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Whole genome sequencing identifies exotoxin and antimicrobial resistance profiles of *Staphylococcus aureus* from Maine dairy farms

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Abstract

Background *Staphylococcus aureus* is a leading cause of mastitis in dairy livestock and is a pathogen with unknown but potential impact on public and herd health in Maine. The primary objective of this study was to describe retrospective trends in *S. aureus* detection at the University of Maine Cooperative Extension Veterinary Diagnostic Laboratory (UMVDL) for milk samples submitted between July 2017 and June 2022. The second objective was to assess the genetic profiles focused on antibiotic resistance and exotoxin genes of 29 *S. aureus* isolates submitted from dairy farms in Maine in 2017 and 2022.

Results Overall, 7.8% of milk samples submitted to UMVDL between July 2017 and June 2022 were positive for *S. aureus*. The 29 isolates collected in 2017 (2 isolates) and between May and July of 2022 (27 isolates) were analyzed by whole genome sequencing and belonged to 8 strain types and 5 clonal complexes typically associated with ruminant species. Across the genomes of the 29 isolates, 14 antimicrobial resistance genes were detected, with antibiotic efflux as the primary resistance mechanism. Each isolate contained 2 to 10 staphylococcal enterotoxin genes representing 15 unique genes. *lukED*, *lukMF'*, Staphylococcal superantigen-like proteins (SSLs), and *hla*, *hlb*, *hld*, *hlgABC* genes were also observed. Antimicrobial resistance and staphylococcal enterotoxin gene carriage mostly clustered with clonal complex and host species of origin.

Conclusions Whole genome sequencing identified ruminant-associated sequence types and antimicrobial susceptibility profiles consistent with other regional reports. Exotoxins with relevance to mastitis and SFP development were also identified. This study provides insight into future opportunities to study *S. aureus* prevalence and to survey dairy production in animal and public health contexts in Maine.

Keywords *Staphylococcus aureus*, Dairy, Mastitis, Whole genome sequencing, Antibiotic resistance, Enterotoxin

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Background

Staphylococcus aureus is a leading cause of mastitis in dairy livestock including cattle, sheep, and goats, and these infections decrease milk quality and quantity [1, 2]. *S. aureus* possesses a range of virulence factors including an array of exotoxins that modulate host immune response and helps the pathogen evade host immune systems. These exotoxins include cytotoxic enzymes like β-hemolysin, cytotoxins such as hemolysins and leukocidins, and staphylococcal enterotoxins (SEs) [3]. The range and variation of these exotoxins provides evidence of adaptation to different host species [4].

β-hemolysin is present and functional in *S. aureus* strains of bovine origin where it acts as a sphingomyelinase, while it is inactive in strains of human origin due to the insertion of the Immune Evasion Cluster (IEC) into this gene [5]. γ-hemolysins and LukMF' can cause lysis of bovine neutrophils, contributing to mastitis development [6]. SEs do not seem to contribute to the development of mastitis in dairy livestock with the exception of SEC. [7, 8]. However, SEs and toxic shock syndrome protein (TSST-1) act as superantigens (SAgs) by binding to variable regions of β-chains of T cell receptors and MHCII molecules to induce T cell activation and cytokine release [9]. Human consumption of milk and dairy products contaminated with *S. aureus* from infected animals can cause staphylococcal food poisoning (SFP) and toxic shock syndrome if the contaminating strain produces SEs and TSST-1. Additionally, SEs are heat and pH resistant, so disease can occur from consumption of pasteurized or unpasteurized dairy products even if *S. aureus* is no longer viable [10].

Antimicrobial-resistant *S. aureus* also poses a risk to public and herd health. The World Health Organization ranks antimicrobial resistance (AMR) as one of the top ten public health threats faced by humanity [11]. Methicillin-resistant *S. aureus* (MRSA) may be the most notable example of antimicrobial resistant *S. aureus* in the human clinical setting, but a range of antimicrobial resistance genes have been detected in *S. aureus* from cases of bovine mastitis around the world [12].

WGS enables comprehensive analysis of complete virulence and antimicrobial resistance gene profiles without the labor and expenses that limit such analysis through traditional biochemical or molecular methods [13], and avoids false-negative results possible in targeted PCR-based identification methods [14]. WGS is particularly advantageous for assessment of SEs since traditional methods were typically limited to identification of classical enterotoxins (SEA, SEB, SEC, SED, and SEE). WGS overcomes this potential reporting bias by identifying *se* gene profiles and subtyping *se* gene variants, by also detecting nonclassical SEs and staphylococcal like proteins (Sels), which do not induce emetic activity [15]. In addition, WGS data can be used to predict multilocus sequence types and clonal complexes, replacing traditional multilocus sequence typing using PCR and Sanger sequencing methods. Whole genomemultilocus sequencing typing (wgMLST) informs our understanding of *S. aureus* epidemiology and host specificity [16].

In the state of Maine in 2022, 171 bovine dairy farms produced 554 million pounds of milk [17], and small ruminant dairy operations further expanded that figure. Maine's dairy farms occupy 700,000 acres of farmland and create thousands of jobs [18]. Antibiotic resistance, herd transmission, and enterotoxigenic potential of *S. aureus* vary among sequence types (STs) of *S. aureus* [19–21], and STs vary by geographic region [22]. Therefore, understanding the frequency of *S. aureus* sequence types can inform our understanding of the relative importance of *S. aureus* as a mastitis pathogen on dairy farms, as well as the potential public health risk. While control of *S. aureus* in dairy production is currently most relevant to cattle health, the public health concern may be emerging as sale of raw milk is legal in Maine except in eating establishments, and the number of licensed raw milk facilities increased from 15 in 2006 to 54 in 2015 [23, 24]. Although *S. aureus* has not been identified as a major cause of foodborne illness outbreaks from dairy products in the US over the last 26 years, foodborne illness from unpasteurized milk is 3.2 times more likely to occur in states where sale of unpasteurized milk is allowed, suggesting future surveillance is warranted [25]. Cases of foodborne illnesses by all pathogens including *S. aureus* are likely underdiagnosed and underreported [26, 27]. It consequently is important to understand the characteristics of any pathogen in dairy production systems, including *S. aureus*, which has potential to cause foodborne illness. Therefore, studying *S. aureus* mastitis epidemiology in Maine is necessary to evaluate its impact on the local dairy industry. The primary objective of this study was to describe retrospective trends in *S. aureus* detection at the University of Maine Cooperative Extension Veterinary Diagnostic Laboratory (UMVDL) for milk samples submitted between July 2017 and June 2022. The second objective was to assess the genetic profiles focused on antibiotic resistance and exotoxin genes of 29 *S. aureus* isolates from dairy farms in Maine submitted in 2017 and 2022.

Results

Objective 1: retrospective trends of *S. aureus* isolation

At UMVDL, 467 of 6,116 (7.8%) milk samples assessed between July 2017 and June 2022 were positive for *S. aureus* (Table 1). This included 430 of 5,919 (7.3%) bovine milk samples from 65 farms, 34 of 189 (18.0%) caprine milk samples from 12 farms, and 3 of 8 (37.5%) of ovine milk samples from 3 farms. One farm had a *S.*

Table 1 *S. aureus* isolation events from milk samples submitted to UMVDL by year and species

Year	Species	Bovine	Caprine	Ovine	Total
2017 (Jul-Dec)	SA isolations	38	1	0	39
	Total Samples	**885**	**11**	**0**	**896**
2018	SA isolations	94	9	2	105
	Total Samples	**1617**	**36**	**3**	**1656**
2019	SA isolations	109	4	0	113
	Total Samples	**1481**	**41**	**3**	**1525**
2020	SA isolations	85	0	1	86
	Total Samples	**944**	**15**	**1**	**960**
2021	SA isolations	65	13	0	78
	Total Samples	**669**	**65**	**1**	**735**
2022 (Jan-Jun)	SA isolations	39	7	0	46
	Total Samples	**323**	**21**	**0**	**341**
Total	SA isolations	430	34	3	467
	Total Samples	**5919**	**189**	**8**	**6116**

aureus-positive result for both a bovine and caprine milk sample. Milk samples submitted to UMVDL were either composite or quarter milk samples. The health status of the animals of origin is unknown.

Using a logistic regression model, where the outcome was a positive *S. aureus* culture among milk sample submissions stratified by sample accession number and month we found the predicted odds of *S. aureus* isolation from submitted milk samples over time decreased 18.5% each year.

The 467 *S. aureus*-positive milk samples originated from 350 unique animals on 79 farms from every county in Maine except Sagadahoc. The frequency of *S. aureus*-positive samples varied among farms. Forty-eight (61%) of the 79 farms had only one *S. aureus*-positive submission over the five-year period. The remaining 31 farms had *S. aureus*-positive submissions on 2 to 26 individual occasions during the five-year period. At the extreme, the 26 submissions of 639 total samples from a single farm resulted in 86 *S. aureus*-positive samples.

At the individual animal level, 83.1% (291/350) of *S. aureus*-positive animals had *S. aureus*-positive samples submitted only once, with the remaining 59 animals having two or more positive submissions. The highest number of *S. aureus*-positive samples from an individual animal was eight, submitted on seven dates over the course of 748 days. The longest interval between two *S. aureus*-positive samples from an individual animal was 840 days.

Antibiotic susceptibility testing was performed via disk diffusion by UMVDL technicians upon submitter request, and results for oxacillin resistance were recorded in the UMVDL mastitis database. Ninety-one *S. aureus* isolates from 51 farms and 83 individual animals had undergone phenotypic antibiotic susceptibility testing, and 25 (27%) were identified as resistant to oxacillin. No methicillin resistance (*mec*) genes were identified among isolates by whiole genome sequencing, and methicillin resistance could not be confirmed as no additional confirmatory tests were performed.

Table 2 MLST types of *S. aureus* stratified by species

Species	CC	ST	Number of Isolates	Total S. aureus from each species
caprine	CC5	6	4	6
caprine	CC133	133	2	
ovine	CC133	8509	1	1
bovine	CC350	350	3	22
bovine	CC97	352	8	
bovine	CC97	2187	5	
bovine	CC97	3028	5	
bovine	CC151	8510	1	

Objective 2: isolate analysis

Isolate sources

Since UMVDL did not routinely store SA isolates in the course of normal diagnostic operations, only *S. aureus* cultured from submitted milk samples between May and July 2022 were available for analysis. This resulted in 27 *S. aureus* isolates from recent UMVDL milk sample submissions, and 2 isolates recovered from storage (frozen at -20˚C since isolation in 2017). Twenty-one isolates were from bovine milk samples, 6 from caprine milk, 1 from ovine milk, and 1 from bovine bulk tank milk. The samples originated from 23 animals on 13 farms. On three occasions, milk samples from different udder quarters or halves of a single animal were submitted to the UMVDL on the same day and resulted in two separate isolations of *S. aureus*. Once, two udder composite samples from a single animal submitted to UMVDL on the same day resulted in two isolations of *S. aureus*. It is unclear if these samples were collected by the farmer at the same time or on different dates. Lastly, two isolates were obtained from milk samples from the same animal on different submission dates. We analyzed all 29 available isolates to expand our descriptive capacity since these samples are not reflective of a single reference population but rather of a broad range of the Maine dairy industry.

MLST

The 29 *S. aureus* isolates were classified into 8 sequence types (STs) within 5 clonal complexes (CCs; Table 2). There was no overlap between STs identified from bovine, caprine, and ovine milk samples. Two novel STs were identified, ST8510 in CC151 is a novel ST related to ST151 with single locus variant at the *arcC* locus ($n = 1$), and ST8509 in CC133 is a novel ST related to ST133 with a single locus variant at the *aroE* locus. ST352 was the most prevalent ST accounting for 27.6% of all samples (8

of 29) and 36.4% (8 of 22) of all bovine samples (including the isolate from bovine bulk tank milk).

Two or more STs were identified from 3 of 13 farms. Two farms from which multiple isolates originated had 1 ST identified. The 8 remaining farms were the source of single isolates, with one ST identified per farm. In the four pairs of isolates which originated from the same animals on the same submission dates but from different teats (for 3 pairs) or from different composite samples (for 1 pair), the isolates belonged to the same STs. The single pair of isolates sourced from the same animal on two different submission dates also belonged to the same ST.

AMR

All 29 isolates were phenotypically susceptible via disk diffusion to amoxicillin, ampicillin, cefoxitin, ceftiofur, cephalothin, erythromycin, gentamicin, oxacillin, penicillin, pirlimycin, streptomycin, and tetracycline. Across the genomes of the 29 isolates, fourteen AMR genes were detected (*arlR*, *arlS*, *FosB*, *GlpT*, *kdpD*, *LmrS*, *mepR*, *mgrA*, *murA*, *norA*, *norC*, *sdrM*, *sepA*, *vanT*). Antibiotic efflux was the primary mechanism of resistance as defined by the Comprehensive Antibiotic Resistance Database (CARD); less frequent mechanisms were target alteration and antibiotic inactivation. Eight genes were detected in all isolates (*arlR*, *LmrS*, *mepR*, *mgrA*, *norA*, *norC*, *sepA*, *vanT*) with a minimum of 10 genes and a maximum of 13 genes detected in any given individual isolate.

AMR gene carriage clustered based on isolate clonal complex and species of origin (Fig. 1). We detected FosB exclusively in CC133. *GlpT* and *murA* were always identified together in the same isolates. Caprine samples had significantly more AMR genes than bovine samples ($P = 0.0238$). A comparison was not conducted with ovine samples because we only had one isolate of ovine origin.

Exotoxin genes

The hemolysin genes *hla*, *hlb*, *hlgA*, *hlgB*, and *hlgC* were identified in all 29 genomes. *hld* was in 22 genomes all belonging to CC5 or CC97. Leukocidin components *lukDE* were identified in all 29 genomes. *lukMF'* were identified in 11 genomes (38%), including all 8 ST352 isolates of bovine origin, 1 isolate of bovine origin from ST151, and two isolates belonging to ST133, one of ovine origin and one of caprine origin.

Chromosomal SE genes *selw* and *selx* were identified ubiquitously in all isolates. Four bovine isolates contained a total of 10 additional SE genes (Fig. 2). Eight additional genes were observed in the single ST8510 isolate in CC151 (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*, *sey*, *sez)* and 7 additional genes were observed in all three isolates in ST350 (*sei*, *sem*, *sen*, *seo*, *seu*, *sel26*, *sel27).* One caprine isolate and the single ovine isolate, both belonging to CC133, possessed the 3 additional genes *sec*, *sel*, and *tst*. However, these genes were not detected in the genome of the other caprine ST133 isolate (UM23). Aside from *sec*, the other classical enterotoxins, *sea*, *seb*, *sed*, and *see*, were not detected in any isolates.

Staphylococcal superantigen-like protein genes *ssl5*, *ssl7*, *ssl9*, *ssl10*, and *sll11* were present in all isolate genomes. *ssl1* was ubiquitous except in the ST8510 isolate, *ssl2* was ubiquitous except in one ST3028 isolate,

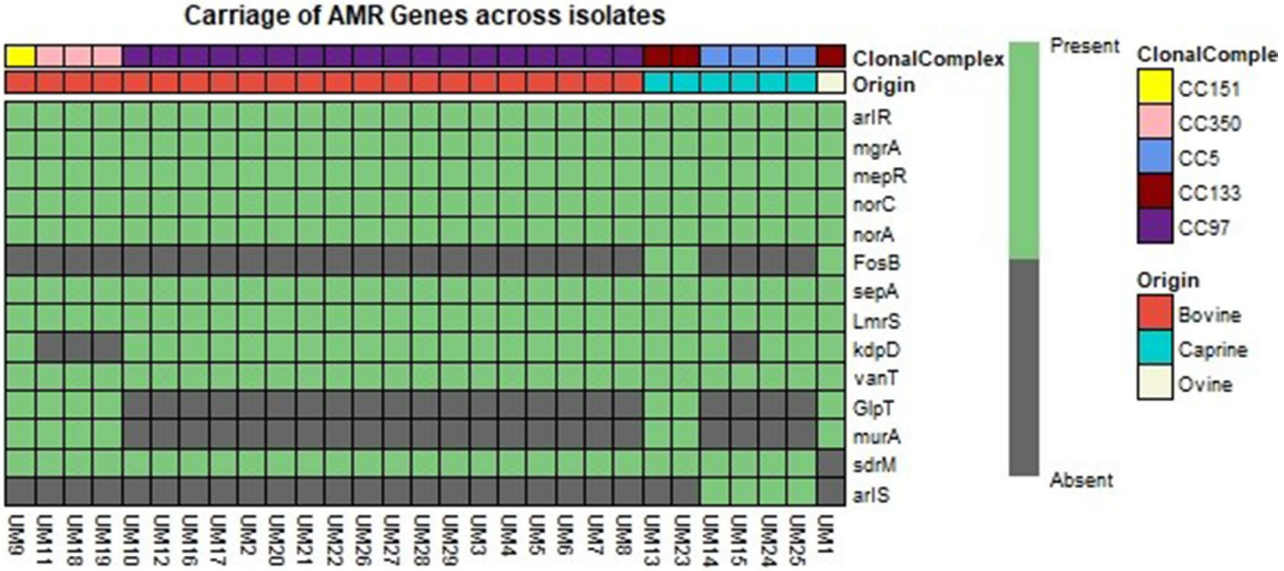

Fig. 1 Heat map of antimicrobial resistant genes identified from whole genome sequencing of 29 *S. aureus* isolates stratified by species host of origin and clonal complex. Light green = gene present, dark green = gene absent

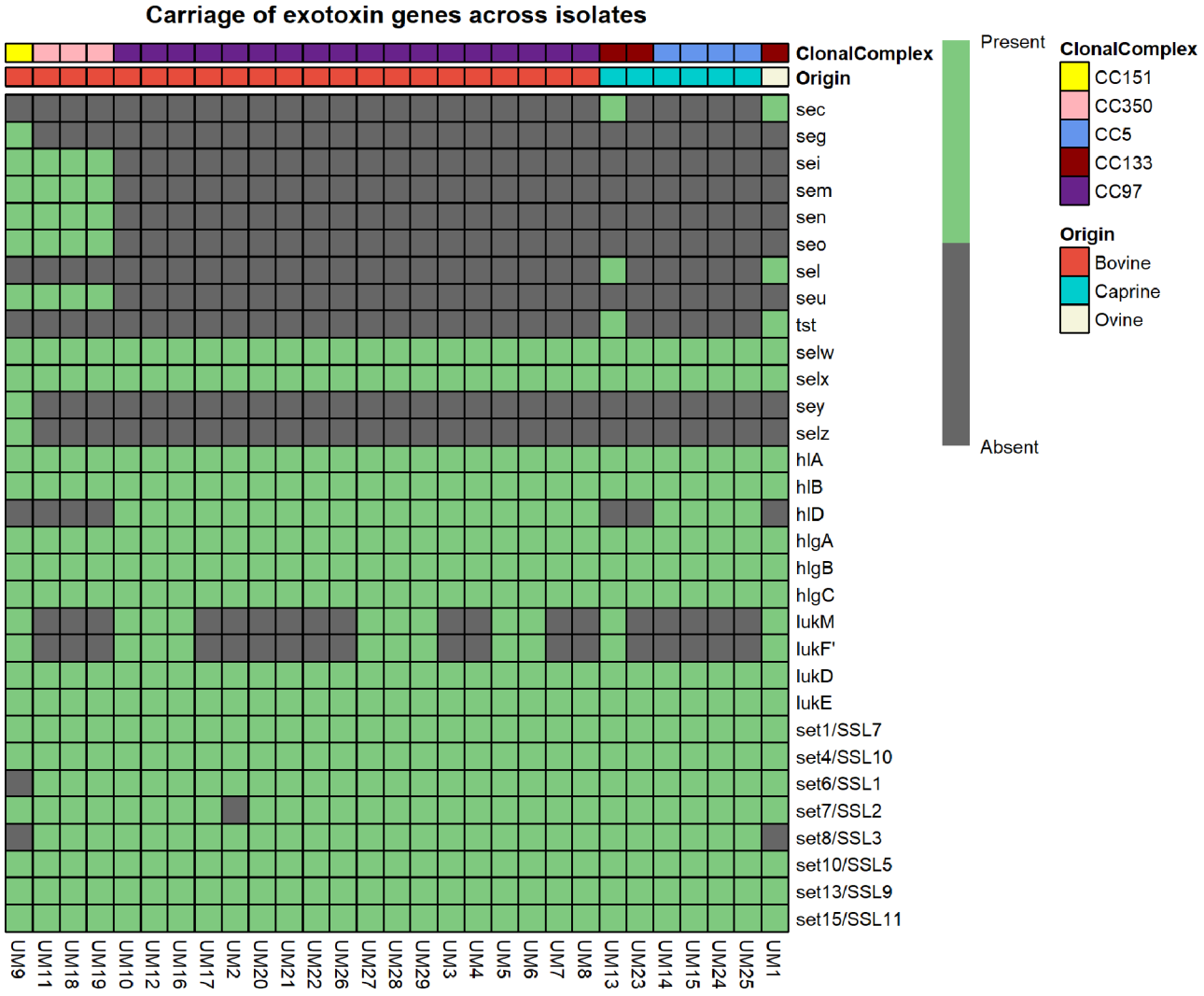

Fig. 2 Heat map of exotoxin genes identified from whole genome sequencing of 29 *S. aureus* isolates stratified by species host of origin and clonal complex. Light green = gene present, dark green = gene absent

and *ssl3* was ubiquitous except in the two ST8509 and 8510 isolates.

Discussion

Objective 1: retrospective trends of *S. aureus* isolation

Here we report a retrospective study describing the frequency and strain diversity of *S. aureus* isolated from milk samples submitted to UMVDL by Maine dairy farms. To the best of our knowledge, there are no previous studies examining prevalence or diversity of *S. aureus* from dairy sources in Maine. Also, in a search of PubMed, we identified no previous publications describing *S. aureus* MLST diversity in Maine. Our primary objective was to report the frequency of *S. aureus* isolation from milk samples submitted to the UMVDL using laboratory records. Recognizing the limited ability to make inferences from these data we elected to augment these data with genomic sequencing results for isolates collected from recent laboratory submissions. There are several limitations with reporting pathogen and disease frequencies from laboratory databases. Key among the limitations is the issue of sampling or ascertainment bias, where we can not determine what the samples submitted to the diagnostic laboratory may represent. For example, the frequencies of *S. aureus* isolation observed by the laboratory should not be used to infer *S. aureus* prevalence among Maine dairy farms. The issue of sampling bias is particularly evident from the observation that there is variation in the number of samples submitted per farm per year. Further, the laboratory records contained limited sample metadata, and in particular lacked key information such as clinical disease state or the reason for sample submission, so we can not infer disease frequency associated with the isolation events. Finally, while the laboratory records allowed us to link the *S. aureus* positive samples to an accession number and associated farm

and individual cow identification, the records lacked these data for submissions where *S. aureus* was not isolated, so we could only model *S. aureus* frequencies from positive sample submissions.

Therefore, while we observed a decrease in odds of *S. aureus* isolation from milk samples submitted each year this does not infer a change in prevalence of *S. aureus* in Maine's dairy herds. The observed decrease may be due to changes in farmer motivation to submit samples, or access to other resources for milk culture. Submissions from the same farm might be expected to show correlations in their outcomes and when modeling pathogen prevalence in regions the hierarchical nature of the data, such as clustering within farms and regions should be considered. Accession numbers are assigned by UMVDL to each sample or set of samples submitted by a individual. Unfortunately, farm information was only available for accession numbers with positive test results, so we were not able to account for clustering within farms in a model of isolation frequency. When modeling the change in isolation frequency over time, we explored an analysis with accession number as a random predictor, but this model did not converge. Repeated submissions of samples from individual animals was also evident, further supporting the value of a modeling approach accounting for clustering of animals within farms, although the majority of submissions were from individual animals. Whether the observed single occurrence of *S. aureus*-positive samples from most individual animals was due to subsequent infection clearance, farmer interventions such as culling *S. aureus* positive cows, or a lack of continued monitoring through follow-up sample submissions is unclear.

At the farm level, since this study was conducted using opportunistic, client-provided samples from a diagnostic laboratory, the motivations behind sample submission may bias the characteristics of *S. aureus* isolated from samples. *S. aureus* CCs differ in virulence properties and ability to cause mastitis [28], and *S. aureus* strains have differing abilities to cause persistent intramammary infections [29]. Previous studies have demonstrated the presence of dominant STs within individual farms [29]. Since the isolates in this study were sourced from milk samples at a mastitis diagnostic laboratory, our results were likely biased to more frequently include those STs that contribute to signs that warrant diagnostic testing. Less virulent strains of *S. aureus* may be present on farms but consequently might not be included in our samples. This potential bias may have been mitigated if some samples were sent to UMVDL regardless of mastitis status as part of routine practices, potentially enabling detection of less pathogenic strains of *S. aureus.* Farmer motivations for sample submissions are not currently known and should be evaluated in future surveys.

Farms with *S. aureus*-positive samples from several animals on multiple dates may have had contagious genotypes of *S. aureus* which are more difficult to eliminate from a herd. In contrast, farms which only had *S. aureus*-positive samples on a single date may have experienced infections with *S. aureus* genotypes that cause sporadic mastitis [21]. Strain typing of *S. aureus* isolates collected from dairy herds in other geographic regions has demonstrated the epidemiology of *S. aureus* was associated with the genotype [19]. Alternatively, farms with several *S. aureus*-positive submission dates may have been more diligent in continuing to monitor their herds for *S. aureus* compared to farms that reportedly had *S. aureus*-positive samples once and failed to submit more samples for follow up. Rather than representing inter-farm differences of *S. aureus* strains, this would reflect farm management practices. A randomized, longitudinal sampling scheme could assess within-farm *S. aureus* ST diversity and statewide *S. aureus* prevalence to better understand these issues.

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Objective 2: isolate analysis

MLST

The majority (81.8% or 18 of 22) of bovine-sourced isolates belonged to CC97 (ST352, ST2187, ST3028), which is a major CC reported in Pennsylvania, Vermont, and Canada [30–32] and was the most prevalent CC reported in a global analysis of bovine *S. aureus* [22]. However, only one isolate belonged to CC151 which has also been frequently detected in these locations and globally [22, 30–32]; this may be attributed to our limited sample size.

Our observed association of ST with milk sample source species is consistent with a study of *S. aureus* from a variety of animal species in four New England states including Maine where ST151, ST2187, and ST352 were found exclusively in samples from cows [33]. ST6, ST133, and the related novel ST8509 were only from small ruminant origin in our study which is consistent with reports that ST133 is rarely identified in bovine sources in New England and Canada [31, 33], and with findings in Victoria, Australia where ovine and caprine samples exclusively belonged to ST133 or a related single locus variant [34].

AMR

The antimicrobial susceptibility profiles of *S. aureus* from Maine dairy farms were consistent with other studies in the Northeast US. The phenotypic susceptibility of our isolates is similar to the susceptibility profiles observed by Thomas et al. in isolates in Pennsylvania, although that study lacked genotypic AMR assessment for comparison with our genotypic results [30]. The AMR genes we detected are similar to those detected in the study of

S. aureus in diverse animal hosts in New England where antibiotic efflux genes *norA* and *lmrS* were in all genomes and *mgrA* was in all but two [33]. Similarly, we detected *FosB* infrequently; this gene was found exclusively in ST133 in our study, and not in ST6 or other STs as previously reported [33]. Our results are also consistent with a Canadian study which found antibiotic efflux genes in all isolates [31].

Notably, none of our isolates contained the *mecA* or *blaZ* genes which confer resistance to methicillin and penicillin respectively. This differs from the studies in Vermont, Pennsylvania, New England, and Canada where *blaZ* was detected, albeit at a low prevalence [30–33, 35] but is similar to finding no *mecA* positive isolates in Vermont [32, 35]. However, most of these studies are non-probability studies and the true prevalence of methicillin-resistant *S. aureus* in dairy-associated samples is unknown. Our observed lack of *mecA* and *blaZ* genes and no phenotypic resistance to oxacillin for the 29 isolates from 2022 studied in Objective 2 is surprising given that the UMVDL reportedly identified oxacillin phenotypic resistance in 27% of samples evaluated over the prior five-year period. From this historical value, assuming a 95% confidence interval that the true prevalence in this discrete population lies between 17 and 37%, we would have expected 4 or more of our 29 isolates to display phenotypic oxacillin resistance. However, there is controversy over the use of disk diffusion methods for oxacillin resistance to identify MRSA and cefoxitin discs are preferred [35]. In addition, among the antimicrobials tested in this study veterinary-specific breakpoints are only available for ceftiofur and pirlimycin, and breakpoints used for other antimicrobials are adapted from human data [36]. For the 2022 isolates, we conducted disk diffusion susceptibility testing to remain consistent with UMVDL procedures, and we were unable to confirm beta-lactam or methicillin resistance. Future prevalence studies should use minimum inhibitory concentration of oxacillin or disk diffusion with cefoxitin to describe the occurrence of oxacillin and methicillin resistance more reliably [35].

There is an apparent discordance between the phenotypic susceptibility of isolates and the genotypic presence of AMR genes which, if expressed, should confer resistance to some of the antibiotics tested. For example, *mep* and *mgrA* encode antibiotic efflux mechanisms which confer multi-drug resistance [37]; however, no phenotypic resistance was observed despite detection of these genes in all 29 isolates. In contrast, other studies have observed the opposite issue where phenotypic resistance was observed but whole genome sequencing failed to find associated genetic markers [30]. Identification of resistance determinants, such as single nucleotide polymorphisms (SNPs), from whole-genome sequence (WGS) data could provide explanations for the observed discrepancies [38].

Exotoxin genes

γ-hemolysins, *lukED*, and *lukMF'* are frequently identified in *S. aureus* from bovine mastitis and cause lysis of bovine neutrophils, thereby contributing to mastitis development [6, 39]. Our ubiquitous identification of *lukED* genes is consistent with previous reports [30, 31]. ST352 in CC97 has also been observed to have a high prevalence of *lukMF'* [30, 40], as have CC133 ovine and caprine isolates [41]. Expression of these genes may vary according to host factors to manifest in clinical or subclinical mastitis [41].

In a study of 57 *S. aureus* STs isolated from bovine mastitis, Wilson et al. reported most bovine *S. aureus* contained a range of 2 to 13 superantigen genes (SAgs) in all genomes [20], which is similar to our detected range of 2 to 10 genes. Our detection of non-chromosomal SEs in 18.2% (4 of 22) of bovine isolates is much lower than the 64.41% prevalence of enterotoxin genes detected in Thomas et al.'s Pennsylvania study and contrasts with a report suggesting that all bovine *S. aureus* typically contain 5 or more superantigen genes [20, 30]. Our less frequent identification of SEs is likely due to a difference in predominant STs. Whereas Wilson et al. included a variety of STs, and the majority of SEs in the Pennsylvanian study were detected in CC151, our isolates mostly belonged to CC97 and lacked enterotoxin genes [20, 30]. Wilson et al. reported CC97 genomes contained the fewest SAgs [20], and CC97 isolates from Naushad et al.'s Canadian study similarly lacked any enterotoxin genes [31], so our results are consistent with these prior reports.

Previously, SEC has been the most frequently detected SE from *S. aureus* animal mastitis isolates [42] and was most frequently identified in bovine, ovine, and caprine raw milk samples from small-scale artisan cheese production in Vermont [43]. In contrast, *sec* was only identified in two isolates in our study which belonged to ST133; *sec* detection in only some ST133 isolates is not without precedent [34]. Perhaps the difference in *sec* detection frequency can be attributed to limitations of traditional phenotypic methods which primarily detected classical SEs, as in D'Amico and Donnelly's assessment in Vermont [43]. Regardless, any presence of *sec* is concerning. SEC may act as a virulence factor in mastitis development [8] and has been associated with clinical mastitis in small ruminants [44], so the presence of SEC-producing *S. aureus* strains within a herd could pose a risk to herd health and milk production. SEC has also been associated with SFP outbreaks [45] and could have implications for public health. In our study, co-detection of *sec* with *sel* and *tst* in two isolates suggests that these isolates contain

the SaPIbov pathogenicity island [46]. The toxic shock syndrome protein (TSST-1) encoded by the *tst* gene is concerning due to its superantigen activity and ability to cause toxic shock [47].

Presence of SE genes does not necessarily correlate with in vivo toxin production [10], although CC has been directly linked to the vSAβ genomic island which contains the enterotoxin gene cluster (*egc)* and can be predictive of SEG and SEI production [48].The *egc* can be composed of a variety of combinations of the genes *seg*, *sei*, *sem*, *sen*, *seo*, and *seu* [9, 48]. *Egc* has been frequently detected in CC151 [20, 30], and our single isolate in CC151 likewise possessed *egc* genes. ST350 isolates were the only others in our study to contain *egc* genes, but this ST was not included in the study by Wilson et al., and the single isolate in Thomas et al.'s study in Pennsylvania belonging to ST350 only possessed *sei* and *sem* [20, 30]. *seg* has been associated with a decreased chance of *S. aureus* establishing persistent intramammary infections [50], so the presence of *seg* in only one of our four isolates containing *egc* genes supports our speculation that our samples are biased towards more pathogenic STs. The role of *egc* enterotoxins in staphylococcal food poisoning is evidenced by SFP outbreaks which lacked detection of classical enterotoxins but did detect *egc* [26], so the presence of these genes could pose a risk to consumers.

Our identification of *selx*, *selw*, *sey*, *selz*, *sel26* and *sel27* adds to the understanding of the diversity of enterotoxins associated with *S. aureus* from dairy sources. There is a discrepancy between the nomenclature and associated sequences of *selw* and *sel26* used in some studies; we referred to *selw* as the chromosomal gene found in studies by Wilson, Nouws, and Aung [15, 20, 51] and *sel26* and *sel27* as the genes identified by Zhang and used in analysis by Aung [51, 52].

A variety of *ssl* genes was similarly observed by Naushad et al., although we observed only 8 rather than 39 of the 40 *ssl* genes [31]. This was likely due to our much smaller sample size (29 vs. 119) and diversity between observed clonal complexes.

Conclusion

This study quantifies *S. aureus* isolation from milk samples submitted to UMVDL by dairy farms in Maine over a five-year period. Whole genome sequencing identified ruminant-associated sequence types and antimicrobial susceptibility profiles consistent with other regional reports. Exotoxins with relevance to mastitis and SFP development were also identified. We provided insight into future opportunities to study *S. aureus* epidemiology and to survey dairy production in animal and public health contexts in Maine.

Methods

Objective 1: retrospective trends of *S. aureus* isolation

In order to summarize the frequency of *S. aureus* isolation events from milk samples submitted to the UMVDL, we conducted a non-probability retrospective observational study of *S. aureus* isolation events reported in the UMVDL custom Mastitis database on Access platform. Milk samples submitted to UMVDL Mastitis Lab were initially cultured (10μL aliquots) on Tryptic Soy Agar with 5% sheep blood following National Mastitis Council mastitis diagnostic procedures [53]. Presumptive *S. aureus* colonies were identified based on colony morphology and were sub-cultured for isolation. *S. aureus* isolates were confirmed by observation of Gram-positive cocci in grape-like clusters, catalase and coagulase positivity, and production of black colonies surrounded by yellow zones on Vogel-Johnson agar. Each milk sample positive for *S. aureus* was considered a unique event. Data about *S. aureus* isolated between July 2017 and June 2022 were extracted from the Mastitis database, then analyzed in Microsoft Excel. Farm names were coded for anonymity in the dataset, and the location of each farm (county only) was noted. Additionally, for each isolation event, the dairy animal species, type of sample (udder composite vs. individual udder quarter or half), and oxacillin antibiotic susceptibility (if tested per submitter request) were compiled. Bulk tank samples were not included in the retrospective analysis.

Objective 2: isolate analysis

Isolate identification

To augment the retrospective analysis by describing the genetic diversity of a recent subset of isolates collected by the UMVDL, 27 *S. aureus* isolates were collected from milk samples submitted to UMVDL between May and July of 2022, and 2 samples had been collected in 2017 and were available from frozen storage. The *S. aureus* positive milk samples originated from dairy cattle, goats, and sheep, and were submitted by organic and conventional farms in Maine. Phenotypic identification of *S. aureus* was conducted at the Mastitis Lab in UMVDL as described above in Objective 1.

Phenotypic antibiotic susceptibility testing

Kirby-Bauer disk diffusion assays were performed for each isolate at UMVDL Mastitis Lab following the American Society for Microbiology's protocol [54]. Susceptibility to the following BD BBL Sensi-Disc Antimicrobial Susceptibility Test Disks was assessed: Amoxicillin/Clavulanic Acid (30 μg), Ampicillin (10 μg), Cefoxitin (30 μg), Ceftiofur (30 μg), Cephalothin (30 μg), Erythromycin (15 μg), Gentamicin (10 μg), Oxacillin (1 μg), Penicillin (10 μg), Streptomycin (10 μg), and Tetracycline (30 μg). Pirlimycin (2 μg) was also tested but sourced

from Oxoid. Isolates were designated as Sensitive, Intermediate, or Resistant to each antibiotic by comparison of measured zone sizes to values from CLSI guidelines [36] or from genus-specific cut points on pamphlets provided with antibiotic disks by manufacturers when specific values were not provided by CLSI. Zone diameter breakpoints in CLSI guidelines are adapted from human data for antimicrobial agents tested except for Ceftiofur and Pirlimycin where veterinary-specific breakpoints were available for bovine mastitis associated *S. aureus* isolates [36]. *S. aureus* ATCC strain 25923 was used as a positive control.

Whole genome sequencing

S. aureus isolates were transported to University of Vermont Quality Milk Research Lab on Nutrient Agar Slants (Northeast Laboratory, ME). Isolates were transferred to tryptic soy broth with 15% glycerol for storage at -80˚C then recovered for subsequent analysis. Isolates were plated on Tryptic Soy Agar with 5% sheep blood (Northeast Laboratory, ME) for 24–48 h at 37˚C, transferred to Tryptic Soy Broth (TSB) and incubated at 37˚C to produce overnight broth culture. DNA extraction was completed from a 100uL aliquot of the overnight culture using MasterPure Gram Positive DNA Purification Kit from Biosearch Technologies. DNA was sent for quality control using Qubit quantification at UVM VIGR CORE facility. Library preparation and sequencing for Oxford Nanopore (ONT) and Illumina sequencing was conducted by the CORE facility. ONT was performed with GridION platform with SQK-LSK109 + EXP-NBD196 kit for library prep, and Illumina was performed with HiSeq platform using150bp paired-end technique with PerkinElmer Nextflex DNASeqKit for library prep.

Genome analysis

ONT long reads and Illumina short reads were assembled using the Unicycler tool to create hybrid genomes [55]. Assembled genomes were submitted to CARD to detect AMR genes, and to VirulenceFinder to detect leukocidin, hemolysin, and enterotoxin genes using default parameters [13, 37, 56–58]. NCBI blastn tool was used to search isolate genomes for *ses*, *set*, *selv*, *selw*, *selx*, *sey*, *sez*, *sel26*, and *sel27* since these were lacking from VirulenceFinder [15]. Exotoxins genes as categorized by Virulence Factor Database (VFDB) [57], including SSLs, α, β, δ, and γ-hemolysins, and leukocidin genes were identified using Rapid Annotation using Subsystem Technology (RAST) [58–60] with the exception of lukMF' which were identified using NCBI blastn tool. Sequence Type was determined using MLST tool [56, 59–65]. The 29 whole genomes are archived at the National Center for Biotechnology Information (NCBI) repository, under BioProject PRJNA993145.

Novel MLST confirmation

For two suspected novel STs, MLST PCR and amplicon sequencing was completed to define the variant alleles for the *aroE* gene from isolate UM1 and *arcC* gene from isolate UM9. Amplified DNA was submitted to UVM VIGR facility for Sanger sequencing. Sequences were then submitted to PubMLST for novel sequence type assignments.

Data management, statistical analysis and data visualization

Data was exported from the UMVDL custom Mastitis database into Microsoft Excel files and descriptive statistics were completed using Microsoft Excel. To test for potential changes in S. aureus isolation frequency over time the probability of a *S. aureus* positive culture was modeled using logistic regression with a logit transformation and date of sample submission (month and year) as the predictor, using R 4.2.2 (R Core Team, 2022). Because the UMVDL database did not record farm or cow numbers for *S. aureus* culture negative submissions, we were not able to include other predictors in the model. The R package "pheatmap" was used to generate presence-absence heatmaps for AMR genes and exotoxin genes in Figs. 1 and 2 [66].

Abbreviations

AMR	Antimicrobial Resistance
CARD	Comprehensive Antibiotic Resistance Database
CC	Clonal Complex
MLST	Multi Locus Sequence Type
MRSA	Methicillin-resistant S. aureus
ONT	Oxford Nanopore
SFP	Staphylococcal Food Poisoning
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
SAg	Superantigen
UMVDL	University of Maine Cooperative Extension Veterinary Diagnostic Laboratory
UVM	University of Vermont
VFDB	Virulence Factor Database
VIGR	Vermont Integrative Genomics Resource
WGS	Whole Genome Sequencing

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Author contributions

E.R. contributed to conceptualization and design of study, laboratory investigations, data curation, formal analysis, data visualization, and manuscript writing. B.K.W. contributed to microbiological diagnostics, data collection and management. A.L. and J.W.B. contributed to conceptualization and design of study, methodology, data curation, resources, supervision, funding acquisition, project administration, and manuscript writing. A.C. and F.M.S.A. contributed to whole genome sequencing and bioinformatics analysis, data visualization, and manuscript review. G.A. contributed to data analysis and manuscript review. All authors reviewed the manuscript.

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Data availability
The datasets (whole genome sequence files) supporting the conclusions of this article are available in the National Center for Biotechnology Information (NCBI) repository, Bioproject PRJNA993145, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA993145

Declarations

Ethics approval and consent to participate
The milk samples were acquired for diagnostic purposes by the University of Maine Veterinary Diagnostic Laboratory. Collection of veterinary bacterial isolates for diagnostic purposes from samples such as milk, and the subsequent use of those isolates in non-commercial research, does not require approval by our local ethics committees at the University of Maine nor at the University of Vermont.

Consent for publication
Not applicable

Competing interests
The authors declare no competing interests.

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