***Discussion***

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

In all 9 herds, there was at least 1 RAPD type of *S. chromogenes* identified to be causing multiple IMI in quarters belonging to different cows. RAPD typing has previously been used to compare ST of different isolates of the same species during outbreaks in order to see if transmission pattern was consistent with infections originating from a common source.In combination with sequencing the 16S rRNA gene for representative isolates, RAPD was used to understand the diversity of ST associated with a multistate outbreak of *Corynebacterium tuberculosis* in several species of animals (Foley et al., 2004), and also for investigation of a *Campylobacter jejuni* outbreak in multiple flocks from 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. In this study, RAPD typing revealed that each cow was infected with a unique strain. These findings confirmed that the observed mastitis outbreak was not due to contagious transmission, but instead was a result of infections from environmental sources. Although the objective of the current study was not to identify the transmission dynamics of *S. chromogenes*, identifying the same RAPD type causing IMI in more than one cow in the same herd suggests cow-to-cow spread is occurring (or transmission 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 and found that a given RAPD type was causing IMI in multiple cows in a herd. Studies using different methods of strain-typing (amplified fragment length polymorphisms: Taponen et al., 2007; PFGE: Gillespie et al., 2009, Mork et al., 2012) have also demonstrated the same *S. chromogenes* strains in IMI from multiple animals in a herd, providing additional evidence that some strains may act in a contagious manner.

Ten different ST (as determined by MLST) were identified for the 30 *S. chromogenes* isolates included in the current study. 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. Furthermore, these 3 ST were identified as single locus variants of ST1 by the MLST 2.0 tool. Isolates in this ST1 cluster were the most commonly found ST 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 a group they identify as nodal cluster 1. ST1 was also commonly found in 105 *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 ST 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 STs among 105 isolates from Sweden. Huebner et al. (2021) found a similar degree of diversity, with 46 ST identified from 120 isolates from 3 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 ST. ST15 was primarily identified in isolates from Vermont and Washington State (16/17 isolates), which Huebner et al. (2021) highlight as an example of geographic variation in the distribution of different STs. Interestingly, although all isolates in the current study are from Vermont, only 2 of 30 isolates belonged to ST15. Only 1 ST15 was found in Sweden (Persson Waller et al., 2023).

Both Persson Waller et al. (2023) and Huebner et al. (2021) observed that ST6 and ST1 were both central nodes of ST clusters, with single- and double-locus variants surrounding them. Both authors suggest this indicates occurrence of a clonal expansion 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 *S. chromogenes* using MLST is a rapidly growing area of research. Four of the 10 ST in the current study had previously not been described, while 43% of all 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 this common mastitis pathogen*.*

*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 of IMI (over a 1 month period) and association with 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 ARG 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 a β-lactamase enzyme which hydrolytically destroys 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. However, within this class, first- and third-generation cephalosporins are the most commonly used, which are more resistant to β-lactamases than penicillin (USDA, 2016; de Campos et al., 2021). In addition to *blaZ*, Persson Waller et al. (2023) identified *strpS194* (conferring resistance to streptomycin) in 7% of their *S. chromogenes* isolates. This ARG was not found 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* included in their study was low (2-3%).

Carriage of *blaZ* was not found to be a significant predictor of whether an isolate would be associated with a persistently high SCC IMI in the current study. Work exploring the association of ARG carriage and clinical characteristics of IMI in *S. chromogenes* is limited, but previous research has identified a link between phenotypic resistance in *S. aureus* and clinical IMI 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*. *S. aureus* isolates in Sol et al. (2000) were associated with clinical mastitis, whereas isolates in Taponen et al. (2003) were from cases of subclinical mastitis. Further, Sol et al. (2000) reported 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. Both Sol et al. (2000) and Taponen et al. (2003) 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 an association between ARG and clinical characteristics of an IMI due to NAS 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 NAS isolates originating from clinical mastitis and only 1/16 (6%) isolates from quarters 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) are genetically linked in this ubiquitous mastitis pathogen.

Although we did not find any support for an association between carriage of *blaZ* and the associated SCC category of an IMI, results from the current study suggest that *blaZ* carriage is likely a function of ST in *S. chromogenes.* For all but 1 of the 5 MLST identified, *blaZ* carriage was uniform across a ST. Numerous studies have identified that resistance profiles for NAS are species-specific (Sampimon, 2009; Persson Waller et al., 2011; Taponen et al., 2016; Nobrega et al., 2018; Fergestad et al., 2021; Taponen et al., 2023), so a genetic basis for carriage of particular AMR determinants at the strain level would not be surprising. For *S. aureus*, carriage of methicillin resistance has been associated with particular clonal complexes both in human medicine (Smith et al., 2021; Garrine et al., 2023) and certain clusters of *spa* ­type for bovine clinical mastitis isolates (Freu et al., 2022). Additionally, in a study comparing isolates from persistent and nonpersistent *S. aureus* IMI*,* Haveri et al. (2007) found that a particular pulsotype associated more with persistent IMI was significantly more likely to harbor the *blaZ* gene. An association between genetic grouping and *blaZ* carriage in *S. chromogenes* was identified in Persson Waller et. al (2023). In their study, all isolates of ST19, ST102, ST103 carried *blaZ*. When analyzing clusters of ST, they found that the two clusters comprised primarily of these 3 ST (clusters III and IV) were significantly more likely to be *blaZ*-positive than other clusters of ST. As isolates belonging to these ST were distributed over different farms and counties in Sweden, the authors suggest that *blaZ-*mediated penicillin resistance is likely a result of the spread of certain lineages of *S. chromogenes,* instead of horizontal gene transfer between different strains or species (Persson Waller et al., 2023). Three of the 4 ST which had uniform *blaZ* carriage in the current study were also distributed over multiple farms. Consistent carriage of *blaZ* from ST originating from different farms may suggest that *blaZ* is located chromosomally for these *S. chromogenes* isolates, instead of on a plasmid. Location of *blaZ* carriage is not well characterized for *S. chromogenes,* but a study of *S. aureus* IMI isolates in Finland and Norway found that 26 out of 34 Finnish isolates (76.5%) and 25 out of 44 Swedish isolates (56.8%) carried *blaZ* on a plasmid (vs. chromosomally) (Bagcigil et al., 2012). They also characterized the diversity of *blaZ* genes among the *S. aureus* isolates, identifying 6 different protein signatures. Studies exploring whether *blaZ* is more likely to be carried chromosomally or on a plasmid for *S. chromogenes* from bovine IMI, as well as characterizing the genetic diversity of the gene present in this population of isolates, would be useful in understanding transmission of penicillin resistance for this predominant mastitis pathogen.

*Virulence genes and associations between VF and SCC category*

The overall number of unique VF identified in the current study (62) from 30 *S. chromogenes* isolates was similar to the findings of Perrson Waller et al. (2023), who identified 57 unique genes among the 105 *S. chromogenes* isolates from Sweden. The average number of VF per isolate reported by Persson Waller et al. (2023) was 30 (SD: 5.4, range: 25-45), which is somewhat lower than the median (44) and range (43-51) reported for isolates in the current study. The database and methodology for identifying VF used in the current study is consistent with Persson Waller et al. (2023), facilitating a direct comparison of these values. In a smaller-scale study of 8 *S. chromogenes* from Finland using a different database, a range of 37-49 VF were identified (Åvall-Jääskeläinen et al., 2018). Although separate species-specific summary statistics are not provided for the 83 *S. chromogenes*, Naushad et al. (2019) found an average of 30 VF genes each for the 441 NAS isolates from 25 different species. They report that the phylogenetic grouping of NAS species with the highest virulence potential (defined by total number of VF) was clade B, which contains *S. chromogenes, S. agnetis,* and *S. hyicus*. A proportionately higher number of exotoxin genes, host evasion genes, and capsular genes contributed to clade B’s high virulence potential in their study.

One of the better-studied virulence genes of NAS is *bap*, encoding a surface protein which is a pathogenic factor of biofilm formation. *bap* was not detected in any of the 83 *S. chromogenes* isolates in Naushad et al. (2019), or any of the 25 isolates of *S. chromogenes* included in a Belgian study of clinical and low-SCC IMI (Wuytack et al., 2020a). It was also rare in Åvall-Jääskeläinen et al. (2018), where it was only found in 1/8 *S. chromogenes* isolates, and was somewhat sporadically found in Persson Waller et al. (2023) in 13/105 isolates. In light of these findings, it was unexpected that *bap* was identified in 28 of the 30 isolates (93.3%) in the current study. It has been suggested that biofilms increase the ability of NAS to persist in the udder (Piessens et al., 2012; Tremblay et al., 2013). As all 30 isolates in the current study are from persistent IMI, finding *bap* in such a high proportion is consistent with the notion that biofilms play a role in the ability of *S. chromogenes* to cause chronic infections. Another staphylococcal virulence factor playing a role in evasion of the host immune response is a polysaccharide capsule which resists phagocytic cell uptake. In Naushad et al. (2019), *S. chromogenes* isolates were seen to have 12 different capsular genes in low frequencies (7-11%). Only 1 of 8 *S. chromogenes* in Åvall-Jääskeläinen et al. (2018) had any capsular genes, and only 2/25 isolates in Wuytack et al. (2020a) was positive for cap5H with PCR. All 30 isolates in the current study contained at least 3 different *cap* genes, with 28 all having *capN, capO, capP.* Two isolates in the current study were missing *capN,* but possessed both *capJ* and *capH.* There is conflicting evidence on the associations between capsule genes and overall virulence of staphylococci. Some evidence exists that staphylococci lacking a capsule are able to better persist in the mammary gland, as encapsulated strains elicit more inflammation and are thereby eliminated faster (Tuchscherr et al., 2005). Other research suggests that the antiphagocytic properties of the capsule allows staphylococci to persist in infected hosts (Thakker et al., 1998). Citing work showing that lack of a capsule is advantageous for *S. aureus* causing chronic IMI, Naushad et al. (2019) argue that the low prevalence of capsule genes in their *S. chromogenes* may explain the how the pathogen has become so widespread in the population of Canadian dairy animals. Finding such a large proportion of isolates carrying multiple capsular genes in the current study of *S. chromogenes* isolates from persistent IMI instead supports the idea that a capsule enhances the ability of the organism to evade the host’s immune response.

Staphyolococcal complement inhibitor (encoded by the gene *scn*) also plays a role in the ability of staphylococci to evade the host’s immune system. *scn* encodes a protein which inhibits the complement system, reducing phagocytosis of the bacterium following opsonization. Although staphylococcal complement inhibitor had been thought to be highly specific to isolates of human origin, Naushad et al. (2019) detected *scn* in a high proportion (88%) of *S. chromogenes* isolates in their study. In agreement with these fidnings, all 30 isolates of *S. chromogenes* in their current study were positive for *scn.* Adenosine synthase A (*adsA*) is an immune evasion factor identified in *S. aureus,* which is responsible for increasing the amount of extracellular adenosine, a potent immuno-suppressive signaling molecule. *adsA* allows staphylococci to survive after being engulfed by neutrophils, giving it the ability to evade the bactericidal activity of host leukocytes. *adsA* was found in a high proportion (99%) of isolates from Naushad et al. (2019), and all 30 isolates in the current study.

Another widely-found VF in *S. chromogenes* is β-hemolysin, a phospholipase C toxin secreted by *S. aureus*. β-hemolysin was the most frequently-found gene in *S. chromogenes* isolates and other species of clade B in Naushad et al. (2019), was found in all 8 isolates in (Åvall-Jääskeläinen et al., 2018), and all 30 of the isolates in the current study. In contrast, the gene encoding exfoliative toxin type C (*etc,* which causes the loss of cell‐cell adhesion in the superficial epidermis in humans) was not identified in any of the *S. chromogenes* isolates in Naushad et al. (2019) or (Åvall-Jääskeläinen et al., 2018), but was present in all 30 of the isolates in the current study. Exfoliative toxins in NAS have been identified in *S. agnetis* and *S. chromogenes* from broiler chickens (as reviewed in Szafraniec et al., 2020), but are not widely reported from isolates of bovine IMI. Another set of toxin genes commonly identified in NAS is the β-type phenol-soluble modulins (PSMs), which have been shown in *S. aureus* to cause lysis of red and white blood cells, contributing to biofilm development and stimulation of inflammatory responses in the host. In Naushad et al. (2019), all *S. chromogenes* isolates possessed a single gene associated with PSMs (*PSMβ4*)*,* which was also widely found in isolates from Persson Waller et al. (2023). All isolates in the current study had the entire suite of PSM-associated genes described in the comprehensive NAS database (*PSMβ1- PSMβ6*), although more research is needed to understand the significance of having a larger number of genetic determinants associated with PSMs for the pathogenesis of *S. chromogenes.*

Two *S. chromogenes* isolates in the current study were positive for *coa,* the gene encoding for the staphylocoagulase enzyme. Staphylocoagulase binds to prothrombin in the host, ultimately forming a fibrin clot which shields the bacteria from the host's defenses and causes localized clotting. *coa* has previously been identified in *S. agnetis* and *S. hyicus* from bovine IMI*,* which are considered coagulase variable (Vanderhaeghen et al., 2015). Except for *S. aureus, S. hyicus,* and *S. agnetis*, coagulase positive staphylococci are rarely isolated from bovine IMI, which is why the coagulase test has been so widely used to classify staphylococci from mastitis into coagulase-positive (primarily *S. aureus*) and coagulase-negative (largely, all other species of staphylococci) (Vanderhaeghen et al., 2015). None of the 441 NAS isolates in Naushad et al. (2019) were *coa-*positive, while 4/4 *S. agnetis* but 0/8 *S. chromogenes* were *coa-*positive in Åvall-Jääskeläinen et al. (2018). Carriage of the *coa* gene by *S. chromogenes* from bovine IMI has not yet been widely reported. Another protein exhibiting coagulating ability, the von Willebrand factor-binding protein, is widely present in NAS bovine IMI isolates. All 30 isolates in the current study were positive for *vWbp,* as were 94% of *S. chromogenes* isolates in Naushad et al. (2019).

In the current study, our hypothesis that total number of virulence genes for each isolate would be a significant predictor of whether it was associated with a HIGH or LOW SCC IMI was not supported. Other researchers exploring virulence potential in NAS of bovine origin have come to similar conclusions. In their study of virulence factors found in 4 different staphylococcal species (4 isolates each of *S. aureus* and *S. agnetis,* 8 isolates each of *S. chromogenes,* and *S. simulans*), Åvall-Jääskeläinen et al. (2018) found no association by visual inspection between the type of mastitis (clinical vs. subclinical) and specific virulence genes, virulence gene profiles, or the cumulative number of virulence genes. As statistical power to analyze these relationships by species in their study was limited by number of isolates, logistic regression analyses of pooled data for all NAS isolates was carried out; still, they did not observe any clear difference in the virulence gene profiles or cumulative number of virulence genes between isolates from clinical and subclinical mastitis (Åvall-Jääskeläinen et al., 2018). Most of the isolates had unique virulence gene profiles, and when two isolates of the same species shared an identical profile, 1 of the isolates was clinical while the other was subclinical (Åvall-Jääskeläinen et al., 2018). When comparing isolates from clinical and subclinical mastitis caused by *S. aureus,* Haveri et al. (2007) found no difference in the cumulative number of VF between the two groups. In a Canadian study of 255 NAS IMI isolates, no association between biofilm formation and SCC associated with the IMI was observed (Tremblay et al., 2013). Similarly, no association was found between the phenotypic ability of a NAS isolate to form biofilm and the persistence of IMI when isolates from 63 persistent and 55 transient were compared (Simojoki et al., 2012). In the same study, researchers found no association between the ability of 114 NAS isolates to form biofilms and the severity of the associated mastitis (as measured by milk N-acetyl-b-D-glucosaminidase activity, an enzyme which reflects tissue damage and is an indicator of inflammation in the udder; Simojoki et al., 2012).

In contrast, other researchers have identified associations between clinical characteristics of an IMI and VF of NAS isolates causing the infections. In a linear regression with all virulence factors considered together, Naushad et al. (2019) did not find that an increase in the overall number of VF for a NAS isolate was associated with an increase in the logSCC of the associated IMI. However, when stratified by type of virulence, the presence of each additional toxin gene for a NAS isolate was associated with a 0.024 increase in logSCC of the associated IMI (Naushad et al., 2019). Similarly, in a logistic regression with ordinal categories for IMI severity (low SCC, medium SCC, high SCC, and clinical mastitis), an overall increase in the number of VFs was not associated with increased severity of an IMI (Naushad et al., 2019). In a regression analysis with VF stratified by functional category, the presence of each additional VF gene associated with host immune evasion increased the odds of having a more severe immune response by 0.074 (Naushad et al., 2019). Naushad et al. (2019) applied various approaches in order to determine whether particular VF distributions had any association with SCC category or occurrence of clinical mastitis, but no clustering of isolates representing low SCC, medium SCC, or high SCC or clinical mastitis was identified. For *S. chromogenes* specifically, Persson Waller et al. (2023) also identified various associations between clinical characteristics of an IMI and VF. They found that a higher number of exoenzyme genes were present in isolates associated with milk samples that had a low CMT vs. a high CMT (Persson Waller et al., 2023). Additionally, isolates from low CMT quarters had higher number of evasion genes than those with high CMT, and the *geh* gene (encoding a lipase) specifically was associated with increased odds of having a low CMT (Persson Waller et al., 2023). As these findings contrasted with those of Naushad et al. (2019) described above, Persson Waller et al. (2023) were unable to identify why they may have observed this association. In the current study, all 30 isolates from both HIGH and LOW SCC categories were positive for *geh,* which is in contrast to Persson Waller et al. (2023) finding it consistently in isolates from IMI with less inflammation occurring. Persson Waller et al. (2023) also found that *capJ* and *capH* were significantly associated with IMI that came from quarters with a lower CMT. This would be consistent with the unencapsulated staphylococci being better able to persist in the udder, as they may cause less of an inflammatory response (Thakker et al., 1998). In contrast, the only 2 isolates carrying *capJ* and *capH* in the current study were associated with a persistently high SCC IMI.

As evidenced by the results of the current study and others failing to find a link between the cumulative number of VF found in staphylococci from a bovine IMI and the degree of inflammation associated with the infection, the expression of disease in an individual animal and the interactions of various VFs with the host’s immune system are complex. Åvall-Jääskeläinen et al. (2018) suggest it is likely that similar symptoms can be caused by several different combinations of virulence factors, rather than by any particular one alone. Similarly, the progression of disease may be determined by the interplay of various VF rather than just the presence of any specific virulence gene. Evidence in support of this was found in Naushad et al. (2019), where they analyzed the relationships between the patterns of VFs associated with isolates from low, medium, and high SCC and clinical mastitis. They were able to demonstrate unique patterns of associations between VFs for low SCC and CM isolates, with many distinct positive and negative association patterns for clinical mastitis isolates in particular. In regards to NAS and IMI, De Buck et al. (2021) write that “pathogenesis is complex and often involves an organized and systematic participation of various VFs to establish disease,” and that “often VFs complement each other to promote pathogen colonization and persistence of disease.” The impact of virulence genes on disease outcomes or development is likely affected by intrinsic (host-level) factors, including the host’s environment, nutritional status and genetics. A particular example of this is the increased concentration of lactoferrin in mastitic milk, which likely inhibits the ability of staphylococci to form biofilms in the udder (as summarized in Simojoki et al., 2012). Extrinsic (environmental) factors, including herd management practices, climatic conditions, the presence of other pathogens in the environment, also play important roles in the successful colonization, persistence, and virulence capability of staphylococci causing intramammary infections.

Similar to *blaZ*, the carriage of VF by isolates in the current study appears to be more a function of phylogeny than a predictor of clinical outcome. The cumulative number of VF identified belonging to the 5 STs with multiple isolates showed little to no variation; total number of VF identified per isolate was uniform for 3 of the 5 ST, 8 of 9 for another ST contained the same number of VF (with the remaining isolate differing by 1 VF), and the remaining ST ranged from 44-48 VF identified per isolate. In a visual assessment of the heat map of VF with isolates organized by ST (Figure 3), many of the limited differences in presence or absence of VF occurred at the ST level. The only isolates lacking *fnbA,* *fnbB*, and *capN*, and possessing *coa, capH* and *capJ* both belong to ST25. Two isolates of singleton STs (ST177 and ST136) which were not classified as a cluster but were grouped together 42% of the time in the phylogenetic analysis were the only 2 isolates positive for the staphylococcal exotoxin genes *set10* and *set24.* Support for an association between phylogeny and VF presence in *S. chromogenes* was also found in Persson Waller et al. (2023). When analyzed at the level of ST cluster, isolates belonging to cluster III, IV, and VII had fewer VF compared to isolates belonging to other clusters, and cluster IV had significantly more exoenzyme genes vs. other clusters. At the strain level, they identified ST59 had higher number of adherence genes vs. other ST. The only gene identified to be associated with phylogenentic grouping was *atl* (autolysin), which was present in clusters V, VI, VII but absent in II, III, and IV (Persson Waller et al., 2023). In the current study, *atl* was consistently found in all 30 *S. chromogenes* isolates. When Naushad et al. (2019) applied various clustering approaches in order to determine whether particular VF distributions had any association with SCC category or clinical mastitis, NAS isolates instead grouped together by their respective species. As the pattern of virulence genes carried by NAS isolates likely is species-dependent, a genetic basis for carriage of VF may also extend to the strain level.

Our ability to explore if pattern and number of VF vary by ST in the current study was limited both by the relatively small number of isolates assigned to most ST identified, as well as complete uniformity in the number and type of VF carried by a particular ST. The number of *S. chromogenes* (30) in this study which were submitted for WGS was a function of financial constraint. Future studies with larger isolate collections, isolates collected from a greater number of farms, and the ability to sequence a larger number of *S. chromogenes* isolates may be better able to explore associations of ARG and VF carriage by ST (as determined by MLST), as larger sample sizes would likely result in a greater diversity of ST and a greater ability to account for the effect of clustering by herd. An additional limitation in the methodology of this and related studies on VF in NAS of bovine origin (Persson Waller et al., 2023; Naushad et al., 2019) is that the database used to identify VF and predict their function was extrapolated from *S. aureus* causing bovine IMI or NAS which were isolated from humans. Until research elucidating the specific pathogenesis for VF identified in NAS isolates of bovine origin is carried out, we are left to infer that VF which are genetically similar to those that are better described in other populations of staphylococci are relevant in bovine IMI. The database compiled and distributed by Naushad et al. (2019) and used in the current study is an extremely valuable contribution to our field, and provides a solid and extensive foundation from which to extend our understanding of VF present in NAS causing IMI in dairy cattle. Lastly, the simple presence or absence of a virulence gene is not indicative of how it is expressed by a pathogen causing an infection in the udder. Previous work on *S. aureus* has shown that the expression level of the VF may influence disease outcome in mastitis (Le Maréchal et al., 2011). Studies exploring gene expression by *S. chromogenes* while causing an IMI would elucidate its pathogenicity *in vivo*.

*Conclusions*

Particular ST (as determined by MLST) of *S. chromogenes* were not associated with persistently HIGH or LOW SCC IMI. Ten different MLST were identified among the 30 isolates, including 4 novel ST. Seven ST were identified in each SCC category, with 4 ST found in both, 3 unique to HIGH, and 3 unique to LOW. The most common STs were ST6 and ST176, with 18 isolates (60%) belonging to 1 of these 2 ST. The only ARG identified was *blaZ,* encoding for resistance to penicillin (33.3% of isolates). Sixty-two unique VF were detected, with a median of 44 VF per isolate, and a range of 43-21. Neither overall number of VF nor *blaZ* carriage was found to be a significant predictor of SCC category. *blaZ* carriage, number and type of VF appears to be a function of ST for *S. chromogenes*, but more research is needed to confirm these findings.

Åvall-Jääskeläinen, S., S. Taponen, R. Kant, L. Paulin, J. Blom, A. Palva, and J. Koort. 2018. Comparative genome analysis of 24 bovine-associated Staphylococcus isolates with special focus on the putative virulence genes. PeerJ 6:e4560.

Bagcigil, A. F., S. Taponen, J. Koort, B. Bengtsson, A.-L. Myllyniemi, and S. Pyörälä. 2012. Genetic basis of penicillin resistance of S. aureus isolated in bovine mastitis. Acta Veterinaria Scandinavica 54(1):69.

Condas, L. A. Z., J. De Buck, D. B. Nobrega, D. A. Carson, S. Naushad, S. De Vliegher, R. N. Zadoks, J. R. Middleton, S. Dufour, J. P. Kastelic, and H. W. Barkema. 2017. Prevalence of non-aureus staphylococci species causing intramammary infections in Canadian dairy herds. J Dairy Sci 100(7):5592-5612.

de Campos, J. L., A. Kates, A. Steinberger, A. Sethi, G. Suen, J. Shutske, N. Safdar, T. Goldberg, and P. L. Ruegg. 2021. Quantification of antimicrobial usage in adult cows and preweaned calves on 40 large Wisconsin dairy farms using dose-based and mass-based metrics. J Dairy Sci 104(4):4727-4745.

Fergestad, M. E., A. De Visscher, T. L'Abee-Lund, C. N. Tchamba, J. G. Mainil, D. Thiry, S. De Vliegher, and Y. Wasteson. 2021. Antimicrobial resistance and virulence characteristics in 3 collections of staphylococci from bovine milk samples. J. Dairy Sci. 104(9):10250-10267.

Foley, J. E., S. J. Spier, J. Mihalyi, N. Drazenovich, and C. M. Leutenegger. 2004. Molecular epidemiologic features of Corynebacterium pseudotuberculosis isolated from horses. Am J Vet Res 65(12):1734-1737.

Freu, G., T. Tomazi, A. F. S. Filho, M. B. Heinemann, and M. V. Dos Santos. 2022. Antimicrobial Resistance and Molecular Characterization of Staphylococcus aureus Recovered from Cows with Clinical Mastitis in Dairy Herds from Southeastern Brazil. Antibiotics 11(4):424.

Garrine, M., S. S. Costa, A. Messa, S. Massora, D. Vubil, S. Ácacio, T. Nhampossa, Q. Bassat, I. Mandomando, and I. Couto. 2023. Antimicrobial resistance and clonality of Staphylococcus aureus causing bacteraemia in children admitted to the Manhiça District Hospital, Mozambique, over two decades. Frontiers in Microbiology 14.

Gillespie, B. E., S. I. Headrick, S. Boonyayatra, and S. P. Oliver. 2009. Prevalence and persistence of coagulase-negative Staphylococcus species in three dairy research herds. Vet Microbiol 134(1-2):65-72.

Haveri, M., A. Roslöf, L. Rantala, and S. Pyörälä. 2007. Virulence genes of bovine Staphylococcus aureus from persistent and nonpersistent intramammary infections with different clinical characteristics. J Appl Microbiol 103(4):993-1000.

Huebner, R., R. Mugabi, G. Hetesy, L. Fox, S. De Vliegher, A. De Visscher, J. W. Barlow, and G. Sensabaugh. 2021. Characterization of genetic diversity and population structure within Staphylococcus chromogenes by multilocus sequence typing. PLoS One 16(3):e0243688.

Kim, S. J., D. C. Moon, S. C. Park, H. Y. Kang, S. H. Na, and S. K. Lim. 2019. Antimicrobial resistance and genetic characterization of coagulase-negative staphylococci from bovine mastitis milk samples in Korea. J Dairy Sci 102(12):11439-11448.

Le Maréchal, C., N. Seyffert, J. Jardin, D. Hernandez, G. Jan, L. Rault, V. Azevedo, P. François, J. Schrenzel, M. van de Guchte, S. Even, N. Berkova, R. Thiéry, J. R. Fitzgerald, E. Vautor, and Y. Le Loir. 2011. Molecular basis of virulence in Staphylococcus aureus mastitis. PLoS One 6(11):e27354.

Mork, T., H. J. Jorgensen, M. Sunde, B. Kvitle, S. Sviland, S. Waage, and T. Tollersrud. 2012. Persistence of staphylococcal species and genotypes in the bovine udder. Vet Microbiol 159(1-2):171-180.

Nobrega, D. B., S. Naushad, S. A. Naqvi, L. A. Z. Condas, V. Saini, J. P. Kastelic, C. Luby, J. De Buck, and H. W. Barkema. 2018. Prevalence and Genetic Basis of Antimicrobial Resistance in Non-aureus Staphylococci Isolated from Canadian Dairy Herds. Front Microbiol 9:256.

Payne, R. E., M. D. Lee, D. W. Dreesen, and H. M. Barnhart. 1999. Molecular epidemiology of Campylobacter jejuni in broiler flocks using randomly amplified polymorphic DNA-PCR and 23S rRNA-PCR and role of litter in its transmission. Appl Environ Microbiol 65(1):260-263.

Persson Waller, K., A. Aspán, A. Nyman, Y. Persson, and U. Grönlund Andersson. 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. Veterinary Microbiology 152(1-2):112-116.

Persson Waller, K., M. Myrenås, S. Börjesson, H. Kim, M. Widerström, T. Monsen, A. K. Sigurðarson Sandholt, E. Östlund, and W. Cha. 2023. Genotypic characterization of Staphylococcus chromogenes and Staphylococcus simulans from Swedish cases of bovine subclinical mastitis. J Dairy Sci 106(11):7991-8004.

Petzer, I. M., C. Labuschagne, L. Phophi, and J. Karzis. 2022. Species identification and cow risks of non-aureus staphylococci from South African dairy herds. Onderstepoort J Vet Res 89(1):e1-e10.

Phophi, L., I. M. Petzer, and D. N. Qekwana. 2019. Antimicrobial resistance patterns and biofilm formation of coagulase-negative Staphylococcus species isolated from subclinical mastitis cow milk samples submitted to the Onderstepoort Milk Laboratory. BMC Vet Res 15(1):420.

Piessens, V., S. De Vliegher, B. Verbist, G. Braem, A. Van Nuffel, L. De Vuyst, M. Heyndrickx, and E. Van Coillie. 2012. Characterization of coagulase-negative staphylococcus species from cows' milk and environment based on bap, icaA, and mecA genes and phenotypic susceptibility to antimicrobials and teat dips. J Dairy Sci 95(12):7027-7038.

Pinho, M. G. 2008. Mechanisms of beta-lactam and glycopeptide resistance in Staphylococcus aureus. Staphylococcus molecular genetics:207-226.

Raspanti, C. G., C. C. Bonetto, C. Vissio, M. S. Pellegrino, E. B. Reinoso, S. A. Dieser, C. I. Bogni, A. J. Larriestra, and L. M. Odierno. 2016. Prevalence and antibiotic susceptibility of coagulase-negative Staphylococcus species from bovine subclinical mastitis in dairy herds in the central region of Argentina. Rev Argent Microbiol 48(1):50-56.

Reydams, H., B. Toledo-Silva, K. Mertens, S. Piepers, F. N. De Souza, F. Haesebrouck, and S. De Vliegher. 2023. Comparison of non-aureus staphylococcal and mammaliicoccal species found in both composite milk and bulk-tank milk samples of dairy cows collected in tandem. J. Dairy Sci. 106(11):7974-7990.

Sampimon, O. 2009. Coagulase-negative staphylococci mastitis in Dutch dairy herds. Utrecht University.

Simojoki, H., P. Hyvönen, C. Plumed Ferrer, S. Taponen, and S. Pyörälä. 2012. Is the biofilm formation and slime producing ability of coagulase-negative staphylococci associated with the persistence and severity of intramammary infection? Veterinary Microbiology 158(3):344-352.

Smith, J. T., E. M. Eckhardt, N. B. Hansel, T. R. Eliato, I. W. Martin, and C. P. Andam. 2021. Genomic epidemiology of methicillin-resistant and -susceptible Staphylococcus aureus from bloodstream infections. BMC Infectious Diseases 21(1):589.

Sol, J., O. C. Sampimon, H. W. Barkema, and Y. H. Schukken. 2000. Factors associated with cure after therapy of clinical mastitis caused by Staphylococcus aureus. J Dairy Sci 83(2):278-284.

Szafraniec, G. M., P. Szeleszczuk, and B. Dolka. 2020. A Review of Current Knowledge on Staphylococcus agnetis in Poultry. Animals (Basel) 10(8).

Taponen, S., A. Jantunen, E. Pyörälä, and S. Pyörälä. 2003. Efficacy of Targeted 5-day Combined Parenteral and Intramammary Treatment of Clinical Mastitis Caused by Penicillin-Susceptible or Penicillin-Resistant Staphylococcus aureus. Acta Veterinaria Scandinavica 44(1):53.

Taponen, S., J. Koort, J. Björkroth, H. Saloniemi, and S. Pyörälä. 2007. Bovine Intramammary Infections Caused by Coagulase-Negative Staphylococci May Persist Throughout Lactation According to Amplified Fragment Length Polymorphism-Based Analysis. J. Dairy Sci. 90(7):3301-3307.

Taponen, S., S. Nykäsenoja, T. Pohjanvirta, A. Pitkälä, and S. Pyörälä. 2016. Species distribution and in vitro antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitic milk. Acta Veterinaria Scandinavica 58(1):12.

Taponen, S., H.-T. Tölli, and P. J. Rajala-Schultz. 2023. Antimicrobial susceptibility of staphylococci from bovine milk samples in routine microbiological mastitis analysis in Finland. Frontiers in Veterinary Science 10.

Thakker, M., J. S. Park, V. Carey, and J. C. Lee. 1998. Staphylococcus aureus serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. Infect Immun 66(11):5183-5189.

Tremblay, Y. D. N., D. Lamarche, P. Chever, D. Haine, S. Messier, and M. Jacques. 2013. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. J. Dairy Sci. 96(1):234-246.

Tuchscherr, L. P., F. R. Buzzola, L. P. Alvarez, R. L. Caccuri, J. C. Lee, and D. O. Sordelli. 2005. Capsule-negative Staphylococcus aureus induces chronic experimental mastitis in mice. Infect Immun 73(12):7932-7937.

USDA. 2016. Dairy 2014: Milk Quality, Milking Procedures and Mastitis in the United States, 2014. Accessed July 12, 2024. <https://www.aphis.usda.gov/sites/default/files/dairy14_dr_mastitis.pdf>.

Vanderhaeghen, W., S. Piepers, F. Leroy, E. Van Coillie, F. Haesebrouck, and S. De Vliegher. 2015. Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. Vet J 203(1):44-51.

Wuytack, A., A. De Visscher, S. Piepers, F. Boyen, F. Haesebrouck, and S. De Vliegher. 2020a. Distribution of non-aureus staphylococci from quarter milk, teat apices, and rectal feces of dairy cows, and their virulence potential. J Dairy Sci 103(11):10658-10675.

Wuytack, A., A. De Visscher, S. Piepers, F. Haesebrouck, and S. De Vliegher. 2020b. Fecal non-aureus Staphylococci are a potential cause of bovine intramammary infection. Vet Res 51(1):32.

Zadoks, R. N., B. E. Gillespie, H. W. Barkema, O. C. Sampimon, S. P. Oliver, and Y. H. Schukken. 2003. Clinical, epidemiological and molecular characteristics of Streptococcus uberis infections in dairy herds. Epidemiol Infect 130(2):335-349.