Short communication: Characterization of an atypical maltose-negative *Staphylococcus aureus* through the use of phenotypic and molecular techniques

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ABSTRACT

The most clinically relevant staphylococci in veterinary medicine are those that are coagulase-positive, namely *Staphylococcus aureus*. During microbiological udder health monitoring (2009–2018), a new *S. aureus* strain (coagulase-positive and maltose-negative) was discovered as an emerging udder pathogen during routine examinations of South African dairy herds. This study challenged the conventional microbiological diagnosis of staphylococci by comparing its results to those of the MALDI-TOF mass spectrometry and 16S rRNA sequencing. Both of these tests confirmed that the maltose-negative staphylococcus (MNS), identified as *Staphylococcus pseudintermedius* by conventional microbiology, was *S. aureus* ST2992. Multi locus sequence typing was performed on 3 of the MNS isolates and indicated that these isolates were of single origin. These strains tested positive for both *MAL*A and *MAL*R genes (control: *S. aureus* ATCC 25923). Although the α -glucosidase gene was present, it was not expressed phenotypically. The latter may be attributed to the abnormal stop codon identified in the *MAL*A gene sequence of *S. aureus* ST2992 (GenBank accession number, MN531305). The newly identified MNS has a field behavior different to that of maltose-positive *S. aureus*, and more similar to the low virulence of non-*aureus* staphylococci.

Key words: *Staphylococcus aureus*, maltose-negative, molecular identification, abnormal stop codon

Short Communication

Staphylococcus aureus remains a leading cause of IMI in dairy cows (Tenhagen et al., 2009). Petzer et

al. (2017) reported that 9.05% of IMI were caused by *S. aureus* in South African dairy herds (2001–2015), and additional studies have reported 10 to 40% in other countries (Basanisi et al., 2017; Liu et al., 2017). More recently, isolates that were all maltose-negative, and formerly identified as *Staphylococcus intermedius* by phenotypic characteristics, were reclassified into 3 clusters—*Staphylococcus intermedius*, *Staphylococcus pseudintermedius* (not yet characterized in dairy cattle), and *Staphylococcus delphini*—based on the nucleotide sequence analysis of the *SODA* and *HSP60* genes (Sasaki et al., 2007).

The main objective of this study was to assess the adequacy of the conventional microbiological identification method. The initial hypothesis tested was to confirm the identity of this maltose-negative *Staphylococcus* (MNS) species.

Milk samples received for routine udder health investigations, taken from all lactating cows in dairy herds, were analyzed using conventional microbiological methods. Thirty-one coagulase-positive, MNS isolates that originated from 10 out of a total of 880 farms in 4 of the 9 provinces of South Africa were examined (2009–2018). These 31 isolates all originated from sub-clinical mastitis cases.

The conventional microbiological identification (on tryptose blood agar) was done at the Milk Laboratory (University of Pretoria, Onderstepoort, South Africa) according to the methods recommended by the National Mastitis Council, as in Petzer et al. (2017). The catalase, coagulase (Staphylase test, Oxoid; Quantum Biotechnologies [Pty] Ltd., Ferndale, South Africa), and maltose agar tests (Merck NT Laboratory Supplies, Halfway House, South Africa) were used for identification of staphylococci (Jorgensen et al., 2015; Petzer et al., 2017). The MALDI-TOF MS identification of these staphylococci was carried out at the National Health Laboratory Service - National Institute for Communicable Diseases (Sandringham, South Africa) using the

Received November 12, 2019.

Accepted April 1, 2020.

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direct transfer method with MALDI Biolayer 3.0 software (Bruker Daltonics, Bremen, Germany; Van Dyk et al., 2016). These isolates were further identified by 16S rRNA Sanger sequencing analysis conducted at Inqaba Biotec (Pretoria, South Africa), amplified as previously described (Weisburg et al., 1991). In this study the JO-MALAR-F (5'-CTATAGGTCTACAAATGGCC-3') and JO-MALAR-R (5'-CAGGAGGTGATTAAATGGTTAC-3') primers were used to amplify the *MALA* and *MALR* gene targets. The Jo-Mal-Int, primer with sequence GATGTAATGACAGCAAC was the internal sequencing primer used.

Three of the 23 maltose-negative *S. aureus* isolates (confirmed by MALDI-TOF MS and 16S), 1 from each 2009, 2013, and 2017, were submitted to Inqaba Biotec for further analysis by utilizing multilocus sequence typing (MLST). The internal fragments of 7 housekeeping genes (*ARCC*, *AROE*, *GLPF*, *GMK*, *PTA*, *TPI*, and *YQIL*) were amplified as previously described (Enright et al., 2000) and sequenced by using Brilliant Dye V3.1 (Nimagen B.V., Nijmegen, Netherlands) and an ABI 3500 XL Genetic analyzer with a 50 cm array and POP-7 (Applied Biosystems, Waltham, MA). The sequence of each locus was compared with allele sequences in the MLST database (<http://saureus.mlst.net>) to define the allelic profile for each isolate and to assign a sequence type, which identified this strain as *S. aureus* ST2992. The absence of maltose activity was evaluated by testing for the presence of the *MALA* and *MALR* genes (Egeter and Brückner, 1995). The sequences of the *MALA* and *MALR* genes of *S. aureus* ST2992 were compared with that of the *S. aureus* ATCC BAA-39 (NCBI database, <https://www.ncbi.nlm.nih.gov/genbank/>), conventionally identified maltose-positive *S. aureus* ATCC 25923 (control), *S. pseudintermedius* ATCC 49444, and coagulase-negative maltose-negative *S. aureus* JN 861808 (Johler et al., 2012).

The conventional microbiology method misdiagnosed the 31 MNS samples as *S. pseudintermedius*. Both the MALDI-TOF MS and 16S identification confirmed that out of the 31 organisms, 23 were *S. aureus*, 4 *Staphylococcus chromogenes*, 1 *Staphylococcus capitis*, and 1 *Staphylococcus xylosus*. The 2 remaining isolates were identified by 16S rRNA sequencing as *Staphylococcus saprophyticus* and *Macrococcus caseolyticus*, but the MALDI-TOF could not identify them. A *Bacillus subtilis* was used as the out-group, while *Macrococcus caseolyticus* was an inbuilt out-group. Further MLST confirmed that 3 of the coagulase-negative *S. aureus* (from the group of 23 identical *S. aureus* strains) were maltose-negative *S. aureus* ST2992, which belongs to clonal complex 97 and were of single origin isolated over time.

The *MALA* and *MALR* genes were present in the maltose-negative *S. aureus* ST2992. However, a stop codon was discovered at base pair 844 of the *MALA* gene caused by a cytosine to thymine transition, which may result in a truncated α -glucosidase protein. Nucleotide sequences of the gene encoding α -glucosidase (*MALA*) that showed the stop codon (Figure 1) and its upstream region in the investigated strain ST2992 have been deposited in GenBank (accession number MN531305).

Both the MALDI-TOF MS and 16S rRNA sequencing tests identified 23 of the total 31 isolates as *S. aureus*. The maltose-negative *S. aureus* ST2992 strain (Schmidt et al., 2017) (classification based on MLST) was shown to be an identical strain of single origin, isolated repeatedly over time. The latter isolates have been shown to belong to the common bovine lineage CC97 (Schmidt et al., 2017). The presence of *FEMA* gene (Kobayashi et al., 1994) further confirmed that these isolates were not *S. pseudintermedius*, but rather a type of *S. aureus* with an unusual biochemical profile. The *MALA* and *MALR* genes were present in both the maltose-negative *S. aureus* ST2992 and the maltose-positive *S. aureus* ATCC 25923 control. The *MALA* gene encodes an α -1,4-glucosidase, or maltase, which also liberates glucose from sucrose. Additionally, it is assumed that the *MALR* gene participates in the regulation of the gene for maltose transport, and would thus be needed for the full expression of these genes (Egeter and Brückner, 1995). The inactivation of *MALA* in a sucrose-6-phosphate hydrolase-deficient *Staphylococcus xylosus* strain has been shown to result in the complete loss of the residual sucrose hydrolase activity (Egeter and Brückner, 1995).

This study discovered a stop codon at base pair 844 of the *MALA* gene where there was a cytosine to thymine transition, which resulted in the early termination of the α -glucosidase protein. This truncated protein is considered to be the most likely cause of the maltose-negative phenotype of *S. aureus* ST2992. The *MALR* gene of the maltose-negative *S. aureus* ST2992 was identical to that of the ATCC strain used. An [coagulase-negative and MNS] isolate from the Netherlands did not show the same mutations in the *MALA* gene sequence as those of the maltose-positive control strains (Johler et al., 2012). Thus, the maltose-negative phenotype is likely caused by various differences occurring in either the *MALA* or *MALR* genes, or in both. Further research is needed to explore these differences.

Although conventional microbiology methods are not entirely correct in the identification of coagulase-positive and MNS phenotypically, it is important to differentiate between maltose-negative and positive *S. aureus* isolates during routine diagnosis of mastitis pathogens because these organisms react differently in

Figure 1. Comparison of the alignment of the *MALA* gene of the maltose-negative *Staphylococcus aureus* ST2992 with that of the *Staphylococcus aureus* ATCC BAA-39 (NCBI database), conventionally identified maltose-positive *Staphylococcus aureus* ATCC 25923, and coagulase-negative maltose-negative *Staphylococcus aureus* JN 861808 (Johler et al., 2012), highlighting the mutation at base pair 844 that resulted in the stop codon.

practice. Petzer et al. (2017) showed that 46.74% ($n = 445$) of quarter milk samples with tentatively identified *S. pseudintermedius* had $\text{SCC} \leq 200,000$ cells/mL and 44.31% ($n = 1,458$) of composite cow milk samples had $\text{SCC} \leq 150,000$ cells/mL in similar samples, compared with 11.2% ($n = 6,087$) and 17.3% ($n = 13,522$) for *S. aureus* (maltose-positive).

Maltose-negative staphylococci represented 1.46% of bacteria isolated from quarter milk samples (2009–2018). Maltose-negative staphylococci have not been isolated from cows more than once, despite repeated testing, nor could udder parenchyma damage be palpated in infected quarters (Petzer et al., 2017). The latter is in sharp contradiction to the prevalence of maltose-positive *S. aureus*, which causes chronic infections and parenchyma damage (Pyörälä and Pyörälä, 1997; Tenhagen et al., 2009). Different levels of virulence (contagiousness and pathogenicity) have also been widely described among typical *S. aureus* (Cremonesi et al., 2015; Leuenberger et al., 2019). Thus, the maltose-negative *S. aureus* ST2992 could be one of the many genotypes of *S. aureus* not contagious and with “environmental” behavior similar to that of non-*aureus* staphylococci (Pyörälä and Taponen, 2009).

The strength of this study lies in proving that the maltose-negative *S. aureus* ST2992 with the abnormal stop codon is different from the conventionally identified maltose-positive *S. aureus*, and that the MNS were of single origin.

ACKNOWLEDGMENTS

The authors thank the producers for allowing them to use data and samples obtained on their farms, the staff of the Milk Laboratory, Department of Production Animal Studies, Faculty of Veterinary Science, Onderstepoort, for the laboratory analyses: J. C. Watermeyer, R. Badenhorst, E. F. Konaite, K. S. Malekane, N. I. Memma, and O. P. Nchako. The authors also thank Olga Perovic and Marshagne Smith of National Health Laboratory Services (NHLS) - National Institute for Communicable Diseases (NICD; Sandringham, South Africa) for the MALDI-TOF MS analysis. This research was partly funded by the National Research Foundation (Pretoria, South Africa). The authors have not stated any conflicts of interest.

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