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Longitudinal study of *Staphylococcus aureus* genotypes isolated from bovine clinical mastitis

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ABSTRACT

Bovine clinical mastitis is an important problem for the dairy industry, and *Staphylococcus aureus* is a common mastitis-causing pathogen in many countries. Detailed knowledge on genetic variation of *Staph. aureus* strains within the bovine population, including changes over time, can be useful for mastitis control programs, because severity of disease and effects on milk production are at least partly strain-associated. Therefore, the major aim of this study was to compare sequence types of *Staph. aureus* isolated from cases of bovine clinical mastitis from 2002 to 2003 with sequence types of a more recent set of isolates collected from 2013 to 2018, using core genome multi-locus sequence typing (cgMLST). We also wanted to compare antibiotic resistance genes of isolates from the 2 sets, to identify changes that may have occurred over time in the *Staph. aureus* population. A total of 157 isolates of *Staph. aureus*, almost equally distributed between the 2 time periods, were subjected to high-throughput sequencing and cgMLST. The results showed that the most prevalent sequence types found among the 2002 to 2003 isolates belonged to the clonal complexes CC97, CC133, and CC151, and that those complexes still dominated among the isolates from 2013 to 2018. However, a population shift from CC133 to CC97 and CC151 over time was observed. Likewise, no important differences in prevalence of antibiotic resistance genes were found between the 2 sets of isolates. As expected, genes belonging to the major facilitator superfamily of transporter proteins, and multidrug and toxic compound extrusion transporters, were very common. Moreover, several genes and mutations conferring resistance to fosfomycin were present, but not in CC97 isolates. The β-lactamase gene *blaZ* was found in only 3 out of 81 isolates from 2002 to 2003 and 1 out of 76 isolates in 2013 to 2018. In conclusion, the results indicate that mastitis-associated *Staph. aureus* strains circulating among dairy cows in Sweden exhibit a remarkable genotypic persistence over a time frame of close to 15 yr.
Key words: milk isolate, core genome multi-locus sequence typing, antimicrobial resistance

INTRODUCTION

Bovine mastitis caused by *Staphylococcus aureus* continues to be a challenge in dairy production, as it can be difficult to detect and treat (Barkema et al., 2006). This can substantially be ascribed to the intracellular preference of the bacteria, irregular shedding, and poor antibiotic accessibility. Some strains of *Staph. aureus* are more commonly isolated from clinical cases of mastitis than others (Capurro et al., 2010a; Lundberg et al., 2014; Ronco et al., 2018). Thus, these strains have a tendency for spreading between animals and herds. Although the prevalence of *Staph. aureus* genotypes varies between herds, it has been found that 1 or 2 strains often dominate within a single herd (Capurro et al., 2010b). Regarding the general distribution of genotypes of *Staph. aureus* within populations of dairy cows, studies have shown that some genotypes are found in bovine mastitis all over Europe, whereas others are unique to certain countries or regions (Cosandey et al., 2016).

Strains of *Staph. aureus* can be represented as pulsotypes, defined by pulsed-field gel electrophoresis (**PFGE**; Linhardt et al., 1992). More recently, genotypes have been defined by multi-locus sequence typing (**MLST**) based on 7 housekeeping genes (Enright et al., 2000) or to even finer detail using whole-genome sequencing, giving access to several thousand gene alleles in so-called core genome MLST (**cgMLST**; Maiden et al., 2013). Using these approaches, *Staph. aureus* isolates are assigned sequence types (**ST**) and are grouped into clonal complexes (**CC**) based on allelic distance.

Gene profiles vary between strains of *Staph. aureus*, and they may contain different sets of antibiotic resistance and virulence genes (Artursson et al., 2016; Nau-

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shad et al., 2020). Some genes, belonging to the core genome, are characteristic for and prevalent in most *Staph. aureus*, whereas others are found only in some isolates, as, for example, the *bla*Z gene, which results in the production of β-lactamase, mediating penicillin resistance. Some virulence factors, such as staphylococcal enterotoxin C, have been correlated with severe clinical cases of bovine *Staph. aureus* mastitis (Kuroishi et al., 2003) and a genetic lineage of the leukocidin gene *LukMF* has also been correlated with clinical mastitis (Hoekstra et al., 2018). Using an alternative typing scheme based on gel electrophoresis readouts of PCR products from the 16S to 23S rRNA intergenic spacer (RS–PCR), a genotype B was defined (Fournier et al., 2008; Boss et al., 2016). This genotype was found to be prevalent in mainland Europe, excluding the Nordic countries, and corresponds mainly to bovine-adapted ST8 strains (Boss et al., 2016). Genotype B strains are considered more contagious than strains of other genotypes (Fournier et al., 2008). However, it is not yet known whether the ability of *Staph. aureus* to be widely disseminated in the population can be correlated with the presence or absence of any specific genes or virulence factors, and defining the prevalence of CC types of *Staph. aureus* isolates over time is one strategy for studying spreading abilities. Furthermore, greater knowledge is needed about the distribution of genotypes in the bovine population to make prevention strategies efficient and reliable. Studies of genetic variation can also detect the introduction of new genotypes and give information on their origins.

To our knowledge, very few longitudinal studies of genetic variation in *Staph. aureus* isolates from cases of bovine mastitis have been performed (Anderson and Lyman, 2006; Grunert et al., 2018; Rossi et al., 2019), and none of these have used cgMLST for characterizing isolates. Several national Swedish surveys on the prevalence of mastitis-causing pathogens and antibiotic resistance of such pathogens have been performed. In one such study of *Staph. aureus* milk isolates collected from cases of bovine clinical mastitis during 2002 to 2003, we found 2 dominating PFGE pulsotypes, together accounting for 64% of the isolates (Lundberg et al., 2014). A subset of isolates from this strain collection was further investigated by a DNA microarray analysis to map the prevalence of different virulence genes (Artursson et al., 2016). The microarray analysis showed that the isolates from the 2 most common pulsotypes were connected to CC133 and CC151, respectively. The third most common pulsotype was connected to CC97. In the present study we continue this work, with the major aim of comparing sequence types of *Staph. aureus* isolated from cases of bovine clinical mastitis from 2002 to 2003 with sequence types of a more recent set of isolates collected from 2013 to 2018, using cgMLST analysis. We also wanted to compare antibiotic resistance genes of isolates from the 2 sets, to identify changes that may have occurred over time in the *Staph. aureus* population.

MATERIALS AND METHODS

Staph. aureus Isolates

Isolates of *Staph. aureus* were collected in 2 nationwide Swedish studies on microbial etiology of bovine clinical mastitis performed from 2002 to 2003 (Ericsson Unnerstad et al., 2009) and 2013 to 2018 (Duse et al., 2021). In short, field veterinarians collected milk samples from dairy cows having macroscopic changes in the milk (with or without swelling of the udder) from the most affected udder quarter. In 2002 to 2003, milk samples were cultured on 5% bovine blood agar at the veterinary practices, and the agar plates were sent to the National Veterinary Institute (Uppsala, Sweden), where growth of microorganisms was confirmed using accredited routines, as briefly described in the following. In 2013 to 2018, milk samples were sent to the National Veterinary Institute for culturing on agar plates containing 5% bovine blood agar with 0.05% esculin. In samples collected from 2002 to 2003, *Staph. aureus* isolates were identified based on colony morphology, through presence of α- and β-hemolysis, and by coagulase reaction. For samples collected from 2013 to 2018, MALDI-TOF MS on a MALDI Biotyper (Bruker Daltonics) was used for identification of *Staph. aureus*. A total of 225 and 229 *Staph. aureus* isolates were collected in 2002 to 2003 and 2013 to 2018, respectively. All isolates were stored at −80°C. For the present study, 90 *Staph. aureus* isolates per study period were randomly selected, under the condition that only one isolate per farm was included. The random selection was independent for the 2 sets and, consequently, not necessarily but possibly from the same farms. However, as it turned out, no farm appeared in more than one time period. From each of the selected isolates, approximately 1 μL of the frozen bacterial stock was cultured on 5% bovine blood agar (Oxoid) plates with 0.05% esculin (Merck) and incubated at 37°C overnight. All cultures were stored at 4°C until further processing for sequencing.

DNA Extraction

Preparation and Extraction. For DNA extraction, the EZ1 DNA Tissue Kit (Qiagen) was used. For each of the 180 isolates, a full white plastic loop (approx. 1 μL) of pure colony was resuspended in a solution

containing 180 μL of digestion buffer G2 from the kit, 20 μL of lysozyme (50 mg/mL; Sigma-Aldrich), and 10 μL of lysostaphin (5 mg/mL; Sigma-Aldrich). The bacterial solution was incubated at 37°C for 1 h and 30 min. The samples were then heated to 95°C for 10 min. Automated DNA extraction was carried out using the EZ1 Advanced or Advanced XL robot (both Qiagen) with the EZ1 Advanced DNA Bacteria card inserted and following the manufacturer's instructions, with a final elution volume of 50 μL. The extracted DNA was immediately stored at −20°C. A negative control was included, using 1 μL of nuclease-free water instead of bacterial sample.

Concentrations of DNA were determined using the Qubit 2.0 fluorometric analysis double-stranded DNA high-sensitivity kit (Thermo Fisher Scientific). The concentrations were adjusted by dilution with nuclease-free water to fall in the range of 5 to 15 ng/μL, suitable for the sequencing instrument.

Sequencing of Staph. aureus Isolates. All library preparation and sequencing was carried out at the Clinical Genomics Stockholm facility at the Science for Life Laboratory (Stockholm, Sweden) using an Illumina Novaseq 6000 instrument with an S4 flow cell. Seven samples failed to produce sufficient numbers of sequence reads or exhibited poor mapping to the reference NCTC 8325 (NC_007795). Of these, 3 were from the 2002 to 2003 subset and 4 from the 2013 to 2018 subset. Thus, 173 samples remained for further bioinformatic analyses. The successfully sequenced samples had a mapping rate in the range 90.7 to 99.3% to the reference strain. The percentage of base pairs with a coverage better than 30 was in the range of 90.2 to 96.4%.

Bioinformatic Analysis of Sequences

Assembly of Genomes and Genotyping. Sequence assembly was carried using the UniCycler pipeline, version 0.4.8-β (Wick et al., 2017). UniCycler employs read error correction and optimizes de novo assembly by SPAdes, version 3.13.0 (Bankevich et al., 2012). In addition, UniCycler removes errors in the assembly using pilon, version 1.23 (Walker et al., 2014). Minimum spanning trees were obtained by SeqSphere+, version 5.1.0 (Kohl et al., 2014), using the assembled contigs obtained from UniCycler with the seed genome with GenBank accession NC_002951.2 and the *Staph. aureus* cgMLST version 1.3, containing 1,861 loci (https://www.cgmlst.org/ncs/schema/141106/). The criteria for identification were 100% aligned length and 90% identity, which were met by 157 isolates (81 from 2002 to 2003 and 76 from 2013 to 2018) for 1,692 loci that, accordingly, were used for creating a minimum spanning tree. The ST were defined by alleles from the following standard set of *Staph. aureus* MLST genes: *arcC*, *aroE*, *gpF*, *gmk*, *pta*, *tpi*, and *yqiL* (Enright et al., 2000). Clonal clusters were defined as groups of ST in which every ST shares at least 5 of the 7 identical alleles with at least one other ST in the group (Feil et al., 2003). With this definition, the sequence types of the isolates were assigned to their usual clonal complex, including the recently described ST71, constituting a divergent subgroup of CC97 (Cormican and Keane, 2018).

Detection of Antibiotic Resistance Genes. The UniCycler sequence assemblies were used to detect antibiotic resistance genes by using Resistance Gene Identifier software (Alcock et al., 2020) to interrogate the Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/analyze/rgi).

Statistical Analysis

Fisher's exact test was employed using python (version 3.7) with the scipy.stats module (version 1.4.0; https://docs.scipy.org/doc/scipy/reference/stats.html#module-scipy.stats) to determine the significance of changes in prevalence of genotypes over the period spanning the collection of the first (2002–2003) and second sets (2013–2018) of *Staph. aureus* isolates.

Comparison with Previous Results

Twenty-nine of the *Staph. aureus* isolates analyzed in this study and collected from 2002 to 2003 had earlier been characterized using a microarray hybridization assay genotyping system (Identibac *S. aureus*, Alere GmbH; Artursson et al., 2016). The microarray assigned *Staph. aureus* isolates to CC and ST. The results of the microarray regarding CC or ST assignment were compared with the corresponding CC and ST assignments by cgMLST in the present study.

RESULTS

Temporal Changes in Prevalence of Sequence Types Isolated from Clinical Mastitis

A cgMLST could be determined for 157 of the isolates, with 81 from 2002 to 2003 and 76 from 2013 to 2018. Overall, most of the isolates belonged to 3 large clusters assigned to the clonal complexes CC97, CC133, and CC151, with 86 and 91% of the isolates belonging to one of these clusters for the 2002 to 2003 and the 2013 to 2018 periods, respectively (Figure 1, Table 1). The most abundant CC of the earlier period was CC133, accounting for 47% of the isolates collected from 2002 to

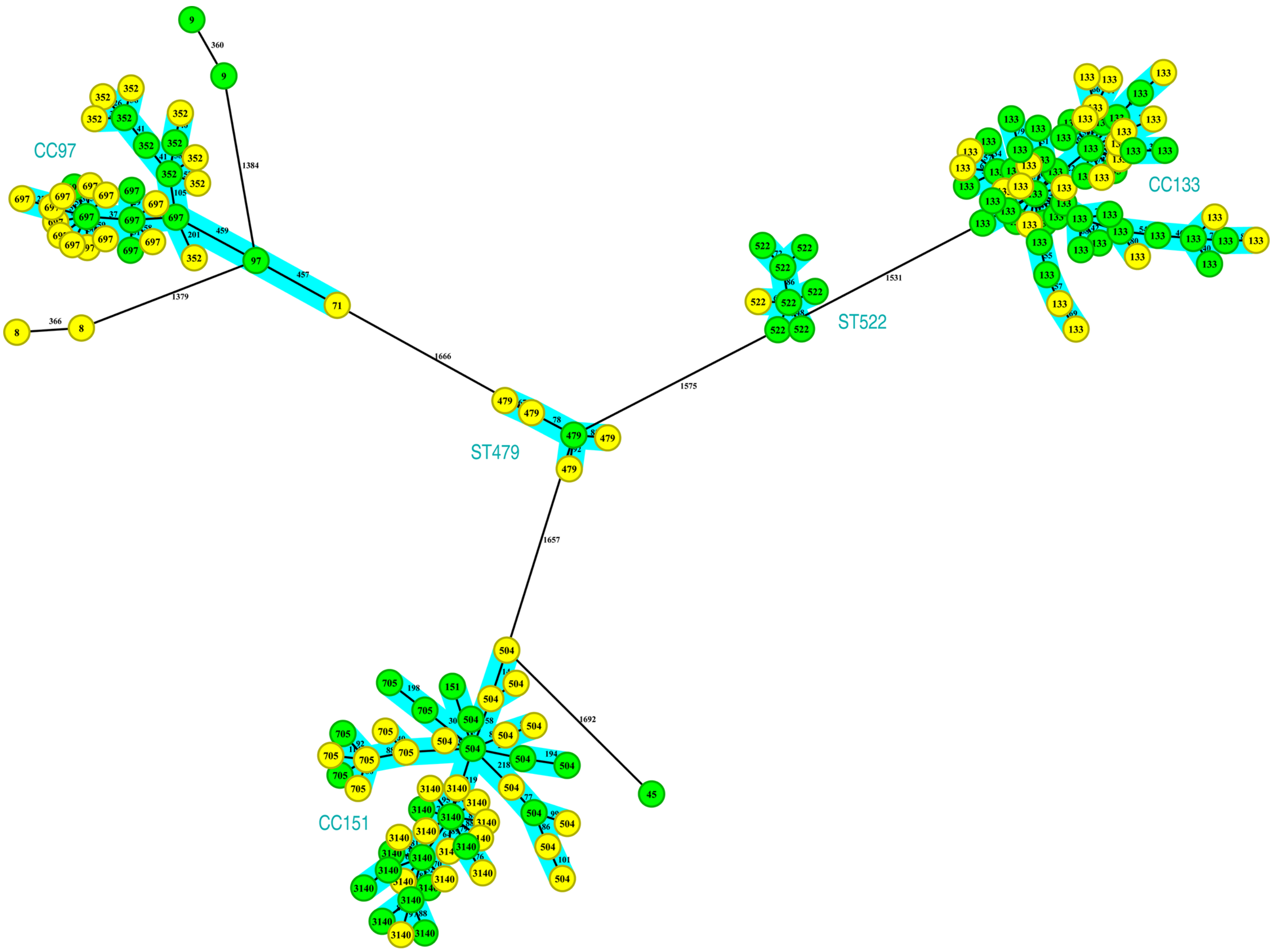

Figure 1. Minimum spanning tree calculated for 81 *Staphylococcus aureus* isolates isolated from cases of bovine clinical mastitis during 2002–2003 (green circles) and 76 isolates isolated during 2013–2018 (yellow circles). The sequence type (ST) of each isolate is shown inside the circles. The tree was created using 1,692 core genome multi-locus sequence typing loci. Member isolates of the designated clonal complexes (CC) CC97, CC133 and CC151 and isolates of ST479 and ST522 are highlighted with blue backgrounds. The allelic distances between nodes are shown.

2003; however, this decreased to 29% during the 2013 to 2018 period. The cluster CC133 was composed of a single sequence type, ST133 (Table 1). The second-largest cluster was CC151, which displayed an increase over time from 26% to 36% of the isolates. The CC151 cluster was composed of sequence types ST151, ST504, ST705, and ST3140, where ST3140 and ST504 were the dominating sequence types. The third cluster, CC97, was composed of ST71, ST97, ST352, and ST697. The proportion of CC97 also increased over time, from 14% of the isolates to 26%. The 2 sequence types ST352 and ST697 dominated this cluster. Two smaller clusters of ST479 and ST522 were also found; their prevalence increased from 1% to 4% and decreased from 7% to 1%, respectively (Figure 1, Table 1). Finally, 2 isolates each of ST8 and ST9 and 1 isolate of ST45 were also found. Isolates not belonging to any of the 3 large clusters will, when appropriate, be referred to as "other isolates."

Detailed information on occurrence of specific CC and ST in the 2 sets of isolates is given in Table 1. The proportion of isolates belonging to CC133 was significantly lower in 2013 to 2018 than in 2002 to 2003 ($P = 0.022$), whereas the increase of CC97 and decrease of ST522 were slightly below the 95% confidence level, with P-values of 0.070 and 0.064, respectively. No other major differences between the 2 sets of isolates were found.

Antibiotic Resistance Genotypes

The antibiotic resistance genes and mutations conferring resistance found in isolates from the 2 time periods

are presented in Figure 2. Genes belonging to the major facilitator superfamily of transporter proteins and regulators, *Lmrs* (Floyd et al., 2010), *norA* with regulators *arlS* and *arlR* (Fournier et al., 2000), and *tet38* with regulator *mgrA* (Truong-Bolduc et al., 2019), were present in virtually all isolates. Also, a multidrug and toxic compound extrusion transporter gene *mepA* with regulator *mepR* (Kaatz et al., 2006) was present in almost all isolates.

Genes and mutations connected to fosfomycin resistance were found to varying degrees and were distinctly linked to certain clonal complexes. The antibiotic fosfomycin acts by interfering with UDP-N-acetylglucosamine enolpyruvyl transferase, which is involved in cell wall synthesis and is encoded by the *murA* gene (Michalopoulos et al., 2011). The fosfomycin thiol transferase gene *FosB* was found in all CC133 isolates except one and, additionally, in the ST8 and ST9 isolates, but not in any other isolates. *Staphylococcus aureus* can also become fosfomycin-resistant by reducing cellular uptake through mutations in the L-a-glycerophosphate transport system encoded by *GlpT* (Michalopoulos et al., 2011; Fu et al., 2016). We found the E291D and T396N mutations in all isolates having *GlpT* mutations conferring resistance to fosfomycin, with 2 exceptions: an ST522 isolate, which had only the T396N mutation, and 1 of the 2 ST8 isolates, which had a unique G257D mutation. The other ST8, the 2 ST9 isolates, and the CC97 isolates had no resistance mutations in *GlpT*, as detected by Resistance Gene Identifier software. A third mechanism for conferring fosfomycin resistance is for mutations directly in *murA* to prohibit interaction with fosfomycin (Michalopoulos et al., 2011; Fu et al., 2016). Closely mirroring the mutations found in *GlpT*, Resistance Gene Identifier detected the A100V mutation in *murA* in all isolates except those of ST8, ST9, and CC97. In addition, all CC151 isolates also had an L27F mutation. In summary, the mutation patterns in *GlpT* and *murA* and the prevalence of the *FosB* gene are linked, except for the CC151 isolates that have *murA* and *GlpT* mutations but lack *FosB*.

The prevalence of the *blaZ* gene, carrying β-lactamase resistance, was very low in both sets of isolates (3 isolates 2002–2003; 1 isolate 2013–2018). The *blaZ*-carrying isolates from 2002 to 2003 belonged to ST133 (n = 2) and ST9 (n = 1), and the isolate from 2013 to 2018 belonged to ST71.

Overall, the antibiotic resistance genes and mutations show a strong association with the clonal clusters, but no loss or gain of genes or mutations among the member strains of the different clonal clusters were observed over the period of this study (Figure 2).

Comparison of Previous Results with Genotyping Microarray

Comparing the CC and ST assignments by the previously used genotyping microarray and the cgMLST showed high agreement (Table 2). For 27 of 29 (93%) isolates, both methods assigned the same ST or CC. However, 2 isolates that were assigned to ST522 by cgMLST were assigned to CC133 by the microarray system.

Table 1. Number (percent prevalence) of *Staphylococcus aureus* isolates collected from cases of bovine clinical mastitis during 2002 to 2003 and 2013 to 2018, assigned to clonal complexes (CC) and multi-locus sequencing types (MLST)

Assignment		N (% prevalence)			
		2002–2003 (n = 81)		2013–2018 (n = 76)	
CC	MLST	CC	MLST	CC	MLST
97	71	11 (14)	0 (0)	20 (26)	1 (1)
	97		1 (1)		0 (0)
	352		4 (5)		7 (9)
	697		6 (7)		12 (16)
133	133	38 (47)	38 (47)	22 (29)	22 (29)
151	151	21 (26)	1 (1)	27 (36)	0 (0)
	504		5 (6)		10 (13)
	705		4 (5)		4 (5)
	3,140		11 (14)		13 (17)
Not assigned	8	11 (14)	0 (0)	7 (9)	2 (3)
	9		2 (2)		0 (0)
	45		1 (1)		0 (0)
	479		1 (1)		4 (5)
	522		7 (9)		1 (1)

Table 2. Comparison of clonal complex (CC) and sequence type (ST) assignment by next-generation sequencing (NGS) and DNA microarray hybridization assay genotyping system (Identibac *S. aureus*, Alere GmbH) of *Staphylococcus aureus* isolated from cases of bovine clinical mastitis

	No. of isolates assigned to the indicated CC or ST (n = 29)	
CC and ST as determined by NGS	No. of isolates	Identified by Identibac *S. aureus*[1]
CC97[2]	7	7
CC133[3]	6	6
CC151[4]	13	13
ST479[5]	1	1
ST522[6]	2	0

[1]Results from Artursson et al. (2016).
[2]NGS data included ST352, ST97, and ST697.
[3]NGS data contained the single sequence type ST133.
[4]NGS data included ST705, ST504, ST151, and ST3140.
[5]For ST522 and ST479, clonal clusters were not assigned.
[6]These 2 ST522 isolates were identified as CC133 by Identibac *S. aureus*.

DISCUSSION

The objective of this study was to determine genetic variability, using cgMLST, in the populations of *Staph. aureus* isolated from 2 randomly selected sets of clinical mastitis cases in Swedish dairy cows, collected approximately 15 yr apart. To the best of our knowledge, this is the first longitudinal study of its kind.

Temporal Changes in Prevalence of Sequence Types Isolated from Clinical Mastitis

The cgMLST results clearly showed that the *Staph. aureus* isolates primarily belonged to a limited number of ST, and the majority of the isolates belonged to 1 of 3 clonal complexes: CC97, CC133, or CC151. The finding of a small number of dominating CC is in line with previous studies in Sweden on pulsotypes among *Staph. aureus* from material collected from 2002 to 2003 (Capurro et al., 2010a; Lundberg et al., 2014). Indeed, the 3 dominating CC found in the present study correspond with the 3 most common pulsotypes identified in those studies. Moreover, the dominance of a small

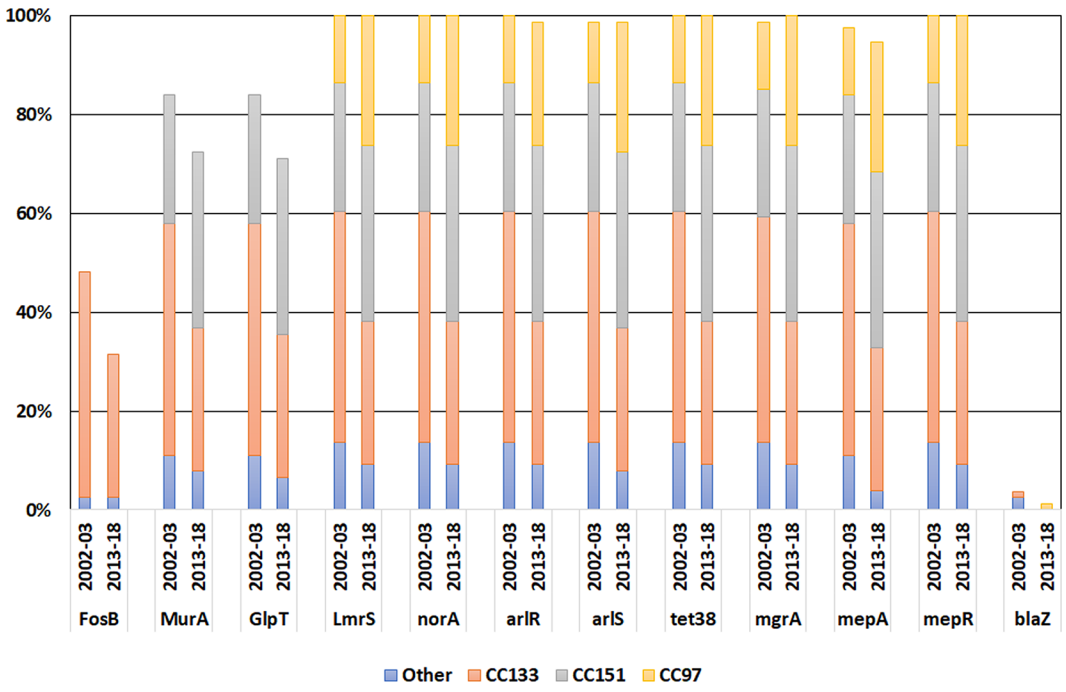

Figure 2. Bar diagram showing the proportions of *Staphylococcus aureus* isolates from cases of bovine clinical mastitis for each collection period [2002–2003 (n = 81); 2013–2018 (n = 76)] carrying antibiotic resistance genes, as detected with the Resistance Gene Identifier tool, using the Comprehensive Antibiotic Resistance Database (Alcock et al., 2020). The assignment of isolates to the clonal complexes (CC) CC97, CC133, and CC151 is shown, with colors as indicated in the figure. Isolates not included in those CC were designated as "other." *FosB* = fosfomycin thiol transferase; *MurA* = UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase (inactivated by fosfomycin; resistance by mutations); *GlpT* = glycerol-3-phosphate transporter, involved in fosfomycin uptake (resistance by mutation). Major facilitator superfamily of transporter proteins: *LmrS* = lincomycin resistance protein, *norA* = quinolone resistance protein (*arlS*, *arlR*; regulators), *tet38* = tetracycline efflux transporter (*mgrA*; regulator). Multidrug and toxic compound extrusion transporter: *mepA* = multidrug export protein (*mepR*; regulator); *blaZ* = β-lactamase.

number of genotypes has also been observed in studies from other Nordic countries (Mørk et al., 2005; Ronco et al., 2018) as well as from other parts of the world (Cosandey et al., 2016; McMillan et al., 2016; Hoekstra et al., 2020). The common genotypes found in the present study have also been found in bovine mastitis in other countries (Boss et al., 2016; Naushad et al., 2020). *Staphylococcus aureus* belonging to CC97 and CC151 (also denoted CC705 in the literature) are often associated with bovine mastitis (Smith et al., 2005; Hata et al., 2006, 2010; Smyth et al., 2009; Schmidt et al., 2017; Ronco et al., 2018) and is generally considered adapted to the bovine host (Weinert et al., 2012; Richardson et al., 2018). Complex CC97 *Staph. aureus* strains have also been isolated from human samples, which suggests zoonotic transfer (Spoor et al., 2013; Schmidt et al., 2017). Interestingly, CC133 was the most commonly occurring CC type among Swedish *Staph. aureus* isolates in the 2002 to 2003 set, although it was partly superseded by the CC97 and CC151 isolates in the 2013 to 2018 set. Cluster CC133 is responsible for a vast majority of *Staph. aureus* mastitis cases in small ruminants such as sheep and goats but has also been associated with bovine mastitis (Ben Zakour et al., 2008; Smyth et al., 2009; Ronco et al., 2018). The CC133 strain does not seem to be as common in central Europe (Magro et al., 2017; Käppeli et al., 2019) as it is in Sweden and elsewhere in northern Europe (Ronco et al., 2018). However, the only temporal change of the *Staph. aureus* populations in Sweden (which is significant at a 95% confidence level in the present work) was a decrease of the CC133 strain from 47% to 29% of the isolates (Table 1). Furthermore, ST522 isolates have been claimed to be exclusive for small ruminants (Matuszewska et al., 2020), but in the present study 7 isolates of this sequence type were found in the earlier sample set and a single isolate in the later sample set. Thus, over a period of 15 yr, a decrease occurred in the proportion of isolates of the sequence types known to be common in small ruminants, ST133 and ST522, and an increase of the proportion of the well-known bovine-adapted sequence types ST97 and ST151.

Changes in the distribution of genotypes may occur for different reasons. Genetic translocations may occur within the *Staph. aureus* genome. In humans, in vivo competition and horizontal gene transfer among *Staph. aureus* lineages can be major drivers for changes during long-term persistence (Langhanki et al., 2018). In dairy production, several factors may influence spread of *Staph. aureus* strains between and within farms, such as trade and herd size. During the time between the 2 study periods, important changes occurred among Swedish dairy herds. For example, the number of dairy farms decreased from 11,270 in 2002 to 3,600 in 2017, and the herd size increased from, on average, 37 to 89 cows per herd during the same period (Statistics Sweden, 2003, 2018). Moreover, loose housing of dairy cows and automatic milking systems have become much more common. The changes in herd number and size may have contributed to reduced contact between small ruminants and dairy cattle, leading to less cross-species transmission of subtypes such as ST522 and ST133. In addition, the extensively bovine-adapted ST97 and ST151 may have a competitive advantage compared with ST133 and ST522. The lack of introduction of novel subtypes might be explained by the very limited import of live dairy cattle to Sweden.

Long-term persistence of a specific genotype of *Staph. aureus* in the population has also been described by Anderson and Lyman (2006), who found that the same *Staph. aureus* strain, as characterized by PFGE, persisted for 13 to 15 years in 3 farms in the United States. In our study, the fact that CC97, CC133, and CC151 were the dominant clonal complexes in both sets of isolates indicates that the presence of these *Staph. aureus* genotypes has been relatively stable in Sweden over at least 15 yr. Thus, only a few new strains of *Staph. aureus* associated with bovine clinical mastitis were introduced between 2002 and 2003 and 2013 to 2018. One new sequence type found among isolates from 2013 to 2018 was ST8. This sequence type is highly associated with the contagious *Staph. aureus* genotype B found in central Europe (Boss et al., 2016). However, further studies (unpublished results) of the 2 ST8 isolates' sequence data revealed no association with genotype B, as both isolates were lacking *sea*, *sed*, and *sej*, whose presence is characteristic for genotype B (Graber et al., 2009).

Antibiotic Resistance Genotypes

As expected, transporter genes belonging to the major facilitator superfamily and multidrug and toxic compound extrusion efflux pumps, which, as reported by the Comprehensive Antibiotic Resistance Database (Alcock et al., 2020), are almost universally found in *Staph. aureus*, were present in all isolates. Efflux of antibiotics is an effective resistance mechanism among antibiotic-resistant *Staph. aureus* (Hassanzadeh et al., 2020).

The fosfomycin resistance gene *FosB*, coding for fosfomycin thiol transferase, was present in almost all CC133 isolates, as well as in the ST8 and ST9 isolates, but in no other isolates. Mutations of the *Staph. aureus murA* and *GlpT* genes, known to confer fosfomycin resistance, were also connected to specific clonal complexes and were found in both these genes for all isolates, with the notable exception of CC97 isolates and ST8 and ST9

isolates. Thus, CC97 isolates are unique in completely lacking mechanisms for fosfomycin resistance.

In line with phenotypic results that 3.8% and 1.3% of the old (Bengtsson et al., 2009) and new isolates (Duse et al., 2021), respectively, were resistant to β-lactam antibiotics, the prevalence of isolates carrying the *blaZ* gene was very low, and no association with sequence types or clonal complexes could be deduced.

Comparisons of Genotypic Methods

Some of the isolates (n = 185) collected from 2002 to 2003 have previously been characterized via PFGE (Capurro et al., 2010a; Lundberg et al., 2014). In these studies, 3 predominant pulsotypes (**PT**) were found, denoted E, Q, and U, with a prevalence of 9% for PTE, 43% for PTQ, and 22% for PTU. These 3 pulsotypes accounted for 74% of all isolates (Lundberg et al., 2014). A subset of 70 isolates from 2002 to 2003, partly the same as analyzed in the present study, were also characterized using a microarray hybridization assay genotyping system (Identibac *S. aureus*, Alere GmbH), which can assign *Staph. aureus* isolates to clonal complexes. The microarray study (Artursson et al., 2016) showed that most isolates of PTE belonged to CC97, isolates of PTQ belonged to CC133, and isolates of PTU belonged to CC151. According to Table 1 in the present study, the total prevalence of isolates of CC97, CC133, and CC151 was 78% of the 81 isolates characterized from 2002 to 2003, which indicates a high degree of concordance in results from the different techniques. When the results of the microarray were compared with those of cgMLST in the present study, the agreement was also very good, as only 2 isolates displayed discordant results (Table 2). For these 2 isolates, sequencing yielded ST522, whereas the Identibac array assigned both to CC133. Two of the 7 alleles are the same for ST522 and ST133, and the lower discriminatory power of the microarray, where results are based on the occurrence of common gene alleles rather than core genome sequences for MLST genes, might explain this discrepancy. A data set based on the 29 isolates available for comparison is too small to make general conclusions about concordance between typing by the Identibac microarray and by next-generation sequencing, but it indicates that the prevalence of ST522 might be underestimated in the bovine population.

CONCLUSIONS

Our results indicate that a stable population of *Staph. aureus* mainly belonging to 3 clonal complexes, CC97, CC133, and CC151, has been associated with bovine clinical mastitis in Swedish dairy herds for over 15 yr. A relative decrease of CC133 and ST522 isolates, known to be primarily small ruminant adapted, and an increase of the established bovine-adapted sequence types CC97 and CC151, might reflect changes in dairy production toward larger and fewer herds, with less contact with small ruminants. Few new sequence types have been introduced during the 15 yr that have passed between the 2 collection periods. Moreover, the antibiotic resistance patterns are virtually unaltered during this time period, and the *blaZ* gene was found in only a few of the isolates. This new knowledge about *Staph. aureus* genotype stability gives important insight into the epidemiology of bovine intramammary infections and is useful for devising strategies for control and prevention of *Staph. aureus* mastitis.

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