***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 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 1). Four novel ST were identified which were not already present in the PubMLST database for *S. chromogenes* (ST174-177). 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 ST’s 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.

***Figures and tables***

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| Table 1. 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 | | | |

Figure S1 caption:

Dendrogram constructed from concatenated nucleotide sequence data for the 20 study isolates as well as 386 publicly-available concatenated MLST sequences for *Staphylococcus chromogenes.* Study isolates which grouped together with a bootstrap value of ≥ 65% were classified as ST clusters, and are highlighted by a red rectangle. The tree was constructed using a maximum-likelihood algorithm with the optimal model and 100 bootstrap replications in MEGA-X (Kumar et al., 2018).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table 2. 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) |
| *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) |
| *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) |
| *Toxins: Phenol soluble modulins* |  |  |  |  |
|  | Phenol soluble modulins beta | *PSMB1* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins beta | *PSMB2* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins beta | *PSMB3* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins beta | *PSMB4* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins beta | *PSMB5* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins beta | *PSMB6* | 15 (100) | 15 (100) |
| *Toxins: Hemolysins* |  |  |  |  |
|  | Beta-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 2.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.