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***Abstract***

*Staphylococcus chromogenes* is the dominant species associated with mammary gland infections in dairy animals and one of the most persistent intramammary pathogens*.* The objectives of the current study were to: 1) identify if distinct strain types (ST) of *S. chromogenes* were associated with intramammary infections (IMI) where quarter SCC is consistently elevated (HIGH SCC IMI) vs. consistently low (LOW SCC IMI), 2) identify if *S. chromogenes* from HIGH SCC IMI are more likely to carry antimicrobial resistance genes (ARG) vs. LOW SCC IMI, and 3) identify if *S. chromogenes* from HIGH SCC IMI possess more genes encoding previously-described staphylococcal virulence factors (VF) vs. LOW SCC IMI. Isolates originate from a longitudinal observational study of 10 organic dairy farms in Vermont (US), where aerobic culture of quarter-milk samples to identify IMI was conducted in parallel with determination of quarter SCC. Two groups were selected from persistent *S. chromogenes* IMI (as confirmed by RAPD PCR): 1) IMI associated with high SCC, where all quarter-day observations had an associated SCC of ≥200,000 cells/mL; and 2) IMI associated with low SCC, where all quarter-day observations had an associated SCC of <200,000 cells/mL. Representative isolates from 15 LOW SCC IMI and 15 isolates from HIGH SCC IMI were submitted for whole genome sequencing and strain-typed according to a 7-locus MLST scheme*.* ARGs and VF were identified from assembled genomes. Separate mixed-effects logistic regression models were made using ST, ARG carriage, and VF number as the predictor, SCC category as the outcome, and herd as a random effect. Ten different ST were identified, including 4 novel ST. Seven ST were identified in each SCC category, with 3 unique to each. In a mixed-effects logistic regression, ST was not a significant predictor of SCC category. The only ARG identified was *blaZ,* encoding for resistance to penicillin (33.3% of isolates; 6/15 in the HIGH category and 4/15 in the LOW category). *blaZ* was not a significant predictor of SCC category in a mixed-effects logistic regression model. *blaZ* was consistently present in all isolates for 4/5 ST with multiple isolates. Sixty-two unique VF were detected (median: 44 per isolate; range: 43-21). Thirty-nine VF were identified which were present in all isolates, including genes associated with iron uptake and metabolism, production of phenol soluble modulins, hemolysins, and an exfoliative toxin. Presence of VF associated with adherence, host immune evasion, type VII secretion system, and production of exoenzymes and exotoxins was variable. In the HIGH category, 677 VF total were identified vs. 670 in the LOW category. In a mixed-effects logistic regression, number of VF identified was not a significant predictor of SCC category. Genes encoding for exfoliative toxin type C (*etc*) and staphylocoagulase (*coa*) were identified in isolates in the current study, neither of which have been widely reported for *S. chromogenes* isolates of bovine origin. *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.

***Introduction***

*Staphylococcus chromogenes* is the leading cause of intramammary infections in dairy cattle worldwide, for both conventional (De Visscher et al., 2016; Condas et al., 2017a; Rowe et al., 2019; Wuytack et al., 2020a) and organic (Peña-Mosca et al., 2023) herds in various countries. *S. chromogenes* is categorized as belonging to a heterogenous group of bacteria known as the non-*aureus* staphylococci (NAS),although not all species within this group behave the same while causing infections of the udder. Within NAS, *S. chromogenes* is of special concern due to its ability to be both persistent and cause an inflammatory reaction increasing quarter somatic cell count (SCC) (Piessens et al., 2011; Supré et al., 2011; Fry et al., 2014), even to the point where the SCC of quarters infected with *S. chromogenes* were no different than quarters infected with a major mastitis pathogen such as *S. aureus* (Wuytack et al., 2020a; Valckenier et al., 2021; Woudstra et al., 2023).

Beyond the marked differences between different of NAS, significant variation in pathogenicity has also been demonstrated by different strains within the same species. Intraspecies variation has been demonstrated in varying effect on SCC (Supré et al., 2011; Fry et al., 2014; Condas et al., 2017a), differences in interaction with host immune cells (Hyvönen et al., 2009; Åvall-Jääskeläinen et al., 2013), persistence of infection (Mork et al., 2012; Valckenier et al., 2021), and effect on milk production (Thorberg et al., 2009). For *S. chromogenes* specifically, studies have demonstrated heterogeneity in populations of isolates causing IMI. Wuytack et al. (2020a) found *S. chromogenes* to be the most prevalent NAS species causing IMI in quarters identified both as healthy (SCC of ≤ 50,000 cells/mL) and infected, but with no observable clinical signs (SCC of > 50,000 cells/mL), as well as one of the three most common species in quarters exhibiting clinical signs of mastitis. Similarly, Condas et al. (2017b) found that among NAS-positive quarters, *S. chromogenes* was isolated with similar frequency from quarters classified as low SCC (< 200,000 cells/mL), high SCC (> 200,000 cells/mL), and those with clinical mastitis. Different strains of *S. chromogenes* have been identified to vary in their interaction with a host’s immune cells and inflammatory response (Breyne et al., 2015; Piccart et al., 2016; Souza et al., 2016), as well as their preferred habitat niche (skin vs. mammary gland; Wuytack et al., 2020b).

Some research has demonstrated an association between different traits associated with clinical signs or pathogenicity for staphylococcicausing IMI*.* Valckenier et al. (2021) describe a link between persistence of infection and associated SCC, where quarters classified as having a transient IMI due to *S. chromogenes* had a mean SCC of 69,000 cells/mL and those classified as having a persistent S. chromogenes IMI had a SCC of 351,000 cells/mL. Wuytack et. al (2020a) found genes encoding various virulence factors associated with staphylococci in 44% of NAS isolates originating from cases of clinical mastitis, while only 19% of isolates associated with infections found in quarters with an SCC of ≤ 50,000 cells/mL. These virulence factors included genes associated with biofilm formation to enhance colonization and evasion of host immune response, various enzymes associated with other virulence proteins, and capsule formation. In a study by Haveri et al. (2005) of 217 *S. aureus* IMI isolates typed using pulsed-field gel electrophoresis (PFGE), researchers were able to identify that a particular pulsotype was significantly associated with severe clinical mastitis symptoms but reduced persistence when compared to the 4 other commonly identified pulsotypes in the study. This association between a specific genotype and consistent expression of a clinical trait associated with an IMI has not yet been widely described for NAS. However, researchers in a large Canadian study investigating the profile of staphylococcal virulence factors for 25 different species of NAS identified 2 rather distinct populations among the 83 *S. chromogenes* included in their study (Naushad et al., 2019). In a cluster analysis looking at the distribution of all 191 virulence factors for the 441 genomes of isolates included in the study, *S. chromogenes* was the only species split into 2 distinct populations: the majority of *S. chromogenes* strains cluster together with profile distinct to their species, but a small number of strains cluster with isolates belonging to different but closely-related species (Naushad et al., 2019). The authors point out that a larger number of *S. chromogenes* isolates were included compared with other species, but suggest the possibility that the finding may represent separate pathotypes of *S. chromogenes* causing bovine IMI.

In a longitudinal study of 10 certified organic dairy farms in Vermont (US), *S. chromogenes* was found to be the most common pathogen causing subclinical mastitis (Jeffrey et al., unpublished manuscript). In agreement with the heterogeneity of SCC observed in Wuytack et al. (2020a) and Condas et al. (2017b), the quarter SCC (qSCC) associated with *S. chromogenes* IMI in our study ranged from 2,000 cells/mL (the lower limit of detection) to 6,100,000 cells/mL (Jeffrey et al., unpublished manuscript). Furthermore, most *S. chromogenes* IMI observed were persistent for at least 60-90 days during the study period. The aim of the current study is to better understand the diversity within *S. chromogenes* causing bovine IMI by identifying if there was a genetic basis for the observed difference in pathogenicity (as measured by qSCC). The specific objectives of the current study are to: 1) identify if distinct strain types of *S. chromogenes* are associated with IMI where qSCC is consistently elevated (HIGH SCC IMI) vs. consistently low (LOW SCC IMI), 2) identify if *S. chromogenes* from HIGH SCC IMI are more likely to carry genes encoding for antimicrobial resistance (as determined by whole-genome sequencing) vs. LOW SCC IMI, and 3) identify if *S. chromogenes* from HIGH SCC IMI possess a larger number of genes encoding previously-described staphylococcal virulence factors vs. LOW SCC IMI.

***Materials and methods***

STROBE-VET (Strengthening the Reporting of Observational Studies in Epidemiology–Veterinary Extension) statement guidelines were followed in the reporting of this study (O'Connor et al., 2016). Animal use for this project was approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC; protocol #19-001).

*Sample origination*

Isolates included in the current study originate from milk samples collected during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US) carried out in Winter 2019-2020. Enrolled farms were a non-probability subsample of certified organic dairies in Vermont which had participated in previous studies, and inclusion criteria included: 1) milking between 35-120 cows and 2) using either a tiestall barn bedded with shavings/sawdust or a bedded pack system to house lactating dairy cows. The inclusive term “bedded pack” is used here to encompass both aerobically composting bedded packs and deep bedded packs, and was defined as an enclosed loose housing facility deeply bedded with organic material (Jeffrey et al., 2024). Around the time of the first farm visit, herd records were captured from the record processing center working with 9 of the participating herds (Lancaster DHIA, Manheim, PA; Dairy One Co-Op. Inc., Ithaca, NY) to obtain freshening date and parity for the current lactation. Freshening date and parity for 1 herd was obtained from personal communication with the producer who kept written records. The goal was to enroll 35 cows of varying parity in early- to mid-lactation from each herd for the duration of the study. In 1 herd with approximately 35 lactating cows, all cows were sampled. In 8 herds with ≥35 cows and with available DHIA data, a stratified random approach was used with cows stratified by SCC, lactation number, and DIM and then randomly selected across these variables. In 1 herd with ≥35 cows and no DHIA data, the producer generated a list of 35 cows in early lactation so that they would continue to be milking for the duration of the study. Cows that were unable to be sampled at a follow-up visit (dried off, left the herd) were replaced with another lactating cow dictated by convenience. At each farm visit, duplicate quarter-milk samples were aseptically collected from each lactating quarter immediately before milking for all enrolled cows according to NMC guidelines (NMC, 2017). Briefly, after routine pre-milking teat disinfection was completed, researchers (wearing clean disposable gloves) scrubbed teat ends and the distal third of teats with 70% isopropyl alcohol-moistened gauze swabs until teat ends were visibly clean, stripped the quarters (discarding 3-5 squirts of foremilk), and sequentially collected approximately 5-6 mL of milk into each of two sterile 11-mL flip-top vials. If any sign of clinical mastitis was present, it was noted and that sample was excluded from the inclusion in this study. Samples were kept on ice in a cooler during transport until stored temporarily overnight at 4°C in the laboratory, where an aliquot was frozen for SCC measurement and the remaining milk sample was processed for bacteriological culture.

*SCC measurement*

Aliquots of frozen quarter-milk samples were sent to the Vermont State Agricultural and Environmental Laboratory, where samples were gradually thawed under refrigeration at time of processing and quarter-level somatic cell count was determined using flow cytometry (Somacount FC, Bentley Instruments).

*Aerobic culture of milk samples and determination of bacteriological status*

Standard aerobic bacteriological culture of quarter-milk was performed in duplicate within 24 hours of collection to identify bacterial species present in the sample. After being homogenized by gentle inversion, tryptic soy agar plates with 5% sheep blood (Northeast Laboratory, Waterville, ME) were inoculated with 10 μL of milk using disposable calibrated plastic inoculating loops. Plates were then incubated in aerobic conditions at 37°C before being read at approximately 24 and 48 hrs.

Aerobic culture results of both samples were then used together to determine the overall bacteriological status of each quarter-milk sample into the following categories: 1) “no significant growth,” when there was no growth on both plates, or ≤200 CFU/mL on one plate and no growth on the other plate, or ≤200 CFU/mL on both plates and morphology of isolates on each plate was different; 2) “pure culture,” when there was ≥100 CFU/mL of a particular isolate identified with the same morphology on both plates; 3) “mixed culture,” when there was ≥100 CFU/mL of two phenotypically-distinct isolates identified, each growing on both plates; 4) “contaminated,” when 1 or both of the 2 samples had more than 2 morphologically distinct isolates growing on a plate; 5) and “indeterminate,” when the set of quarter-milk samples did not meet the criteria for any of the previous categories (e.g., missing duplicate).

*Identification of bacterial isolates to species*

Isolates from both pure and mixed culture quarter-milk samples were selected and grown in pure culture on blood agar. Standard benchtop tests were done to presumptively identify bacteria following NMC procedure guidelines, including differential growth on selective media, colony morphology, hemolytic pattern, catalase reaction, Gram stain, and coagulase testing (NMC, 2017). Isolates were preserved in tryptic soy broth with a final concentration of 15% glycerol in cryovials and stored at -80°C. Frozen isolates were sent overnight on ice to the University of Missouri for speciation using MALDI-TOF mass spectrometry (Microflex, Bruker Daltonics) with Flex Control software (Bruker Daltonics). The protocol for identifying bacterial isolates with MALDI-TOF mass spectrometry has been described previously (Haw et al., 2024). Briefly, generated spectra were assigned a score based on similarity with spectra in the manufacturer’s database (MBT 8468 MSP Library), as well as the University of Missouri laboratory custom database (Adkins et al., 2018). The confidence levels used for NASM species identification were applied as previously described (Cameron et al., 2017), in which ≥1.7 was used for species-level identification and <1.7 was classified as inconclusive. Suspect staphylococci and mammaliicocci isolates unable to be identified to the species level and those identified as *Staphylococcus agnetis* or *Staphylococcus hyicus* by MALDI-TOF were speciated using *tuf*gene sequences with a cut-off of 98% identity as previously described (Hwang et al., 2011).

*Determination of IMI status and selection of isolates*

Using the bacteriological status and speciation information, a quarter-day IMI status was assigned to each quarter observation: 1) “healthy,” when there was no significant growth; 2) “infected with *S. chromogenes* only,” when ≥100 CFU/mL of *S. chromogenes* was identified in pure culture on both plates (interpretation in series; Dohoo et al., 2011); 3) “mixed infection with *S. chromogenes*,” when ≥100 CFU/mL of *S. chromogenes* and an additional species were identified in mixed culture on both plates; 4) “infected with pathogen other than *S. chromogenes*,” when ≥100 CFU/mL of a species besides *S. chromogenes* was identified in pure or mixed culture on both plates; and 5) “unknown” if the sample status had been identified as contaminated or indeterminate as previously described. Quarter-day observations were eligible for inclusion in further analysis if they had an associated quarter-level SCC measurement and the IMI status was classified as infected with *S. chromogenes* only.

A given quarter was considered to have a potentially persistent *S. chromogenes* IMI if: 1) it had ≥ 2 quarter-day observations (from sequential sampling events approximately 30 days apart); 2) IMI status could be determined for all sampling events associated with that quarter; and 3) it was infected with *S. chromogenes* only for all associated quarter-day observations throughout the study. Two groups were then selected from all potentially persistent *S. chromogenes* IMI: 1) IMI associated with high SCC, where all quarter-day observations had an associated SCC of ≥200,000 cells/mL; and 2) IMI associated with low SCC, where all quarter-day observations had an associated SCC of <200,000 cells/mL. Any potentially persistent *S. chromogenes* IMI that did not fit into one of these two categories was excluded from further analysis (e.g., had an SCC of <200,000 cells/mL for one quarter-day observation and an SCC of ≥200,000 cells/mL for the next).

*Strain-typing and selection of isolates*

All isolates associated with each potentially persistent high and low SCC *S. chromogenes* IMI were strain typed using random amplification of polymorphic DNA (RAPD)-PCR. DNA was extracted using a commercial kit from overnight broth culture following the manufacturer’s instructions (DNeasy Blood and Tissue Kit, Qiagen) and then stored at -20 °C until further analysis. RAPD-PCR was performed as described by Wuytack et al. (2020b) using the primer set D11344 (as described by Fitzgerald et al., 1997) with the following PCR conditions: 4 cycles of 94 °C at 5 min, 36 °C at 5 min, and 72 °C at 5 min and 30 cycles of 94 °C at 1 min, 36 °C at 1 min, and 72 °C at 2 min. A negative control (no DNA) was included for each amplification. Amplified DNA fragments were then separated on 1.5% (wt/vol) agarose gels stained with SYBR Safe (0.1 µL/mL; ThermoFisher Scientific), at 120 V for 75 min, and then photographed by UV transillumination (Image Lab, Bio-Rad). The RAPD PCR product of all isolates from a given persistent IMI were analyzed in the same PCR amplification and were run side-by-side on the same gel. The images were inspected visually, and isolates with the same banding pattern, number, and size of bands were considered to be the same RAPD type. If theisolates from all quarter observations of an *S. chromogenes* IMI belonged to the same RAPD type, the quarter was considered persistently infected with the same strain.

In order to describe the diversity of *S. chromogenes* RAPD types among persistent IMI within each herd, one representative isolate was selected from each confirmed persistent IMI for strain comparison. The RAPD PCR products from all representative isolates within a herd were run side-by-side on a gel and imaged (as described above) along with a 1 kb bp ladder for image standardization. The gel images were imported into BioNumerics version 7.5 (AppliedMaths, Sint-Martens-Latem, Belgium) and analyzed with the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) with both optimization and position tolerance set at 1.0%. Isolates from the same herd with 100% similarly were considered the same RAPD type.

From among the confirmed persistent *S. chromogenes* IMI, 15 quarters with a persistently low SCC IMI (LOW) were selected to match the 15 quarters with a persistently high SCC IMI (HIGH). LOW quarters were matched to HIGH quarters belonging to the same cow (different quarter) when possible. If this was not possible, LOW and HIGH quarters were matched on farm, or facility type (bedded pack vs. tiestall) when same farm was not possible. When LOW and HIGH quarters were paired between different cows, quarters were matched as closely as possible to ensure a similar DIM and parity. From each of the 15 HIGH and 15 LOW IMI, a representative isolate was chosen to undergo whole-genome sequencing (WGS). For each persistent IMI which had 3 associated quarter-day observations, the middle isolate in the series was submitted for WGS. For persistent IMI which had 2 associated quarter-day observations, 1 of the 2 isolates in the series was haphazardly selected for WGS.

*DNA extraction, whole-genome sequencing, assembly, and annotation*

Each of the 30 *S. chromogenes* isolates selected for WGS were grown from frozen stock on blood agar in aerobic conditions at 37°C, and read at approximately 24 and 48 hrs. All plates were then inspected to ensure purity, and a single colony was selected and passed to a new blood agar plate. After again being incubated at 37°C, read at approximately 24 and 48 hrs, and checked for contamination, 48-hr growth plates were wrapped in Parafilm (Amcor). Wrapped plates were sent overnight to a commercial sequencing facility (SeqCoast Genomics; Portsmouth, NH, USA) for DNA extraction, library preparation, long read sequencing using GridION Oxford Nanopore, paired-end sequencing using Illumina, assembly, and annotation. DNA extraction was performed on colony material collected from the agar plates with a commercial kit using bead beating lysis (MagMAX Microbiome Ultra Nucleic Acid Isolation Kit, Applied Biosystems). Library preparation was completed using Illumina DNA Prep tagmentation kit (Illumina), and paired-end sequencing (2x150bp) was run on the Illumina NextSeq2000 platform (Illumina). During Illumina sequencing, 1-2% PhiX control was spiked into the run to support optimal base calling, and read demultiplexing, read trimming, and run analytics were performed on the instrument using DRAGEN v3.10.12. Library preparation for long-read sequencing was completed using the Oxford Nanopore Technologies SQK-LSK114 native barcoding kit, and sequencing was performed on the GridION platform (FLOW-MIN114 Spot-ON Flow Cell, vR10). Quality-trimming of raw reads was completed using Trimmomatic v0.39 (Bolger et al., 2014) and Porechop v.0.2.4 (https://github.com/rrwick/Porechop) for reads from Illumina and Oxford Nanopore sequencing, respectively. Unicycler v0.4.4 (Wick et al., 2017) was used for hybrid assembly of all genomes. Briefly, the trimmed Illumina reads were assembled using SPAdes v3.14.0 (Bankevich et al., 2012) and then mapped with trimmed error-corrected Oxford Nanopore reads using Bowtie2 (Langmead and Salzberg, 2012) and SAMtools (Li et al., 2009). The polishing of the final hybrid assembly was done using Pilon (Walker et al., 2014), and annotation was completed using BAKTA v1.5.1 (Schwengers et al., 2021).

*Bioinformatic analyses, in silico multilocus sequence typing, and detection of ARG genes and VF*

Whole genome multilocus sequence types were predicted *in silico* from the annotated genomes for the 7-locus scheme described for *S. chromogenes* (Huebner et al., 2021) using the MLST 2.0 tool (Center for Genomic Epidemiology, Technical University of Denmark, Kongens Lyngby, Denmark; software v2.0.9, database v2023-06-19; MLST allele sequence and profile data obtained from PubMLST.org). Any novel alleles identified by were confirmed using PCR and Sanger sequencing. The 7-locus concatenated nucleotide sequence data were then combined with all 386 available concatenated MLST sequences for *S. chromogenes* in PubMLST. The resulting FASTA file was used for the construction of phylogenetic trees by maximum-likelihood algorithm with the optimal model and 100 bootstrap replications in MEGA-X (Kumar et al., 2018). Isolates which grouped together with a bootstrap value of ≥ 65% were classified as clusters.

ARGs were identified from assembled genomes using ABRicate v1.01, which draws from 5 different databases [ResFinder from Center for Genomic Epidemiology (Camacho et al., 2009; Bortolaia et al., 2020), Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020), MegaRES v3.0 (Bonin et al., 2023), ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation), and AMRFinderPlus from NCBI (Feldgarden et al., 2021)] using the default settings (https://github.com/tseemann/abricate). Virulence factors were identified from assembled genomes using the VFDB tool (Chen et al., 2016) and a “blastp” search against a published comprehensive dataset of staphylococci virulence factors (Naushad et al., 2019). After the blast search, the best hit of virulence genes for each genome was chosen based on *H* values, as described by Naushad et al. (2019). Briefly, an *H* value was calculated to determine homology between query protein sequences and blast hits (Fukiya et al., 2004). *H* values (in units of amino acids) between protein sequences were calculated using the following formula: *H* = *VFid × Lm/Lq*, where *VFid* represents the percent similarity between the VF query sequence and the identified protein sequence (expressed as proportion between 0 and 1), *Lm* represents the alignment length, and *Lq* denotes the length of the query sequence (Fukiya et al., 2004). A cutoff was established for sequence similarity of 30% and a query length coverage of 50%, with any hits having values below these cutoffs discarded from the data set. Hits from each query sequence were then arranged according to their *H* value, and the hit with the largest value (highest sequence similarity and query length coverage) was selected in order to prevent one VF query returning hits to two different genes within a given genome. The list of remaining VF were classified into five functional categories, as outlined in Naushad et al. (2019): 1) adherence, 2) exoenzymes, 3) host immune evasion, 4) iron uptake and metabolism, and 5) toxins (including hemolysins, leukocidins, leukotoxins, toxic shock syndrome toxin, exfoliative toxins, type VII secretion system genes, phenol-soluble modulins, enterotoxins, and exotoxins).

*Statistical analysis*

A spreadsheet (Microsoft Excel, Redmond, WA) with isolate identification, associated metadata, and outcome variables was made and imported into the R Statistical Programming Environment (R Development Core Team, 2023) for analysis. Descriptive statistics were generated to compare parity and DIM of the cow, quarter location, and average SCC associated with each persistent IMI between the two SCC categories (HIGH vs. LOW). Normality of the data was checked using a Shapiro test. For outcomes which were not normally distributed (parity, DIM, average SCC), a Mann Whitney U test was used to compare metrics between the HIGH and LOW groups. For outcomes which were normally distributed (quarter location), Fisher’s Exact test was used to compare the two groups. Statistical significance for these tests were declared at P ≤ 0.05.

Separate mixed-effects logistic regression models were made using ST, ST cluster, *blaZ* carriage, and VF number as the predictor, SCC category as the outcome, and herd as a random effect using the “lme4” package (R Development Core Team, 2023). The variable representing the number of VF genes identified per isolate was centered and scaled by subtracting the mean and dividing by the standard deviation. Significance of predictors in these mixed-effects logistic regression models was assessed using a cutoff of ≤0.05 for the *P-*value associated with the z-statistic.

*Data availability*

The raw reads from ONT and Illumina for all 30 genomes are available under NCBI Bioproject accession number PRJNA1130504 (Biosamples SAMN42232476 to SAMN42232505) in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

***Results***

*Descriptive results, MLST and phylogenetic analyses*

In total, 136 potentially persistent *S. chromogenes* IMI were identified from the dataset. There were 91 potentially persistent IMI which were associated with 3 sequential quarter-observations and 45 which were associated with 2. There were 15 potentially persistent IMI where all quarter-day observations had an associated SCC of ≥200,000 cells/mL, 60 where all quarter-day observations had an associated SCC of <200,000 cells/mL, and 61 which had an associated SCC both above and below 200,000 cells/mL. Of the 60 LOW IMI, 45 were associated with 3 sequential quarter-observations (135 associated isolates), and 15 were associated with 2 sequential quarter-observations (30 associated isolates). Of the 15 HIGH IMI, 3 were associated with 3 sequential quarter-observations (9 associated isolates), and 12 were associated with 2 sequential quarter-observations (24 associated isolates). One hundred and ninety-eight isolates associated with 75 potentially persistent *S. chromogenes* IMI underwent RAPD-typing, with 74 of the 75 IMI determined to be caused by the same strain type. The median number of persistently high and low SCC IMI per herd was 8 (Table 1; range: 3-14), and the median number of RAPD types associated with these IMI was 5 (range: 2-9).

The representative isolates from 15 HIGH and 15 LOW IMI which were selected for WGS originated from 7 of the sampled organic herds, with 16 coming from a herd using a bedded pack facility and 14 from tiestall. Thirteen were associated with 3 sequential quarter-observations and 17 were associated with 2 sequential quarter-observations. Isolates in the HIGH group were from 6 different farms (8 bedded packs and 7 tiestalls), while isolates in the LOW group also come from 6 different farms (8 bedded packs and 7 tiestalls). The median parity and DIM of the cow from which the isolate originated was 2 (range: 1-6) and 281 days (range: 58-438 days) for the HIGH group, and 2 (range: 1-6) and 229 days (range: 41-438 days) for the LOW group, respectively. Parity group (first, second, third, fourth and above), DIM, and quarter position did not differ between the HIGH and LOW group (p = 0.88, 0.14, 0.88, respectively). The median of the average SCC associated with each IMI was 410,000 cells/mL (range: 230,000-2,798,000 cells/mL) for the HIGH group, and 98,500 cells/mL (range: 28,000-185,000 cells/mL) for the LOW group. The average SCC associated with the IMI in the HIGH group was greater than that of the LOW group (p <0.001).

Ten different multilocus sequence types were identified among the 30 representative isolates which underwent WGS, with 7 ST identified in each the HIGH and LOW SCC categories (Table 2). Four novel ST were identified which were not already present in the PubMLST database for *S. chromogenes* (ST174 through ST177). Four ST were found in both SCC categories (ST 5, ST6, ST48, ST176), 3 were unique to the HIGH category (ST25, ST136, ST177), and 3 were unique to the LOW category (ST51, ST174, ST175). The most common STs identified were ST6 and ST176, with 18 isolates (60%) belonging to 1 of these 2 ST (9 isolates, or 30%, belonging to each ST6 and ST176 respectively). In a dendrogram constructed from concatenated nucleotide sequence data for the study isolates as well as 386 publicly-available concatenated MLST sequences for *S. chromogenes*, five ST clusters were identified where study isolates which grouped together with a bootstrap value of ≥ 65% (Supplemental Figure S1). Ninety percent of isolates (27/30) were able to be assigned to 1 of these 5 ST clusters. The 3 remaining isolates represented ST with only a single isolate.

*Analysis of associations between ST (or ST cluster) and SCC category*

In a mixed-effects logistic regression, ST was not found to be a significant predictor of whether an isolate would be in the HIGH or LOW SCC category (*P* <0.05). As 5 isolates were singleton STs, and the 2 isolates belonging to ST25 were both in the HIGH category, this model was run for a dataset containing the remaining 23 isolates (belonging to 4 different ST). Similarly, cluster was not found to be a significant predictor of whether an isolate would be in the HIGH or LOW SCC category (*P* <0.05) for a dataset containing the 25 isolates able to be grouped into 1 of the 5 ST clusters identified. Three separate models (with all 30 isolates in the dataset for each) were run to see if belonging to ST176, ST cluster 1, or ST6 predicted the SCC category of an isolate. However, all three models found that belonging to each of these 3 groupings was not a significant predictor of SCC category (*P* = 0.69 for ST176; *P* = 0.37 for ST1 cluster; *P* = 0.69 for ST6).

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

The only resistance determinant identified among the 30 *S. chromogenes* isolates was *blaZ*. Ten of the 30 (33%) *S. chromogenes* isolates were positive for *blaZ,* 6/15 (40%) in the HIGH category and 4/15 (26.7%) in the LOW category (Figure 1). *blaZ* gene carriage was not found to be a significant predictor of SCC category (HIGH vs. LOW) for an isolate in a mixed-effects logistic regression model (*P* = 0.44). As *blaZ* carriage was consistently present in all isolates for 4 out of the 5 ST with multiple isolates (Table 3), statistical analysis exploring if ST predicted *blaZ* carriage was not possible. All isolates belonging to ST5 and ST48 were *blaZ* positive, while no isolates belonging to ST25 and ST176 carried the gene. Only isolates belonging to ST6 were varied in *blaZ* carriage.

*Virulence genes identified and analysis of associations between VF and SCC category*

There were 62 different VF detected among the 30 *S. chromogenes* isolates (Table 4). There were 39 VF identified which were present in 100% of isolates (Figure 3), which included all genes associated with iron uptake and metabolism, and those associated with production of phenol soluble modulins, hemolysins, and an exfoliative toxin. Presence of VF associated with adherence, host immune evasion, type VII secretion system, and production of exoenzymes and exotoxins varied between isolates. Some patterns of presence or absence of VF was specific to particular ST. This included the presence of *capJ*, *capH* (both related to capsule formation), and *coa* (staphylocoagulase enzyme), and the absence of *fnbA, fnbB* (both related to adherence), and *capH* for both isolates belonging to ST25; and the presence of *set21* (exotoxins) in both isolates belonging to ST48. The full complement of genes associated with the type VII secretion system (*esaA, esaB, essA, essB, essC, esxA*) were only found in isolates from ST48 and ST177, which were not clustered together in the phylogenetic analysis.

A total of 677 VF were identified among the 15 isolates in the HIGH category, compared to 670 total VF for the 15 LOW isolates. The median number of VF found in both categories was 44, while the range for the HIGH category 44-51 and the range for the LOW category was 43-50. There were 61 different VF detected in isolates belonging to the HIGH category, and 57 different VF found in the LOW category. Five VF were unique to the isolates in the HIGH category: *coa, set10, set34, capH* and *capJ.* The two isolates positive for *coa, capH* and *capJ* in the HIGH group were both ST25, which was an ST unique to the HIGH SCC category. The two isolates positive for *set10* and *set34* in the HIGH group belonged to ST136 and ST177, which were both unique to the HIGH SCC category. In the phylogenetic analysis, ST136 and ST177 clustered together 42% of the time, which was below the 65% cutoff used to identify clusters of STs. Only 1 VF was unique to an isolate in the LOW category (*sdrD,* a gene associated with fibrinogen binding proteins rich in aspartic acid and serine). This isolate belonged to ST5, 2 of which were in the HIGH category and 1 of which was in the LOW category.

In a mixed-effects logistic regression, number of VF identified was not found to be a significant predictor of whether an isolate would be in the HIGH or LOW SCC category (*P* = 0.54). As the number of VF identified was fairly consistent across all isolates in a given ST, statistical analysis exploring if a particular ST (or ST cluster) was a significant predictor of VF number was not feasible. All isolates belonging to ST6 (n=9) and ST25 (n=2) had 44 VF identified, both isolates in ST48 (n=2) had 50 VF identified, and 8 of the 9 isolates belonging to ST176 have 44 (one has 43). The 3 isolates of ST5 had some variation in number of VF (44, 47, 48 genes each).

***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., 2017a) 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.

***Figures and tables***

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| --- | --- | --- |
| Table 1. Diversity of RAPD types causing persistently high and low SCC *Staphylococcus chromogenes* intramammary infections by farm. An *S. chromogenes* IMI was considered persistent if it had ≥ 2 quarter-day observations (from sequential sampling events approximately 30 days apart) and it was infected with *S. chromogenes* only for all quarter-day observations. Persistent IMI were then selected where all quarter-day observations had an associated SCC of ≥200,000 cells/mL or <200,000 cells/mL. All isolates associated with each IMI were strain typed using RAPD to ensure persistency; 74 out of 75 IMI were found to be caused by the same strain of *S. chromogenes.* | | |
| Farm | No. persistent IMI | No. RAPD types |
| Farm A | 8 | 3 |
| Farm C | 14 | 9 |
| Farm D | 6 | 3 |
| Farm E | 7 | 5 |
| Farm F | 3 | 2 |
| Farm G | 8 | 5 |
| Farm H | 13 | 7 |
| Farm I | 3 | 2 |
| Farm J | 11 | 6 |

|  |  |  |  |
| --- | --- | --- | --- |
| Table 2. Number of *Staphylococcus chromogenes* isolates associatedwith persistent bovine intramammary infections belonging to each strain type (ST; as determined by multilocus sequence typing), stratified by SCC category. All isolates in the HIGH category come from IMI which had an SCC of ≥200,000 cells/mL associated with all quarter-day observations, and all isolates in the LOW category come from IMI which had an SCC of <200,000 cells/mL associated with all quarter-day observations. A phylogenetic tree was constructed from concatenated nucleotide sequence data for the study isolates as well as 386 publicly-available concatenated MLST sequences for *S. chromogenes.* Study isolates which grouped together with a bootstrap value of ≥65% were classified as ST clusters. | | | |
| SCC category | Strain type | ST cluster | No. isolates |
| HIGH | 5 | 5 | 1 |
|  | 6 | 6 | 5 |
|  | 25 | 25 | 2 |
|  | 48 | 48 | 1 |
|  | 136 | - | 1 |
|  | 176\* | 1 | 4 |
|  | 177\* | - | 1 |
| LOW | 5 | 5 | 2 |
|  | 6 | 6 | 4 |
|  | 48 | 48 | 1 |
|  | 51 | - | 1 |
|  | 174\* | 1 | 1 |
|  | 175\* | 1 | 1 |
|  | 176\* | 1 | 5 |
| \* Indicates a novel strain type of *S. chromogenes* not previously identified in PubMLST | | | |

|  |  |  |
| --- | --- | --- |
| Table 3. *blaZ* gene carriage by strain type (as determined by multilocus sequence typing) and ST cluster for 30 *Staphylococcus chromogenes* isolates associatedwith persistent bovine intramammary infections. | | |
| Strain type | ST cluster | Prop. isolates + for *blaZ* (%) |
| 6 | 6 | 4/9 (44%) |
| 176 | 1 | 0/9 (0%) |
| 5 | 5 | 3/3 (100%) |
| 25 | 25 | 0/2 (0%) |
| 48 | 48 | 2/2 (100%) |
| 51 | - | 1/1 (100%) |
| 136 | - | 0/1 (0%) |
| 174 | 1 | 0/1 (0%) |
| 175 | 1 | 0/1 (0%) |
| 177 | - | 0/1 (0%) |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table 4. Presence of potential virulence factors and related genes for 30 *Staphylococcus chromogenes* isolates from subclinical bovine intramammary infections, stratified by SCC category. All isolates in the HIGH category (n = 15) come from IMI which had an SCC of ≥200,000 cells/mL associated with all quarter-day observations, and all isolates in the LOW (n = 15) category come from IMI which had an SCC of <200,000 cells/mL associated with all quarter-day observations. Grouping scheme adapted from Naushad et al., 2019. Bolded virulence factors with an asterisk represent related genes that were unique to an SCC category. | | | | |
| Type of virulence |  |  | SCC category | |
| *Adherence* | Virulence factor | Related genes | LOW  n (%) | HIGH  n (%) |
|  | Autolysin | *atl* | 15 (100) | 15 (100) |
|  | Biofilm-associated surface protein | *bap* | 14 (93.3) | 13 (86.7) |
|  | Fibronectin binding proteins | *fnbA* | 15 (100) | 13 (86.7) |
|  | Fibronectin binding proteins | *fnbB* | 15 (100) | 13 (86.7) |
|  | Ser-Asp-rich fibrinogen binding proteins | *sdrC* | 1 (6.7) | 1 (6.7) |
|  | **Ser-Asp-rich fibrinogen binding proteins\*** | *sdrD* | 1 (6.7) | 0 (0) |
|  | Ser-Asp-rich fibrinogen binding proteins | *sdrE* | 1 (6.7) | 1 (6.7) |
|  | Ser-Asp-rich fibrinogen binding proteins | *sdrG* | 1 (6.7) | 1 (6.7) |
| *Host immune evasion* |  |  |  |  |
|  | **Capsule formation\*** | *capH* | 0 (0) | 2 (13.3) |
|  | **Capsule formation\*** | *capJ* | 0 (0) | 2 (13.3) |
|  | Capsule formation | *capN* | 15 (100) | 13 (86.7) |
|  | Capsule formation | *capO* | 15 (100) | 15 (100) |
|  | Capsule formation | *capP* | 15 (100) | 15 (100) |
|  | Staphylococcal complement inhibitor | *scn* | 15 (100) | 15 (100) |
| *Iron uptake and metabolism* |  |  |  |  |
|  | ABC transporter (siderophore receptor) | *htsA* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *htsB* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *htsC* | 15 (100) | 15 (100) |
|  | Iron-regulated surface determinant protein | *isdF* | 15 (100) | 15 (100) |
|  | Iron-regulated surface determinant protein | *isdG* | 15 (100) | 15 (100) |
|  | Iron-regulated surface determinant protein | *isdI* | 15 (100) | 15 (100) |
|  | Staphyloferrin B synthesis-related genes | *sbnA* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *sfaA* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *sfaB* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *sfaC* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *sfaD* | 15 (100) | 15 (100) |
|  | Staphyloferrin A synthesis-related | *sirA* | 15 (100) | 15 (100) |
|  | Staphyloferrin A synthesis-related | *sirB* | 15 (100) | 15 (100) |
|  | Staphyloferrin A synthesis-related | *sirC* | 15 (100) | 15 (100) |
| *Exoenzymes* |  |  |  |  |
|  | Adenosine synthase A | *adsA* | 15 (100) | 15 (100) |
|  | Aureolysin | *aur* | 15 (100) | 15 (100) |
|  | **Staphylocoagulase\*** | *coa* | 0 (0) | 2 (13.3) |
|  | Lipase | *geh* | 15 (100) | 15 (100) |
|  | Lipase | *lip* | 15 (100) | 15 (100) |
|  | Thermonuclease | *nuc* | 15 (100) | 15 (100) |
|  | von Willebrand factor-binding protein | *vWbp* | 15 (100) | 15 (100) |
| *Exotoxins* |  |  |  |  |
|  | **Staphylococcal exotoxin 10\*** | *set10* | 0 (0) | 2 (13.3) |
|  | Staphylococcal exotoxin 15 | *set15* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 16 | *set16* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 18 | *set18* | 1 (6.7) | 2 (13.3) |
|  | Staphylococcal exotoxin 20 | *set20* | 13 (86.7) | 10 (66.7) |
|  | Staphylococcal exotoxin 21 | *set21* | 1 (6.7) | 1 (6.7) |
|  | Staphylococcal exotoxin 26 | *set26* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 3 | *set3* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 30 | *set30* | 15 (100) | 15 (100) |
|  | **Staphylococcal exotoxin 34\*** | *set34* | 0 (0) | 2 (13.3) |
|  | Staphylococcal exotoxin 40 | *set40* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 6 | *set6* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 8 | *set8* | 1 (6.7) | 2 (13.3) |
| *Toxins: Phenol soluble modulins* |  |  |  |  |
|  | Phenol soluble modulins (β) | *PSMB1* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB2* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB3* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB4* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB5* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB6* | 15 (100) | 15 (100) |
| *Toxins: Hemolysins* |  |  |  |  |
|  | β-hemolysin | *hlb* | 15 (100) | 15 (100) |
| *Toxins: Exfoliative toxins* |  |  |  |  |
|  | Exfoliative toxin type c | *etc* | 15 (100) | 15 (100) |
| *Toxins: Secretion system* |  |  |  |  |
|  | Type VII secretion system | *esaA* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *esaB* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *essA* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *essB* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *essC* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *esxA* | 1 (6.7) | 2 (13.3) |



Figure 1.Example of dendrogram of the RAPD fingerprints of 13 *Staphylococcus chromogenes* isolates representative of an IMI associated with a persistently high (≥200,000 cells/mL) or low (<200,000 cells/mL) SCC, originating from Farm H. RAPD types were assigned an arbitrary lower case letter based on the clustering. Seven RAPD types were identified from the 13 isolates causing IMI.



**n = 9**

**n = 11**

**n = 6**

**n = 4**

Figure 2.Carriage of the *blaZ* gene (encoding for the production of β-lactamase) for 30 *Staphylococcus chromogenes* isolates from subclinical bovine intramammary infections by SCC category. All isolates in the HIGH category (n = 15) come from IMI which had an SCC of ≥200,000 cells/mL associated with all quarter-day observations, and all isolates in the LOW (n = 15) category come from IMI which had an SCC of <200,000 cells/mL associated with all quarter-day observations.



Figure 3.Distributions of putative virulence genes for 30 *Staphylococcus chromogenes* isolates from subclinical bovine intramammary infections by virulence type. Isolates of the same strain type (ST; determined by multilocus sequence typing) are listed sequentially. Scheme for grouping putative virulence genes by type adapted from Naushad et al., 2019.

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