RFLP test for *groEL* gene has been developed, but it is time consuming (Santos et al., 2008). Similarly, amplicon PCR sequencing-based tests based on the *tuf* (Hwang et al., 2011), *sodA* (Poyart et al., 2001) and *rpoB* genes are time consuming and expensive. In this study, we used the well-conserved gene *cydB* together with MALDI-TOF MS to identify CoNS to the species level. The *cydB* primer sets developed provide a new approach to complement MALDI-TOF and other methods for the rapid and accurate identification of CoNS to the species level.

2. Materials and methods

2.1. Sample collection

A total of 320 milk samples were obtained from all 4 quarters of 80 randomly selected female buffaloes on a dairy farm located in Sao Paulo State, Brazil during the period of November 2013 to April 2014. Animals were sampled once. A mammary gland physical examination of each animal was first performed (Radostitis et al., 2007) and the milk from each mammary quarter was submitted to a strip cup test and a California Mastitis Test (CMT) (Schalm and Noorlander, 1957). Prior to collection, teats were aseptically cleaned with 70% ethanol. Samples were also collected with sterile swabs (Pro-Lab Diagnostics) from the hands of consenting milkers (n = 16), from nares and mouths of calves (n = 20), and from liners (n = 32) and stored in peptone water as described previously (Silva et al., 2000). In addition, water samples (n = 8) from two water hoses used to clean the teats were collected in sterile plastic bottles. This study was approved by the Ethics Committee on Animal Use (CEUA) Sao Paulo State University protocol number 02663/14.

2.2. Staphylococcus spp. isolation and identification

A total of 10 μL of milk was streaked on 5% sheep blood agar and incubated at 37 °C for 24 and 48 h. Isolation and identification of *Staphylococcus* spp. were performed according to the guidelines of the National Mastitis Council (National Mastitis Council, 2004). Cultures results were classified as positive based on the presence of three or more identical colonies. After colony morphology and Gram-stain reaction analysis, the Gram-positive bacterial isolates were separated into *Streptococcus* spp. or *Staphylococcus* spp. based on the catalase test. *Staphylococcus* spp. (catalase positive) further underwent the coagulase test and coagulase negative isolates were designated as CoNS. Isolates were preserved in brain heart infusion broth (BHI, Becton, Dickinson and Company, Sparks, MD, USA) with 20% glycerol at −70 °C for further analyses.

2.3. MALDI-TOF MS

The identification of CoNS at the species level was performed using a MALDI Bruker Biotyper system (Bruker Daltonics Inc., Billerica, MA, USA) at the Animal Health Laboratory, University of Guelph, Guelph, Ontario, Canada. Results were analyzed using Biotyper 3.0 software which included over 5500 reference spectra. For MALDI-TOF analysis, pure cultures of CoNS were grown on 5% sheep blood agar for 24 h. A small amount of bacterial growth was transferred to the spots of an MSP 96 polished steel BC target plate (Microflex LT, Bruker Daltonics/BD, Germany, USA) using a disposable loop, then 1 μL of Matrix [α-cyano-4-hydroxycinnamic acid (HCCA)] was added and the plates were air-dried 1 to 2 min at room temperature. The resultant mass spectra were analyzed and compared with the reference spectra. Bacterial identification to the species level was achieved if the score value was equal or greater than 2.00. A score value between 1.70 and 1.99 was accepted as accurate genus level identification and presumptive species level identification.

2.4. cydB qPCR

qPCR primer pairs were designed based on the *cydB* (cytochrome d ubiquinol oxidase subunit II) gene sequences of 13 coagulase negative *Staphylococcus* spp. (i.e., *S. epidermidis* (NZ_JZUL01000001.1-1), *S. haemolyticus* (NZ_JFOJ01000002.1), *S. warneri* (ACPZ01000027.1-1), *S. xylosus* (NZ_CP007208.1), *S. chromogenes* (JMJF01000001.1), *S. saprophyticus* (AP008934.1-1), *S. caprae* (GL545272.1), *S. agnetis* (NZ_CP009623.1), *S. hominis* (NZ_AKGC01000038.1-1), *S. equorum* (NZ_CAJL01000020.1), *S. sciuri* (NZ_LDTK01000063.1), *S. pasteuri* (CP004014.1), *S, hyicus* (NZ_CP008747.1)) obtained from the NCBI nucleotide database. The *cyd*B gene sequences of the above-mentioned *Staphylococcus* spp. were first aligned using the basic local alignment search tool Blast (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) and CLC Sequence View 7 software to look for unique species-specific regions (Fig. 1). Based on these analyses, primers were designed using PrimerQuest software (Integrated DNA technologies, Inc. http://www.idtdna.com) to specifically amplify *cydB* genes in the above species (Table 1). Analyses for the detection of the *cyd*B gene were also done by "*in silico*" PCR using the program http://insilico.ehu.es/PCR/ for the species that had sequences available in the program database. The predicted size and sequence (forward and reverse) of one amplicon from each primer pair was confirmed by DNA sequencing (Laboratory Sciences Division, Agriculture and Agrifood Laboratory, Guelph). 16S rRNA gene sequencing was also done to confirm the identity of one *cydB* qPCR positive *S. hyicus* and *S. agnetis* isolate.

2.5. DNA extraction

For DNA extraction, well isolated colonies were used to inoculate tubes containing 5 mL of BHI broth. After incubation at 37 °C for 18 h, genomic DNA extraction was done using a modified method of Kuramae-Izioka (1997). One mL of bacterial culture was transferred to 2.0 mL microfuge tubes, centrifuged at 13,400 × *g* for 2 min, and the cell pellet re-suspended in 700 μL of extraction buffer [160 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 20 mM NaCl and SDS 0.5% (w/v)]. The cell suspensions were then homogenised by vortexing and incubated in a water bath at 65 °C for 40 min with vortexing every 10 min. Next, 300 μL of 5 M potassium acetate was added and the homogenates were incubated in an ice bath for 30 min with inversion every 15 min. Following this incubation, the samples were removed from ice and 650 μL of a chloroform and isoamyl alcohol [24: 1(v/v)] was added. The samples were then mixed by inversion for two min and centrifuged at 13,400 × *g* for 10 min at 4 °C. Following centrifugation, the supernatants were transferred to new tubes and the DNA was precipitated by the addition of 1000 μL of ice-cold absolute ethanol. The DNA precipitate mixtures were mixed by inversion and placed at −20 °C overnight prior to recovery of the DNA by centrifugation at 13,400 × *g* for 10 min at 10 °C. The resultant DNA pellets were air dried for 30 min and suspended in 30 μL 10 μM Tris-HCl, pH 7.3, 0.1 μM EDTA (TE).

2.6. Real time PCR (qPCR) and amplicon sequence

Real-time PCR primers (Table 1) were used at a concentration of 10 pmol/μL in 25 μL reaction mixtures containing 5 μL DNA and 20 μL Light Cycler 480 (LC480) Probe Master mix (Roche Diagnostics, Indianapolis, IN). Amplification was done in an LC480 real-time PCR instrument using the following cycling parameters: 1 cycle of 95 °C for 10 min, followed by 40 cycles consisting of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 25 s. Ramp rates were 4.4 °C/s, 2.2 °C/s, and 4.4 °C/s,

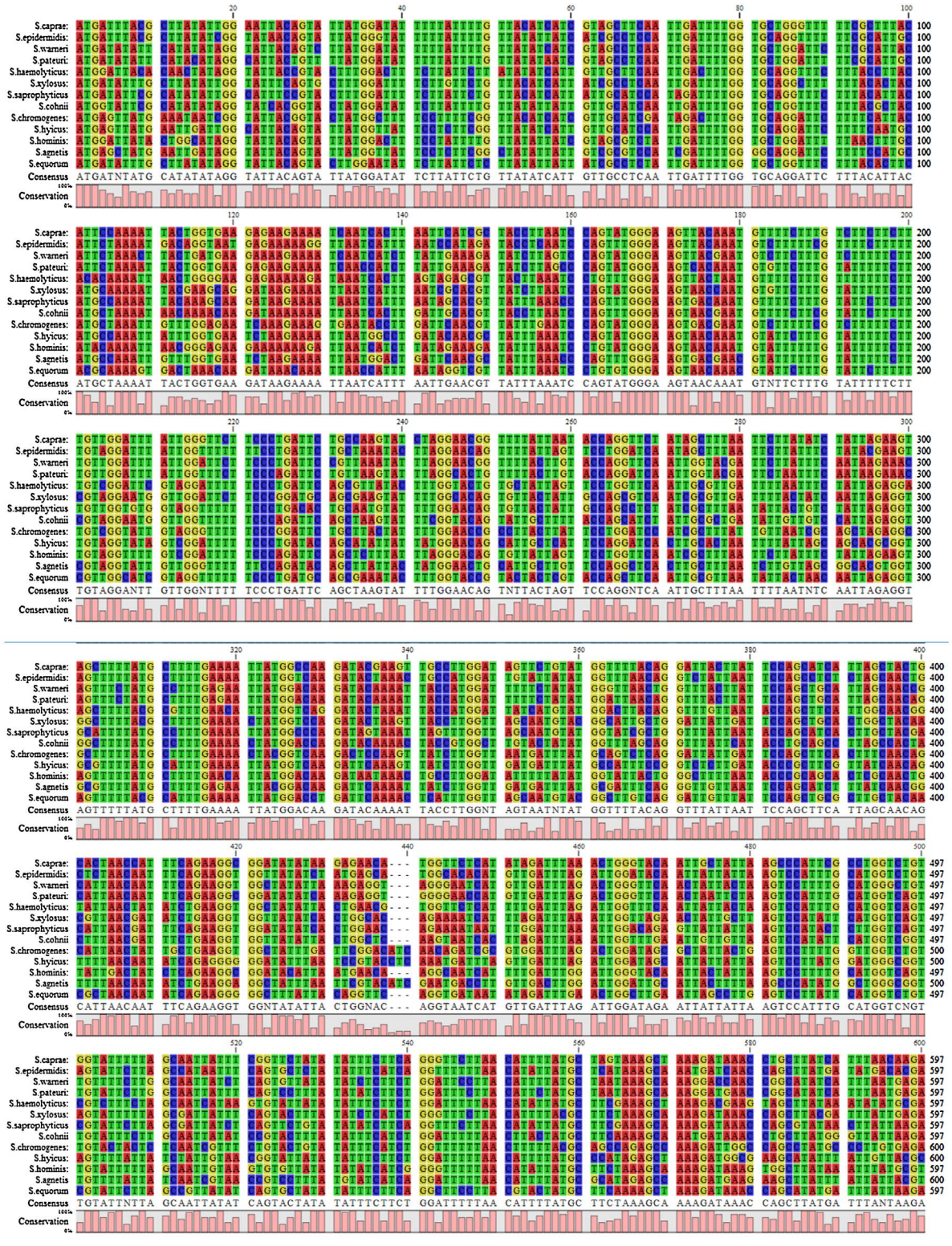

Fig. 1. Alignment of *cydB* genes from selected CoNS.

respectively, negative (*Streptococcus suis*), and water controls were included with each primer pair. In addition, a melting step (predicted primers melting temperature of 62 °C) was done at the end of each run to confirm the presence of a single product.

3. Results

3.1. Microbial isolation

In about 33.7% of 320 samples of milk, abundant colonies sugges-

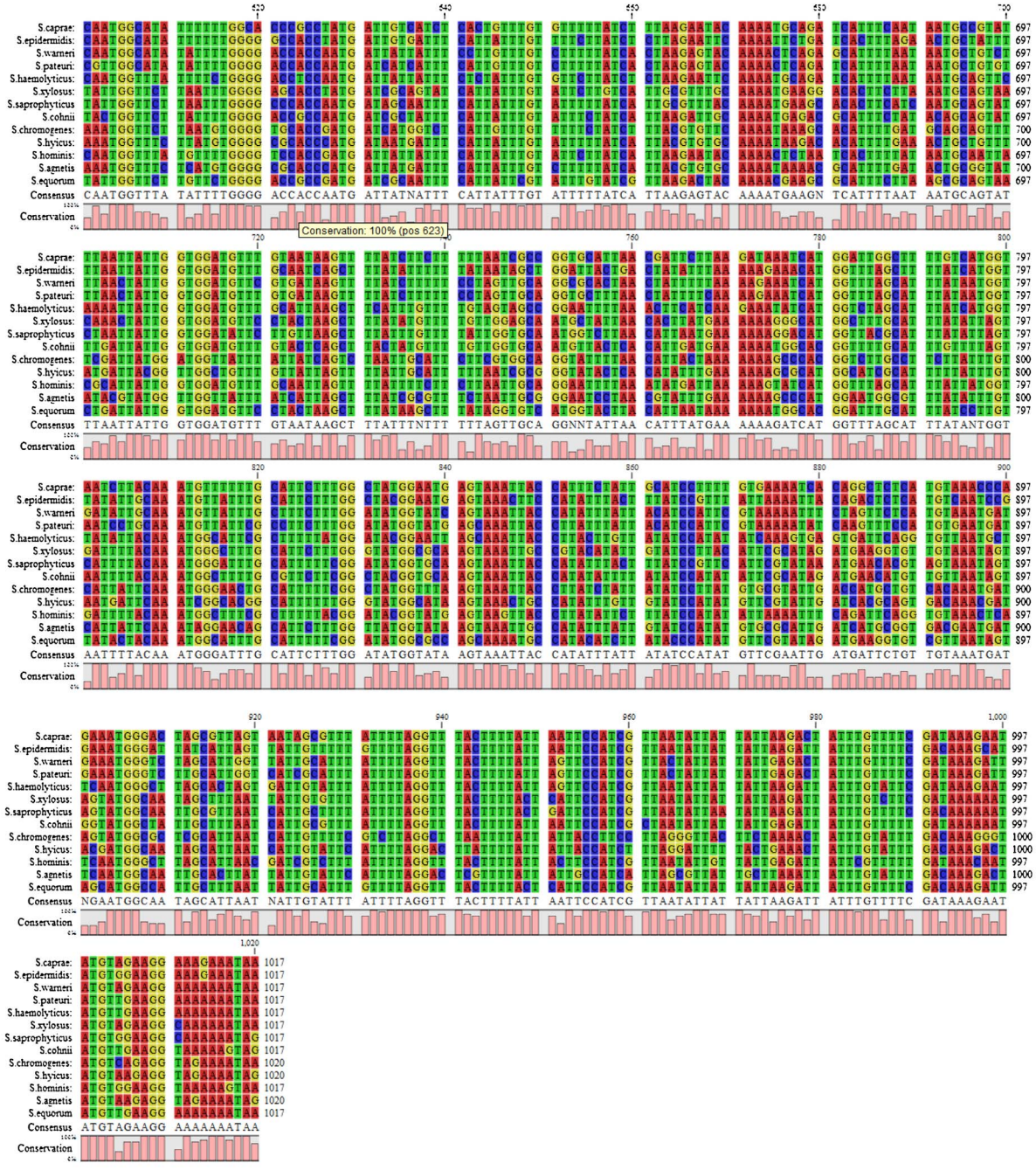

Fig. 1. (*continued*)

tive of *Staphylococcus* spp. were observed. Single colonies were steaked onto TSA agar for further testing. Based on their Gram, catalase and coagulase test reactions, 84 independent coagulase negative *Staphylococcus* spp. were isolated from milk (n = 57), liners (n = 10), milker hands (n = 12), and calves (n = 5).

3.2. Species level identification by MALDI-TOF MS

Analysis of the 84 CoNS samples by MALDI-TOF MS revealed 75 belonged to one of 11 different *Staphylococcus* species (i.e., had a score value > 1.99) namely: *S. chromogenes* (n = 47), *S. caprae* (n = 1), *S. epidermidis* (n = 6), *S. equorum* (n = 2), *S. haemolyticus* (n = 2), *S. hominis* (n = 1), *S. hyicus* (n = 10), *S. pasteuri* (n = 1), *S. sciuri* (n = 3), *S. saprophyticus* (n = 1), *S. warneri* (n = 1). Nine CoNS with scores of 1.7 to ≤1.99 were assigned to genus with a presumptive species identification of *S. hyicus* (n = 4), *S. sciuri* (n = 2) or *S. chromogenes* (n = 3).

3.3. cydB gene alignment

Alignment of available gene sequences revealed that the *cydB* gene is well conserved in the genus *Staphylococcus*, but there is sufficient nucleotide variation to allow for the design of species-specific primers (Fig. 1). All of the PCR products generated using the species-specific *cydB* primers had 97–99% nucleotide sequence identity with the reference sequences in the NCBI nucleotide database.

3.4. Real time PCR results

There was generally good correlation between the MALDI-TOF MS

Table 1
Primer pairs for amplification of the *cydB* gene in CoNS.

Gene	Primer	Sequence (5′-3′)	Product (bp)
cyd-caprae	Forward	ACGCAAGTAAAGCACAAGATAAG	206
	Reverse	CGTTAAAGCACCTGCAATTAGG	
cyd-epidermidis	Forward	GGCTGTTAATTCCAGCTTCTCT	235
	Reverse	TGCCATTGTCGTGTCATATCAT	
cyd-warneri	Forward	AAAGGATGAACCGGCATATCA	268
	Reverse	ACCATATCCAAAGAATGCGAATAAC	
cyd-pasteuri	Forward	GTTTGGGAAGTAACCAACGTATTT	229
	Reverse	ATGCAGCCGGAATCAACA	
cyd-haemolyticus	Forward	GCATGGTCAGTCGTCTTTCT	420
	Reverse	GCCCATTGAAGCATTAACACC	
*cyd-*xylosus	Forward	GTACGGCATTGCTGGATTATTG	282
	Reverse	GATACTGCGATCATAGGTGCTC	
cyd-saprophyticus	Forward	GCCAGCCTCTATCGCTTTAATA	250
	Reverse	CGCTAAGAATACGACTGACCAA	
cyd-sciuri	Forward	TACAGTGCTTTGGCTCTTTCT	430
	Reverse	GTGAACGCCATCACTTGTTTC	
cyd-chromogenes	Forward	CGCGTGGATTTAGACTGGATAG	304
	Reverse	CTGCCACGAAGAATGCAATTAG	
cyd-hyicus	Forward	CTCTTGATACCCGCTTCGTTAT	210
	Reverse	TTCGCCATCTTTAGCTCTATGG	
cyd-hominis	Forward	CACTCGCAACTGTATTGACTATCT	226
	Reverse	CAGAACATAAACCATTGACGCATAA	
cyd-equorun	Forward	TCCCTGATGCAGCGAAATAC	293
	Reverse	AACGGCTAAGAATACGACAGAC	
cyd-agnetis	Forward	CGCATAGAGCCAAAGATGAAGA	204
	Reverse	ACGTTAGGATTCCCGCAATTAG	

Table 2
Identity of coagulase negative *Staphylococcus* species by MALDI-TOF MS versus *cydB* qPCR results.

Identity by qPCR	No. of Isolates	MALDI-TOF MS		
		Genus	Species	Correlation
S. chromogenes	50	3	47	94%
S. epidermidis	6	0	6	100%
S. hyicus	0	4	10	0%
S. agnetis	14	0	0	0%
S. pasteuri	1	0	1	100%
S. sciuri	5	2	3	60%
S. hominis	1	0	1	100%
S. haemolyticus	2	0	2	100%
S. warneri	1	0	1	100%
S. caprae	1	0	1	100%
S. saprophyticus	1	0	1	100%
S. equorum	2	0	2	100%

and the *cydB* qPCR results (Table 2). However, all of the isolates that were classified as *S. hyicus* by MALDI-TOF MS (n = 10) were identified as *S. agnetis* by *cydB* q*PCR*. Their identity as *S. agnetis* was confirmed using16S rRNA gene sequence as the gold standards. As well, 2 of 5 *S. sciuri* and *3* of the 50 *S. chromogenes* isolates were identified only at the genus level by MALDI-TOF MS. Confirmation of their identity as *S. sciuri* and *S. chromogenes* was also verified by 16S rRNA gene sequencing.

4. Discussion

In this study, 84 CoNS isolates obtained from 320 buffalo milk and milking environment samples were evaluated by MALDI-TOF MS and a novel *cydB* qPCR test. Although MALDI-TOF MS appears to be a reliable tool for the rapid and accurate diagnosis of *S. chromogenes* and *S. epidermidis*, *S. agnetis* may be misidentified as *S. hyicus* because reference spectra are not present in all commercially available databases. That said, the protein spectra of these species might be so similar that they may be difficult to differentiate by MALDI-TOF, but that remains to be established. When compared to *cydB* real-time PCR the identification of CoNS to the species level by MALDI-TOF MS was in agreement in 75 of 84 cases (89.3%) (Fig. 2); nine CoNS samples that had MALDI-TOF MS scores of less than 1.99 were identified to the species level by *cydB* qPCR and 16S rRNA gene sequencing. These findings are comparable to those of a study of 108 CoNS obtained from bovine intramammary infections. In this investigation, Tomazi et al. (2014) observed 95.4% agreement of MALDI-TOF MS and *groEL* PCR-RFLP results. However, problems were noted with the identification of *S. saprophyticus* and *S. haemolyticus* by MALDI-TOF. In another study, Zhu et al. (2015) reported that of 216 samples of *Staphylococcus* isolates identified by biochemical tests and 16S rRNA gene sequence analysis, 75% of the *S. sciuri* and 60% of *S. caprae* produced low but accurate MALDI-TOF MS Biotyper scores. Similar results were reported using MALDI-TOF for characterization of human clinical samples. Previous reports indicated that correct identification rates ranged from 74.2% to 99.3% (Dupont et al., 2010; Spanu et al., 2011; Loonen et al., 2012). In these studies, the variability of CoNS identification rates could be explained by different conditions of bacterial growth, preparation of samples, number of reference spectra present in the software database, version of the software, and design of the studies (Loonen et al., 2012; Tomazi et al., 2014)

The most common isolates detected in the current study were *S. chromogenes* (n = 50), *S. agnetis* (n = 10) and *S. epidermidis* (n = 6). Small numbers of other staphylococcal spp. (i.e., *caprae, equorum, heamolyticus, hominis, pasteuri, sciuri, saprophyticus* and *warneri*) were also obtained. Isolation of *S. chromogenes* in milk samples and milking environment might be attributed to the fact that these species seem to be very well adapted to the bovine (and perhaps buffalo) mammary gland (Thorberg et al., 2009). These findings are comparable to previous reports which suggested that *Staphylococcus* spp. (Ali et al., 2008; Oliveira et al., 2011; Medeiros et al., 2013) including *S. chromogenes*, *S. hyicus*, *S. simulans* and *S. epidermidis* (Capurro et al., 2009) are the most commonly isolated species from cases of buffalo mastitis and buffalo milk. Although, caution should be exercised when extrapolating findings from one species to another and the fact that one buffalo herd may not be representative for herd comparison, these findings are also similar to the reports of CoNS in bovines (Santos et al., 2008). As well, *S. chromogenes* along with *S. haemolyticus, S. epidermidis,* and *S. simulans* is reported to be associated with persistent intramam-

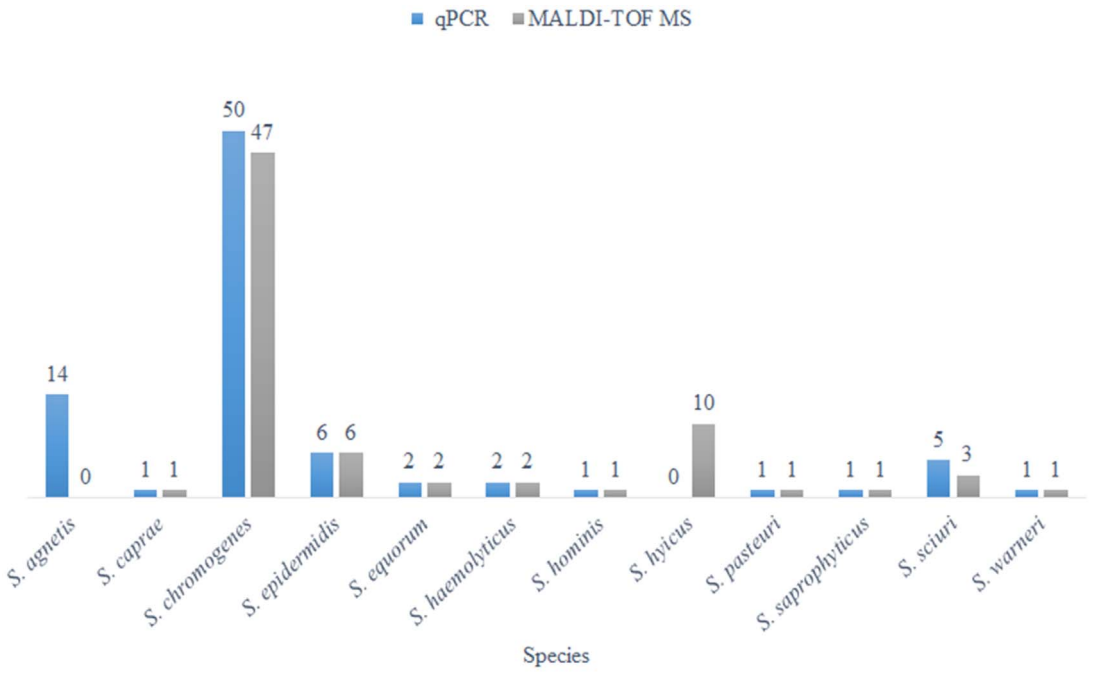

Fig. 2. Identification of CoNS by MALDI-TOF MS and *cydB* qPCR amplification.

mary infections and an increase in somatic cell count (Fry et al., 2014) in dairy cattle. *S. equorum, S. sciuri, S. cohnii*, and *S. saprophyticus* (of environmental origin) may be acting as opportunistic mastitis pathogens (Piessens et al., 2011)

5. Conclusions

The results of the current study suggest that MALDI-TOF MS can be a reliable tool for the rapid and accurate diagnosis of *S. chromogenes* and *S. epidermidis*, but currently *S. agnetis* may be misidentified as *S. hyicus*. As well, MALDI-TOF MS may be a good method for the identification of *S. pasteuri*, *S. hominus*, *S. haemolyticus*, *S. warneri*, *S. caprae*, *S. saprophyticus* and *S. equorum,* but additional isolates should be analysed before this assertion can be made with confidence. To complement (or where not available, replace) MALDI-TOF MS, speciation of CoNS can be done using *cydB* PCR amplification.

Conflict of interests

The authors confirm that they have no conflicts of interest.

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