***Discussion***

*Diversity of strain type as determined by RAPD and MLST*

In all 9 herds, there was at least one RAPD type of *S. chromogenes* identified to be causing an IMI in quarters belonging to different cows. RAPD typing has been used to compare strain types of different isolates of the same species during outbreaks of bacterial infections among animals, in order to see if the transmission pattern was consistent with a pathogen acting in a contagious manner (or originating from a common point source), or the increased number of infections were caused by multiple strains which were not derived from a common source.In combination with sequencing the 16S rRNA gene for representative isolates, RAPD was used to understand the diversity of strain types causing a multistate outbreak of *Corynebacterium tuberculosis* in multiple species (Foley et al. 2004), and also for investigation of a *Campylobacter jejuni* between different flocks on a single broiler farm (in combination with sequencing the 23S rRNA gene; Payne et al., 1999). RAPD alone was used by Zadoks et al. (2003) to identify transmission dynamics of the mastitis isolate *Streptococcus uberis* within a single herd, which revealed that each cow was infected with a unique strain, confirming that the observed mastitis outbreak was not due to contagious transmission, but instead was resulted from infections from environmental sources. Although the objective of the current study was not to identify the transmission dynamics of *S. chromogenes* within a given dairy herd, finding the same RAPD type in more than one cow in the same herd suggests that cow-to-cow spread is occurring within a herd (or spread is occurring via a common point source). These findings are consistent with Wuytack et al. (2020b) and (Reydams et al. 2023), who also used RAPD typing for *S. chromogenes* isolates. Studies using amplified fragment length polymorphisms (AFLP) (Taponen et al. 2007) and pulsed-field gel electrophoresis (PFGE) (Gillespie et al. 2009; Mork et al. 2012) have also demonstrated the same *S. chromogenes* strains have been found in multiple animals from the same farm, suggesting some strains may act in a contagious manner.

In the current study, 10 different strain types determined by MLST were identified for the 30 *S. chromogenes* isolates. As the MLST scheme for *S. chromogenes* was described fairly recently (Huebner et al. 2021), the number of studies describing strain-typing results using this scheme to date is limited (Petzer et al. 2022; Persson Waller et al. 2023). In the phylogenetic analysis, study isolates belonging to ST174, ST175, and ST176 were identified as being closely related to ST1 isolates from PubMLST, and were identified as single locus variants of ST1 by the MLST 2.0 tool. ST1 was the most commonly found strain type in the current study (11/30 isolates, 36.7%). This agrees closely with the work of Huebner et al. (2021), who determined MLST for 120 *S. chromogenes* isolates from Belgium, Vermont (US), and Washington state (US). They found 39/120 (32.5%) of isolates strain-typed belonged to a nodal cluster centered around ST1. For the 48 isolates in Huebner et al. (2021) from Vermont, 36 (75%) belonged to nodal cluster 1. ST1 was also commonly found in 118 *S. chromogenes* isolates from bovine subclinical IMI in Sweden, in a study from Persson Waller et al. (2023), although ST6 and a related novel ST (ST109) were more predominant. For Huebner et al. (2021), ST1 was the only strain type found in all three geographical locations. ST6 was the second most commonly found ST in the current study (9/30 isolates, 30%), and the third most common (15/120, 12.5%) in Huebner et al. (2021). Persson Waller et al. (2023) identified 47 different strain types among 105 isolates from Sweden. Huebner et al. (2021) found a similar degree of diversity, with 47 ST identified from 120 isolates from three geographical locations. After ST1, ST15 was the second most commonly identified by Hubener et al. (2021), with 17/120 (14.2%) of isolates belonging to this strain type. ST15 was primarily identified in isolates from Vermont and Washington state (16/17 isolates), which Huebner et al. (2021) highlight as an example of how the distribution of different strain types varies geographically. Interestingly, 2 of 30 isolates belonged to ST15 in the current study, while only 1 ST15 was found in Sweden (Persson Waller et al., 2023).

Both Persson Waller et al. (2023) and Huebner et al (2021) saw a pattern where ST6 and ST1 as central nodes of ST clusters, with single- and double-locus variants surrounding these two ST. Both authors suggest this indicates a clonal expansion is occurring for *S. chromogenes* isolates belonging to these 2 ST. Results of the current study would support this, as the 3 ST in ST cluster 1 (ST174, ST175, ST176) were all newly-identified single-locus variants of ST1. Describing the diversity of strain type using MLST is an incredibly active area of research. Four of the 10 ST in the current study had previously not been described, while 43% of isolates belonging to 33 new ST were identified by Persson Waller et al. (2023). These results highlight the importance of contributing to publicly-available databases in order to improve our ability to better understand the diversity of *S. chromogenes.*

*Associations between ST and SCC category*

Our initial hypothesis that ST may be a significant predictor of SCC phenotype (HIGH vs. LOW category) was not supported. Persson Waller et al. (2023) also explored associations between genotypes and phenotypic qualities, such as persistency (over a 1 month period) and association with a CMT score at sampling. Although they found no association between ST or ST cluster and persistency, isolates belonging to their cluster VII were significantly more likely to be associated with a high CMT score, indicating a larger inflammatory reaction was occurring in the gland. Isolates belonging to ST6 (the most prevalent ST in cluster VII, and only ST also found in the current study) tended to be more likely to have a high CMT score vs. other STs in the cluster. However, this difference did not achieve the cut-off used for statistical significance.

*Antimicrobial resistance genes and associations between ARG and SCC category*

Overall, both phenotypic resistance and resistance genes are relatively rare in *S. chromogenes* when compared to other non-aureus staphylococci (NAS), with the important exception of the *blaZ* gene (Sampimon 2009; Persson Waller et al. 2011). Our findings support this principle, as the only ARG identified in the 30 *S. chromogenes* isolates was *blaZ. blaZ* encodes β-lactamase enzymes which hydrolytically destroy β-lactam antibiotics, and is the primary determinant of phenotypic resistance to benzylpenicillin in staphylococci (Pinho 2008).In the current study, 10/30 (33.3%) of isolates were *blaZ-*positive, which is higher than the 10% reported for *S. chromogenes* isolates in a Canadian study (Condas et al. 2017) and the 22% reported in Persson Waller et al. (2023), but much less than the 87% of *S. chromogenes* in a Flemish study (Sampimon 2009). Resistance to β-lactam antibiotics is the predominant type of AMR present in staphylococci, and the reported proportion of NAS isolates exhibiting β-lactamase resistance can be fairly high depending on geographical location (51.6% in Argentina, Raspanti et al. 2016; 63% in South Africa, Phophi et al. 2019; 23% in Belgium and Norway, Fergestad et al. 2021; 14% in Korea, Kim et al. 2019). β-lactam antibiotics are among the few choices for treating mastitis in the US, with first- and third-generation cephalosporins being the most commonly-used mastitis treatments (USDA 2016; de Campos et al. 2021). In addition to *blaZ*, Persson Waller et al. (2023) found strpS194, which confers resistance to streptomycin, in 7% of their *S. chromogenes* isolates. This ARG was not identified in isolates from the current study. Nobrega et al. (2018) identified various other ARG in *S. chromogenes* isolates, including genes associated with aminoglycoside resistance [ant(3’’), ant(4’), ant(6)], resistance to amphenicols (fexA), and resistance to tetracyclines (tetK, tetL). However, the estimated prevalence of these genes in the population of *S. chromogenes* tested in their study was low (2-3%).

Carriage of the *blaZ* gene was not found to be a significant predictor of whether an isolate would be associated with a persistently high SCC IMI. Not much is known about the association of ARG carriage by *S. chromogenes* and clinical characteristics of IMI, but previous work has identified a link between phenotypic resistance in *S. aureus* and clinical outcome. Both Sol et al. (2000) and Taponen et al. (2003) found that penicillin-resistant strains of *S. aureus* (those which produced β-lactamase) had a lower bacteriological cure rate *in vivo,* despite use of an appropriate intramammary antibiotic that the isolate was susceptible to *in vitro*. The *S. aureus* isolates in Sol et al. (2000) were associated with clinical mastitis, whereas the isolates in Taponen et al. (2003) were from cases of subclinical mastitis. Further, Sol et al. (2000) report that IMI due to penicillin-resistant *S. aureus* were associated with a more persistently elevated SCC, indicating the IMI was associated with a higher degree of inflammation. The authors of both studies conclude that either 1) the penicillin-resistant strains of *S. aureus* were more virulent than susceptible strains, due to a possible relationship between production of β-lactamase and other virulence factors, or 2) that any antibiotic used to treat mastitis caused by penicillin-resistant strains works less efficiently, due to unidentified pharmacokinetic or pharmacodynamic factors. A more recent example of a possible association between ARG and clinical characteristics of an infection due to NAS staphylococci is described in Wuytack et al. (2020a). When comparing NAS isolates associated with IMI which had an SCC of ≤50,000 cells/mL to isolates from cases of clinical mastitis, Wuytack et al. (2020a) identified *mecA* (a methicillin-resistance gene) in 21/43 (49%) of the NAS isolates originating from clinical mastitis and only in one isolate 1/16 (6%) in IMI from a quarter with an SCC of ≤50,000 cells/mL. Based on these findings, the authors suggest that *mecA* in NAS isolates from bovine IMI may be linked to virulence genes or pathogenicity islands, supposedly both present on a mobile genetic element (*SCCmec,* staphylococcal cassette chromosome *mec).* Of the 22 NAS isolates identified as *mec*-positive in Wuytack et al. (2020a), none were *S. chromogenes.* Further research into exploring associations between ARG and clinical characteristics of IMI including a larger number of *S. chromogenes* are certainly warranted, in order to better understand if particular undesirable traits (e.g., penicillin resistance and a greater inflammatory response) may be genetically linked in this ubiquitous mastitis pathogen.

*Antimicrobial resistance genes and associations between ARG and SCC category*

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