***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.

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