Some coagulase negative Staphylococcus spp. isolated from buffalo can be misidentified as Staphylococcus aureus by phenotypic and Sa442 PCR methods

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Abstract
Objective: Staphylococcus aureus is a commonly reported cause of buffalo mastitis. However, its prevalence may be overestimated. The aim of this study was to compare S. aureus identification by conventional phenotypic and genotypic assays versus Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and novel real-time quantitative PCR tests for the cytochrome oxidase subunit D II (cydB) and staphylocoagulase (coa) genes.

Results: From 408 samples obtained from buffalo milk/milking environment, 32 putative S. aureus strains were identified based on characteristic growth on Baird Parker agar, positive catalase reaction, ability to clot rabbit plasma, and positive Sa442 PCR assay. However, in further testing, only 10 of these strains were positive in latex agglutination tests and by MALDI-TOF MS, only eight of the 32 strains were S. aureus while the rest were S. chromogenes (19), S. agnetis (3), S. cohnii (1), or S. xylosus (1). All eight strains identified as S. aureus by MALDI-TOF analysis and confirmed by 16S RNA gene sequencing were positive in a S. aureus-specific cydB PCR test. As well, 7/8 S. aureus strains were PCR positive in a real-time coa PCR test as were 2/69 S. chromogenes and the lone S. xylosus strain tested.

Keywords: Mastitis, Staphylococcus aureus, Species-specific PCR tests, cydB PCR

Introduction
Buffalo milk and its derivatives have become increasingly important worldwide [1] and Staphylococcus aureus is one of the most significant pathogens responsible for contagious mastitis in dairy buffaloes [2]. Antibiotic treatment of S. aureus mastitis is often unsuccessful and treatment failures can lead to spread of the infection. As a result, animals with chronic S. aureus infection are often culled [3].

The initial identification of S. aureus is based on culture and phenotype on specific media; other assays commonly used to identify S. aureus are the Sa442 PCR, nuc gene PCR, and latex agglutination tests. The Sa442 PCR test, developed by Martineau et al. [4] targets a chromosomal DNA fragment thought to be specific for S. aureus, the nuc gene encodes a species-specific thermonuclease, while commercially available latex agglutination kits such as the Staphaurex latex test are based on the interaction of S. aureus surface-anchored proteins with human IgG and fibrinogen bound to latex particles [5]. However, these tests may not be accurate and can lead to erroneous identification, and in turn, to unnecessary culling [5, 6]. Here we describe how common testing approaches can lead to misidentification of “non-S. aureus” strains as S. aureus and the development of new a cydB real-time PCR assay that can be used for accurate S. aureus identification.

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Main text

Methods

Sample collection

Milk samples (n = 320) were collected from 80 randomly selected female buffaloes from a private dairy farm located in Sao Paulo State, Brazil from November 2013 to April 2014. After physical examination of the mammary glands [7], teats were cleaned with 70% alcohol and milk from each quarter was evaluated by strip cup and California mastitis tests [8]. Hand samples from 16 consenting milkers and 64 samples from liners were collected using sterile swabs (Pro-Lab Diagnostics) and stored in peptone water as described previously [9].

CoPS isolation and identification

Isolation and identification of S. aureus was done according to compendium of methods for the microbiological examination of foods [10]. Strains with positive egg yolk reactions [11] were tested for Gram and catalase reactions, haemolytic activity, and ability to clot rabbit plasma using a Coagu-Plasma kit (Laborclin, Pinhais, Brazil) according to manufacturer’s instructions with S. aureus ATCC 25293 and S. epidermidis ATCC 12228 as the positive and negative controls respectively. In addition, isolates were tested with the Staphyclin latex test (Laborclin, Pinhais, Brazil) according to the manufacturer’s instructions.

DNA extraction

For DNA extraction, well-isolated colonies were inoculated into BHI broth, incubated at 37 °C for 18 h and extracted according to the method of Kuramae-Izioka [12] with minor modifications as described previously [13].

Sa442 detection

Isolates were characterized using the PCR assay of Martineau et al. [4] with minor modifications. PCR primers (Table 1) were used at 10 pmol/µL in 25 µL reaction mixtures with 50 ng/µL of DNA and 20 µL LightCycler® 480 SYBER Green Master mix (Roche Diagnostics, Indianapolis, IN). Amplification parameters were: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 12 s in a Roche LightCycler® 480 (LC480) thermocycler. The ramp rates were 4.4, 2.2, and 4.4 °C/s, respectively. Staphylococcus chromogenes and Streptococcus suis DNAs and water were used in negative control reactions and S. aureus strains COL, NewMan, MW2, Mu50 and ATCC 25923 were used as positive controls. A melting step (62 °C) was done to confirm single product.

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI–TOF MS)

Identification of putative Staphylococcus aureus strains was done using a MALDI Bruker Biotype system (Bruker Daltonics Inc., Billerica, MA, USA) at the Animal Health Laboratory, University of Guelph, Guelph, Ontario, Canada, as described previously [13].

16S rRNA gene sequencing

16S rRNA gene sequencing (~1000 bp) was also done at the Animal Health Laboratory and sequences were compared with the 16S rRNA gene of Staphylococcus aureus MCRF184 (CP014791.1) and other Staphylococcus spp. sequences using blastn.

Design of cydB species-specific primers

PCR primers to the S. aureus cydB gene (cytochrome D ubiquinol oxidase subunit II; NCBI accession number NC_007795.1) were designed as described previously [13] and produced a 432 bp amplicon. The amplicons were confirmed by DNA sequencing as described above (Table 1). As well, an additional 84 putative staphylococci from buffalo milk/milking environment were evaluated using the cydB test [13].

Design of coagulase gene primers

Published coa primers [14] generate products of different sizes so a new primer pair (coaF and coaR; Table 1) was designed using PrimerQuest software (Integrated DNA Technologies, San Diego, CA).

Table 1

| “Gene” | Primer | Sequence (5’–3’) | Product (bp) | Reference |
|--------|--------|-----------------|--------------|-----------|
| coa    | Forward | GTCTAAAAGTAGCTCACATCTAAACTTG | 228 | This study |
|        | Reverse | ATCCAAATGTCCATCTGTATTC | | |
| cyd-aureus | Forward | CCCATTTGCTTGGTCTGTAGTA | 432 | This study |
|        | Reverse | GTCCAGCCATTATGGATTA | | |
| Sa442  | Forward | AATCTTGTGGTACAGGATATTCCAG | 108 | Martineau et al. [4] |
|        | Reverse | CGTAATGAGATTTCCAGGATAATACAACA | | |
technologies, Inc. http://www.idtdna.com) to the coagulase gene of *S. aureus* JCSC 7638 (AB488509.1). A conserved region of the gene was identified by aligning 103 strains in GenBank using CLC Sequence View 7 software (Additional file 1: Fig. S1).

**Real-time PCR**

Real-time PCR primers (Table 1) were used at 10 pmol/µL in 25 µL reaction mixtures containing 50 ng/µL of DNA and 20 µL Light Cycler 480 (LC480) SYBER Green Master mix (Roche Diagnostics, Indianapolis, IN). For cydB and coa gene amplifications the parameters were: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 25 s in a Roche Light Cycler 480 (LC480). Ramp rates were 4.4, 2.2, and 4.4 °C/s, respectively; *Streptococcus suis* DNA and water were used as negative controls. In addition, a melting step (62 °C) was done to confirm a single product. *S. aureus* strains COL, NewMan, MW2, Mu50 and ATCC 25923 were used as positive controls. All real-time PCR were performed in triplicate.

**Results**

**Staphylococcus spp. isolation and preliminary identification**

Thirty-two putative *S. aureus* strains were selected based on their characteristic phenotype on Baird Parker agar. These Gram positive strains were catalase positive and were positive in the *S. aureus* species specific Sa442 PCR assay of Martineau et al. [4]. In further testing, 24 samples were consistently positive in the coagulase test; while eight gave at least one discordant result. Also, 21 of the 32 putative *S. aureus* strains were β-haemolytic, two were α-haemolytic and nine were non-haemolytic.

**Latex test, identification by MALDI-TOF and 16S rRNA sequencing**

Only ten of the 32 strains gave a positive latex agglutination result and MALDI-TOF MS analysis revealed that only eight of the 32 were *S. aureus* with the remainder being *S. chromogenes* (n=19), *S. agnetis* (n=3), *S. xylosus* (n=1), or *S. cohnii* (n=1) (Table 2). All eight strains identified as *S. aureus* by MALDI-TOF analysis had 100% identity with the 16S rRNA gene of *Staphylococcus aureus* MCRF184 (NZ_CP014791.1). The two latex false positive strains were *S. agnetis* and *chromogenes* by 16S sequencing and MALDI_TOF.

**cydB gene analysis and detection**

Alignment of gene sequences (https://www.ncbi.nlm.nih.gov/genbank/) suggested that the *cydB* gene is well conserved in *Staphylococcus* and thus allowed for the design of species-specific primers (Additional file 2: Fig. S2). The eight *S. aureus* isolates were positive for the *S. aureus* specific *cydB* primers and resultant amplicons had 99–100% identity with *S. aureus* NCTC 8325. Further, the *S. aureus* specific *cydB* primers did not amplify any of the other CoPS tested in this study nor the 84 strains of other putative staphylococci evaluated by Pizauro et al. [13].

**coa gene analysis**

Alignment of 103 coagulase gene sequences (https://www.ncbi.nlm.nih.gov/genbank/) revealed that although

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**Table 2 Comparison of Staphylococcus spp. identification by MALDI-TOF MS and positive rabbit plasma clotting, Sa442 PCR, latex agglutination, coa and S. aureus cydB PCR tests**

| Species               | MALDI-TOF MS | Plasma clotting | Sa442 PCR | Latex test | cydB PCR | coa PCR |
|-----------------------|--------------|-----------------|-----------|------------|----------|---------|
| *S. agnetis*          | 17           | 3               | 3         | 1          | 0        | 0       |
| *S. aureus*           | 8            | 8               | 8         | 8          | 8        | 7       |
| *S. caprae*           | 1            | 0               | 0         | 0          | 0        | 0       |
| *S. equorum*          | 3            | 0               | 0         | 0          | 0        | 0       |
| *S. epidermidis*      | 8            | 0               | 0         | 0          | 0        | 0       |
| *S. haemolyticus*     | 2            | 0               | 0         | 0          | 0        | 0       |
| *S. hominis*          | 1            | 0               | 0         | 0          | 0        | 0       |
| *S. pateur*           | 2            | 0               | 0         | 0          | 0        | 0       |
| *S. saprophyticus*    | 1            | 0               | 0         | 0          | 0        | 0       |
| *S. sciuri*           | 1            | 0               | 0         | 0          | 0        | 0       |
| *S. warneri*          | 1            | 0               | 0         | 0          | 0        | 0       |
| *S. xylosus*          | 1            | 1               | 1         | 0          | 0        | 1       |
| *S. chromogenes*      | 69           | 19              | 19        | 1          | 0        | 2       |
| *S. cohnii*           | 1            | 1               | 1         | 0          | 0        | 0       |
| **Total**             | **116**      | **32**          | **32**    | **10**     | **8**    | **10**  |

* Data from Pizauro et al. [13]
coa genes possess many polymorphic areas, a region between 1300 and 1600 bp has sufficient homology to be used for detection in S. aureus strains (Table 1, Additional file 1: Fig. S1). All of the S. aureus positive control strains tested [COL (NC_002951.2), NewMan (NC_009641.1), MW2 (NC_003923.1), Mu50 (NC_002745.2) and Staphylococcus aureus ATCC (25923)] were positive using the coaF and coaR primer pair.

Coagulase test and coa gene detection
Twenty-four strains clotted rabbit plasma (Table 2). Eight strains gave discordant results with at least one negative and one positive. Of these strains, seven of the S. aureus (n = 8) and two of the S. chromogenes (n = 19) and one S. xylosus strain were positive for the coa gene while none of the S. agnetis (n = 3) nor the S. cohnii were positive for the coa gene. The sequence of the PCR products had 98% identity with the S. aureus coa gene from strain JCSC 7633 (accession number AB488507.1) and 99% identity with the coa gene in S. aureus strain MW2 genome (accession number BA000033.2).

Discussion
A number of typically coagulase negative Staphylococcus spp. including more than a quarter of S. chromogenes isolates (19/69) and at least some S. xylosus (1/1), S. cohnii (1/1), and S. agnetis (3/17) were coagulase positive. This finding is consistent with studies of Santos et al. [15] in which 23/42 CoNS strains clotted rabbit plasma. These authors suggested that this phenotype was related to specific PFGE-types, but not with the clumping factor test. The presence of coagulase is an indicator of pathogenicity since it enables bacteria to resist phagocytosis and cause chronic infections [16]. Host specific adaptations can be acquired through mobile genetic elements (MGEs) [16, 17] from nearby S. aureus [16] or other CoPS such as S. pseudointermedius [15]. Thus, a coa gene in CoNS with the newly described coa primers may be the result of such transfer. Coagulase activity in the current study may also be related to another gene such as the one described in S. chromogenes that shares 41% identity with the predicted coagulase gene of the S. pseudointermedius [15].

In this study, non-S. aureus stains able to clot plasma (19/19 S. chromogenes, 3/3 S. agnetis, 1/1 S. cohnii and 1/1 S. xylosus) were also Sa442 positive. This is the first report of false positive reactions with these species; however, further stains/herds should be tested to know whether these findings can be generalized. When the sequences of the Sa442 primers [4] were compared with the available genome of S. chromogenes MU 970 strain (NZ_JMF00000000.1), no significant homology was detected; however, this draft whole shotgun sequence could be missing the region containing Sa442 sequences. Apart from having been established as unique for S. aureus, the Sa442 fragment has not been further characterized [18]. As well, Klaassen et al. [18] and Heilmann et al. [19] have reported false negative results with the Sa442 test and the nuc PCR is subject to strain variation [6].

Latex agglutination tests may also be problematic. In previous studies, false positive results have been observed at relatively low frequencies (e.g., 7.9% [20] and 9.3% [21]). The greater false positive reaction in this study (20%) may have been related to the population structure and/or the relatively small sample size.

Given the impact that S. aureus can have on both human and animal health, its diagnosis is important [22, 23]. Misidentifying more benign Staphylococcus species as S. aureus, though arguably less serious, is not without significant economic consequences. In the current study, the eight S. aureus strains (as identified by MALDI-TOF/16S rRNA sequencing) were positive with our novel S. aureus-specific cdB PCR test while no amplification was observed with the Sa442 and plasma clotting-positive S. chromogenes (n = 19), S. agnetis (n = 3), S. xylosus (n = 1) or S. cohnii (n = 1) strains tested. Also, it might be noted that S. caprae, S. hyicus, S. hominis, S. epidermidis, S. haemolyticus, S. warneri, S. equorum, S. sciuri, and S. pasteuri were negative in the S. aureus-specific cdB real-time PCR test in a complementary study [13] (Table 2).

Conclusions
In summary, a significant number of CoNS strains could clot rabbit plasma and were positive for the Sa442 PCR test and so, could be misclassified as S. aureus. The bases of these phenotypes remain to be determined, but they could be the result of horizontal gene transfer or to the fact that these species are less homogenous than previously thought. On the other hand, MALDI-TOF and a species-specific real-time PCR test for the cdB gene may permit accurate identification of CoNS.

Limitations
This study used samples from one buffalo herd which limits the generalisation of the results. In addition, it was beyond the scope of this study to determine the basis of the abnormal coagulase positive phenotype or to determine if there had been horizontal gene transfer to coagulase negative Staphylococcus strains.

Additional files

Additional file 1: Fig. S1. S. aureus coagulase gene (coa) alignment using CLC Sequence View 7 software.

Additional file 2: Fig. S2. Alignment using CLC Sequence View 7 software of the cdB gene of 18 closely related Staphylococcus spp. in this study.
Abbreviations
CoNS: coagulase negative Staphylococcus; MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS); qPCR: real-time quantitative PCR; PCR: polymerase chain reaction; CoPS: coagulase positive Staphylococcus.

Authors’ contributions
CCA, LJLP and GAS developed the proposal, collected the samples in the field, did the laboratory work and drafted the manuscript. DS, FAA, JMP and JMV corrected the proposal, supervised the sample collection and laboratory work, analyzed the data, interpreted the results and provided technical advice and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets generated during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study was approved by the Ethics Committee on Animal Use (CEUA) of the School of Agricultural and Veterinarian Sciences, Jaboticabal—FCAV/UNESP/Jaboticabal Campus—Brazil (Protocol Number 013737/13). Dairy farm owner have given verbal consent to use the animals in this work. Farm workers have also given verbal consent to participate in this work.

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