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Genomic investigation of Danish *Staphylococcus aureus* isolates from bulk tank milk and dairy cows with clinical mastitis

Short title: Analysis of *S. aureus* isolates from dairy Farms

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Highlights

- *S. aureus* isolates from bulk tank milk and clinical mastitis had similar genetic background.
- Dairy cows can be carriers of subtypes that can cause clinical mastitis under right conditions.
- Three mobile genetic elements were primarily found among closely related ST151 isolates.

Abstract

Staphylococcus aureus is one of the most common pathogens that cause mastitis in dairy cows. Various subtypes, virulence genes and pathogenicity islands have been associated with isolates from bulk tank milk and clinical mastitis. So far, no Danish cattle associated *S. aureus* isolates have been whole-genome sequenced and further analyzed. Thus, the main objective was to investigate the population structure and genomic content of isolates from bulk tank milk and clinical mastitis, using whole-genome sequencing. This may reveal the origin of strains that cause clinical mastitis.

S. aureus isolates from bulk tank milk (n=94) and clinical mastitis (n=63) were collected from 91 and 24 different farms, respectively and whole-genome sequenced. The genomic content was analyzed and a phylogenetic tree based on single nucleotide polymorphisms was constructed.

In general, the isolates from both bulk tank milk and clinical mastitis were of similar genetic background. This suggests that dairy cows are natural carriers of the *S. aureus* subtypes that cause clinical mastitis if the right conditions are present and that a broad range of subtypes cause mastitis. A phylogenetic cluster that mostly consisted of ST151 isolates carried three pathogenicity islands that were primarily found in this group. The prevalence of resistance genes was generally low. However, the first ST398 methicillin resistant *S. aureus* isolate from a Danish dairy cow with clinical mastitis was detected.

Keywords

Whole-genome sequencing, *Staphylococcus aureus*, bovine mastitis, bulk tank milk, population structure, virulence and resistance genes

Introduction

Staphylococcus aureus is an opportunistic pathogen that may cause severe infections in both humans and livestock and is a major cause of mastitis in dairy cows (Holmes and Zadoks, 2011)(Agersø et al., 2012)(Larsen et al., 2015). Bovine mastitis results in reduced animal welfare, milk quality and milk production which is the reason for remarkable economic losses worldwide (Halasa et al., 2007)(Haran et al., 2012)(Barkema et al., 2009). A variety of different sequence types (STs) (ST97, 126 133, 151, 479 and 771) (Holmes and Zadoks, 2011)(Zadoks et al., 2011) and *spa*-types (t518, t519, t524 t528, t529 and t543) have been associated with bovine mastitis and cattle worldwide (Hasman et al., 2010)(Ikawaty et al., 2009)(Sakwinska et al., 2011). Previous studies have shown that few types of strains belonging to specific genotypes are successful at causing persistent mastitis and strain RF122 (ST151) has been reported as one of the most common clone types involved in clinical mastitis (CM) (Kapur et al., 1995)(Reinoso et al., 2004)(Haveri et al., 2005)(Fritzgerald et al., 1997). This strain carries various mobile genetic elements (MGEs) that contain virulence genes and other types of genes related to host adaption. Most of these genes are found within specific types of MGEs known as *S. aureus* pathogenicity islands (SaPIs) (Herron-Olson et al., 2007). In general, various types of virulence genes have been detected in clinical and subclinical mastitis isolates and in bulk tank milk (BTM). These virulence factors are involved in: Host colonization (*cap, clfA/B, cna, fib* and *sak*), toxin production (*tst, sea-j, hla/b/g, lukD/E/FS, etA/B*) and biofilm formation (*icaD, fnbB*) (Fueyo et al., 2005)(Bardiau et al., 2016)(Xu et al., 2015)(Fournier et al., 2008). Many of these virulence genes encode toxins that are also harmful to humans. For example, staphylococcal enterotoxins (encoded by *se* genes) cause food poisoning, the toxic shock syndrome toxin-1 (encoded by *tst*) causes toxic shock syndrome and leukocidins (encoded by *lukD/E/FS*) are involved in various types of clinical infections (Asao et al., 2003)(Umeda et al., 2017)(Deurenberg et al., 2005) (Lina et al., 1999).

Methicillin resistant *S. aureus* (MRSA) belonging to ST398 has been observed among bovine mastitis isolates across the globe but has not disseminated among Danish herds of dairy cows (Holmes and Zadoks, 2011)(Zadoks et al., 2011). However, in Denmark this lineage has primarily been found in pigs and is now an increasing cause of human infections (Agersø et al., 2012)(Larsen et al., 2015). Previously, various studies of Danish *S. aureus* isolates associated with bovine mastitis have been carried out (Katholm et al., 2012)(Aarestrup et al., 1995a)(H. D. Larsen et al., 2000)(Larsen et al., 2002)(Larsen et al., 2000) but no Danish isolates from BTM and CM have so far been whole-genome sequenced. Thus, the main objective of this study was to investigate the genomic content and population structure of Danish *S. aureus* isolates from BTM and CM, using whole-genome sequencing. A further objective was to investigate possible differences between the BTM and the CM isolates.

Materials and Methods

S. aureus isolates

In 2016, CM isolates (n=63) were all sampled from different cows on 24 different Danish farms. The aseptic foremilk samples were collected from dairy cows with CM according to the National Mastitis Council's guidelines. Samples of mastitis secrete or plates with growth were submitted to the Danish Veterinary Institute for *S. aureus* verification using Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF).

Isolates from BTM (n=94) were sampled from 91 different Danish dairy farms. The farms were selected based on the yearly BTM samples taken under a surveillance program for *Streptococcus agalactiae* as previously described (Katholm et al., 2012). Samples were analyzed with the Mastit4 real-time PCR test (DNA diagnostic A/S, Risskov, Denmark) at an analytic laboratory (Eurofins, Vejen, Denmark). Based on the PCR test result, 100 herds with the lowest Ct-value (ranging from 21-27) were selected and samples submitted to the Danish Veterinary Institute. The BTM samples were cultured by streaking 10 µl on blood agar (Columbia agar base (Oxoid, CM0331, Hampshire, UK) supplemented with 5% calf blood) and on *S. aureus* selective ager (SA Select, bioMérieux, Marcy-l'Étoile, France). Colonies suspected for being *S. aureus* were further sub-cultured and verified as *S. aureus* using MALDI-TOF.

Both BTM and CM isolates were sampled from different farms distributed in all parts of the country.

DNA purification and whole-genome sequencing

S. aureus colonies were grown overnight on blood agar at 37°C and single colonies were cultured in 5 ml trypticase soy broth (Becton-Dickinson and Company, Franklin Lakes, USA) under the same conditions. DNA was purified using a Maxwell 16 LEV Blood DNA Kit (Promega, Madison, USA) according to manufacturer's instructions, with an additional lysis-phase including 200 µg/ml lysostaphin per sample (Sigma-Aldrich, St. Louis, USA). Subsequently, a Nextera XT kit (Illumina, San Diego, USA) was used for building DNA libraries according to manufacturer's instructions. The DNA libraries were paired-end sequenced applying Illumina's NextSeq platform with a read length on 2×151bp. The Illumina sequence reads have been deposited in NCBI's short read archive with the study accession no. SRP119902.

De novo assembly and subtyping

The quality of the Illumina raw reads was analyzed in FastQC 0.11.5 and bases of low quality were trimmed in CLC bio's Genomics Workbench (GW) v10.0 (CLCbio's, Aarhus, Denmark) using default settings. Subsequently, *de novo* assembly was performed in CLC bio's GW on default settings and a minimum contig size of 500 nt. MLST was performed at PubMLST (Jolley et al., 2004) and MLST v1.8 (Larsen et al., 2012) whereas *spa*-types were determined using spaTyper v1.0 (Bartels et al., 2014).

Identification of genomic content

Resistance and virulence genes were identified in *de novo* assembled contigs using ResFinder v2.1 (Zankari et al., 2012) and VirulenceFinder v1.5 (Joensen et al., 2014), respectively. Few virulence genes (*fib*, *hla*, *icaD* and *nuc*) were extracted from strain Sa52 (Ronco et al., 2017). Subsequently, the genes were identified in the assemblies using the BLASTN (Altschul et al., 1997) implementation in CLC bio's Main Workbench (MW) v7.7.3 and in general, if genes were located on > 1 contig CLC bio's MW was used to identify these. The presence of ORFs that belonged to eight different SaPIs (Table S1-S8) was investigated using CLC bio's GW.

Statistics

Statistical analyses were performed using GraphPad Prism v5.02 (GraphPad Software Inc., San Diego, USA). Differences in the presence of STs, *spa*-types and virulence/resistance genes between BTM and CM isolates were investigated applying a Chi-square test for independence. In cases of ≤ 5 observations, a Fisher's exact test was used. The confidence interval was 95% and the difference considered significant when $P < 0.05$.

Identification of single nucleotide polymorphisms

To investigate the relationship between the 157 isolates single nucleotide polymorphisms (SNPs) were identified using CSI Phylogeny v1.4 (Kaas et al., 2014) with *S. aureus* strain ED133 as reference chromosome (accession no. NC_017337). The SNPs were identified with a quality of ≥ 30, a minimum depth of ≥ 10 × and a distance between SNPs of ≥ 10. Subsequently, a phylogenetic tree was visualized using iTOL v3.6.1 (Letunic and Bork, 2011).

Results

MLST and *spa*-typing

All isolates had an average sequencing depth of >50 fold except a single that had 47 fold. Statistical analyses showed no significant differences in distributions of STs or *spa*-types between BTM and CM isolates, except for ST1 and ST97 that were significantly associated with CM isolates (Table 1). Thirty different STs were found and 12 of these were new and subsequently registered at PubMLST (Jolley et al., 2004). Among BTM isolates 27 different STs were observed whereas 15 were found among the CM isolates. The most prevalent of the new STs were ST3891 and ST3897 found in 17% (27/157) and 5% (8/157) of all isolates, respectively (Table 1). Of the remaining STs, the prevalence of the six most commonly found (ST50, 71, 97, 133, 151 and 479) ranged 5-19% with ST151 as the most prevalent (Table 1). Among all isolates, 15 different *spa*-types were observed. However, 24 BTM and 15 CM isolates were identified as being of unknown *spa*-type. The prevalence of the six most often observed *spa*-types (t519, t524, t528, t529, t543 and t1403) ranged 5-27%, with t529 as the most prevalent (Table 1).

Resistance and virulence genes

In general, all genes were identified with thresholds of 90% nucleotide identity and 90% coverage of the query sequence length. Statistical analyses showed no significant differences in distributions of resistance genes between BTM and CM isolates. Ten different antibiotic resistance genes were observed. The *norA* gene was found in all isolates except a single one, whereas the second most prevalent resistance gene, *blaZ* was observed in 17% (27/157) of the isolates. Only 9% (14/157) of all isolates carried other types of resistance genes than *blaZ* and *norA* (Table 2). Altogether, 82% (129/157) of all isolates carried no other resistance genes than *norA* (data not shown). Among 62 of the 63 CM isolates only *blaZ* and *norA* were found whereas a single ST398 isolate carried a wide range of resistance genes (*blaZ, ermB, lnuB, mecA, norA, tetK*, and *tetM*) (data not shown)

Twenty-nine different virulence genes were identified and they could be divided into three groups according to prevalence among all 157 isolates. One group consisted of the 13 most prevalent genes (*aur*, *hla*, *hlb hlgB*, *splA*/*B*, *lukD*/*E*, *hlgA*/*C*, *nuc*, *fib* and *icaD*) found in ≥ 81% of all isolates. In the second group, the prevalence of six enterotoxin genes (*seg, sei*, *sem*, *sen*, *seo* and *seu*) ranged 45-69%, whereas the prevalence of the ten remaining genes (*sec*, *seh, sek, sel*, *seq*, *sea*/*sep, splE, tst*, *scn* and *sak*) in the third group ranged 2-16% (Table 2). According to statistical analyses five enterotoxin genes (*sei*, *sem*, *sen*, *seo* and *seu*) were significantly associated with BTM isolates whereas a serine protease gene (*spl*E) and an enterotoxin gene (*seh*) were significantly associated with CM isolates (Table 2). When looking at the combination of virulence genes found among isolates within the eight most prevalent STs, no ST97 isolates carried any enterotoxin genes whereas 1/8 of the ST71 and 1/14 of the ST133 isolates carried a single enterotoxin gene, *sei* (Table S9). The only types of enterotoxin genes that were found among the eight most prevalent STs were the six most prevalent types (*seg*, *sei*, *sem*, *sen*, *seo* and *seu*) (Table S9).

Identification of SaPIs

In seven different SaPIs, open reading frames (ORFs) were primarily identified with thresholds of > 80% nucleotide identity and 90% coverage of the query sequence length. In some cases the ORFs were identified with thresholds of > 70% nucleotide identity and a SaPI was only considered present if ≥ 80% of its ORFs were present. Our analyses showed that some types of SaPIs were primarily present among isolates with closely related genetic background (Table 3). The three SaPIs; *φ*12bov, *υ*SaBov and *φ*SaBov-*υ*-Sa*βφ* were only identified among a group of closely related isolates that primarily belonged to ST151, except for *υ*SaBov that was also found in a single ST7 isolate. Isolates in this group that did not belong to ST151 were either single locus variants (SLVs) (ST3899, 3900 and 705) or double locus variants (DLVs) (ST504) of ST151. Additionally, SaPIbov1 and SaPIbov-*υ*Sa*α* were primarily found in six ST504 isolates but also in a single ST705 and a single ST71 isolate. In contrast, SaPIbov4 and SaPIbov5 were found among isolates from many different STs that were not closely related and none of the 157 isolates carried SaPIbov2 (Table 3). A visual overview of the distribution of SaPIs among all isolates can be found in Fig 1.

Phylogenetic analysis

The SNP analysis included 38782 variant positions and 67.8% of the reference chromosome was covered by all isolates. In general, the phylogenetic tree showed that both CM and BTM isolates clustered together into groups of identical or closely related STs (Fig 1). The largest cluster in the tree consisted primarily of 30 ST151 isolates whereas the remaining isolates were either SLVs (ST3899, 3900 and 705) or DLVs (ST504) of ST151. The second largest cluster consisted of 41 isolates whereof eight belonged to ST50 and the post prevalent of the remaining new STs were 26 ST3891 isolates (SLVs of ST50). The third largest cluster consisted of nine ST479 isolates, eight ST3897 isolates and five ST1380 isolates. Furthermore, smaller clusters primarily including isolates that belonged to ST133, ST97 and ST71 were present (Fig 1).

Discussion

Here, we carried out whole-genome sequencing to investigate the population structure and genomic content of 157 Danish *S. aureus* isolates from BTM and dairy cows with CM. To our knowledge it is the first time that such types of Danish isolates have been whole-genome sequenced and made publicly available. Statistical analyses showed no significant differences in the distribution of *spa*-types or STs between the two isolate groups except for ST97 and ST1 that were significantly more associated with CM isolates compared to BTM isolates (Table 1). Only four of all isolates belonged to ST1 and therefore it is difficult to conclude further on this finding. Isolates from BTM samples may originate from subclinical infected quarters, but also from extra-mammary sites such as teat skin, teat canal and the cow environment or from milking staff (Haveri et al., 2008). This could be the reason for finding a more diverse composition of STs in BTM samples (27 different STs) compared to CM samples (15 different STs). Furthermore, the BTM isolates were collected from farms that had shown an increased concentration of *S. aureus* in BTM (PCR Ct-values: 21-27). A study suggests that Ct-values < 32 very likely can be interpreted as reflecting *S. aureus* intra-mammary infections (Mahmmod et al., 2017). Therefore, it is likely that the isolates from BTM were partly from cows with subclinical mastitis which is a mild form of mastitis that requires further testing to be recognized by the farmer. It has been described that a high strain heterogeneity can be interpreted as evidence of environmental mastitis (Klaas and Zadoks, 2017). Thus, the BTM isolates that showed increased strain heterogeneity compared to the CM isolates, could be associated with environmental mastitis

The phylogenetic analysis showed that the BTM isolates clustered together with CM isolates of identical or closely related STs. A large cluster of isolates that primarily belonged to ST151 was observed and the majority of the isolates in this cluster carried three SaPIs (*φ*12bov, *υ*SaBov and *φ*SaBov-*υ*-Sa*βφ*) found in strain RF122 (Herron-Olson et al., 2007). These three SaPIs were exclusively found in this cluster except for a single ST7 isolate that also carried one of these SaPIs (Fig 1). Strain RF122 belonged to ST151 and has been reported to be a commonly observed mastitis causing clone type (Fritzgerald et al., 1997). The SaPIs originating from RF122 contained various virulence genes and therefore it has been suggested the these SaPIs play an important role regarding the CM pathogenesis and successful adaption to dairy cows (Herron-Olson et al., 2007). SaPI *φ*SaBov-*υ*Sa*βφ* carries leucocidin genes (*lukE*/*D*), serine proteases (*spl*C/*E*/*F*) and enterotoxin genes (sec/g/i/m/n/o) whereas *υ*SaBov carries streptolycin genes

(Table S6 and S7). The streptolycin genes encode leucocidin homologs that originates from *Streptococcus pyogenes* (Herron-Olson et al., 2007) and many of the virulence genes carried by *φ*SaBov-*υ*Sa*βφ* have previously been found among mastitis isolates (Fueyo et al., 2005)(Bardiau et al., 2016)(Fournier et al., 2008)(Kot et al., 2016). Additionally, these three SaPIs (*φ*12bov, *υ*SaBov and *φ*SaBov-*υ*-Sa*βφ*) contain many hypothetical and phage related genes (Table S6-S8) that encode proteins of unknown functions and further studies could reveal which potential role they play. Both statistical and phylogenetic analyses showed that the BTM and CM isolates in general were of identical genetic background. This correspond to other studies (Boss et al., 2016)(Conceicão, 2017)(Jørgensen et al., 2005) which found that STs and *spa*-types that were often associated with bovine mastitis are also present in healthy cows and BTM. These findings indicate that dairy cows are natural carriers of *S. aureus* subtypes that can cause CM, for example if the cows appear immunocompromised combined with poor milking practices and hygiene etc.

Some of the most prevalent STs (ST97, 133, 151 and 479) found in this study have previously been associated with bovine mastitis (Holmes and Zadoks, 2011)(Zadoks et al., 2011)(Boss et al., 2016) whereas others (ST50 and 71) have been related to healthy cows and BTM (Smith et al., 2005)(Hata et al., 2010). In addition, the two most prevalent of the 12 new STs ST3891 and ST3897 were SLVs of ST50 and ST479, respectively. The most prevalent *spa*-type was t529 and observed in 27% (43/157) of the isolates, followed by t1403 and t519 that were both found in 10% (16/157) of the isolates. These three *spa*-types have all been associated with bovine mastitis (Ikawaty et al., 2009)(Sakwinska et al., 2011)(Boss et al., 2016)(Johler et al., 2011) but also healthy cows (Hasman et al., 2010). All 30 ST151 isolates belonged to *spa*-type t529 which correspond to a previous study (Sakwinska et al., 2011). Many isolates (39/157) were identified as being of unknown *spa*-type using spaTyper (Bartels et al., 2014). The main reason for this was that the *spa* genes were located on >1 contig and therefore not all repeats were identified by spaTyper (Bartels et al., 2014). Assembly and sequencing error could also explain why the order of the *spa* repeats was not determined correctly. However, it was beyond the scope of this study to register new *spa*-types according to guidelines found at the Ridom SapServer (http://www.spaserver.ridom.de/).

In general, the prevalence of resistance genes was low which correspond to a previous study of Danish mastitis isolates where 81% of 105 isolates were susceptible to 11 antibiotics (Aarestrup et al., 1995a). However, the first ST398 livestock-associated (LA) MRSA isolate (Sa52) from a Danish dairy cow with CM was discovered. The fact that strain Sa52 carried many other resistance genes than the rest of the CM isolates that only carried *blaZ* or *norA*, indicates that it has been transmitted to the dairy cow from an environment with a different selective pressure in regard to antibiotics. Interestingly, previous studies suggest the ST398 lineage has the ability to efficiently jump between humans and livestock and cause severe humans infections (Larsen et al., 2015)(Price et al., 2012). Additionally, it has been suggested that human epidemic MRSA clones originate from isolates within CC97 that have jumped from cows to humans (Spoor et al., 2013). This makes it important to further monitor cattle herds to avoid potential problems regarding LA-MRSA with zoonotic potential, even though the transmission of for example

ST398 strains between humans and dairy cows seems to occur less frequently (Sakwinska et al., 2011)(Boss et al., 2016). LA-MRSA isolates belonging to ST398 have primarily been associated with pigs but strain Sa52 was sampled on a farm where no pig farming had taken place (Larsen et al., 2015)(Price et al., 2012). Thus, it is unlikely that this strain was directly related to pig farming but it could have been transmitted by a visitor or farm worker carrying the clone. Seven-teen % of all isolates carried *blaZ* which correspond to a previous Danish study where 17% of 105 isolates produced beta-lactamases (Aarestrup et al., 1995a). Remarkably, *norA* was found in all isolates except a single one. This gene encodes a multidrug drug resistance efflux pump that mediates resistance to quinolones and a variety of other antiseptic compounds (Santos Costa et al., 2015)(Kaatz and Seo, 1995). Since fluorquinolones are not used to treat Danish dairy cows for CM (https://www.foedevarestyrelsen.dk/Leksikon/Sider/VetStat.aspx) the presence of this gene must be driven by other factors. It may be suggested that *norA* caused resistance to antiseptic compounds used to increase the hygiene in the Danish dairy industry.

Six enterotoxin genes (*seg, sei, sem, sen, seo* and *seu*) were found more frequently than the rest of the enterotoxin genes and were additionally the only types found among isolates from the eight most prevalent STs (Table S9). Five of these genes were significantly more associated with BTM isolates compared to CM isolates. Previously, many of these genes have been found in *S. aureus* isolates from bovine mastitis (Fueyo et al., 2005)(Xu et al., 2015)(Fournier et al., 2008)(Kot et al., 2016). However, the role of enterotoxins in the mastitis pathogenesis is not clear and studies indicate that they are not essential (Larsen et al., 2002)(Larsen et al., 2000). Enterotoxins are heat-stabile and may therefore be found in various dairy products such as milk after heat treatment (Jørgensen et al., 2005)(Hennekinne et al., 2012). Previously, enterotoxins have been reported to be associated with staphylococcal food poisoning caused by cow milk or other dairy products and even with mastitis in humans (Asao et al., 2003)(Jørgensen et al., 2005)(Hennekinne et al., 2012)(Franck et al., 2017). Interestingly, isolates from the highly prevalent ST97 and ST133 that have been found to be strongly associated with CM (Holmes and Zadoks, 2011)(Zadoks et al., 2011), carried almost no enterotoxins genes (Table S9). Concordantly, a previous PCR investigation of 106 Danish *S. aureus* isolates from subclinical and CM showed that none of the isolates carried any enterotoxin genes (Aarestrup et al., 1995b). Currently, more than 20 types of enterotoxin genes have been identified (Hennekinne et al., 2012) but in this study only 12 types were investigated.Thus, it is possible that the isolates carried other enterotoxin genes than those investigated. Furthermore, a high proportion (≥81%) of all isolates carried the leukotoxin encoding genes *lukD* and *lukE*. These genes are often found in isolates associated with bovine mastitis (Fueyo et al., 2005)(Bardiau et al., 2016) but have also been detected in clinical isolates from humans (Yoong and Torres, 2014).

Conclusion

In summary, both statistical and phylogenetic analyses showed that isolates from BTM and CM generally were of similar genetic background. This suggests that dairy cows can be natural carriers of, or subclinicallly infected with, *S. aureus* subtypes that can cause CM if the right conditions are present. A

large cluster primarily consisting of ST151 isolates carried three SaPIs that were almost only found in this group and probably are involved in host adaption and the mastitis pathogenesis. A high proportion of all isolates carried leukotoxin genes and other toxin genes whereas five enterotoxin genes were significantly more associated with BTM isolates compared to CM isolates. Thus, both BTM and CM isolates carried genes that encode toxins that are harmful to humans. The prevalence of resistance genes was in general low but the first ST398 LA-MRSA isolate from a Danish dairy cow with CM was detected. Further surveillance of the Danish dairy cows is therefore important in order to avoid dissemination of zoonotic pathogens.

Conflicts of interest statement

All authors declare no conflicts of interest

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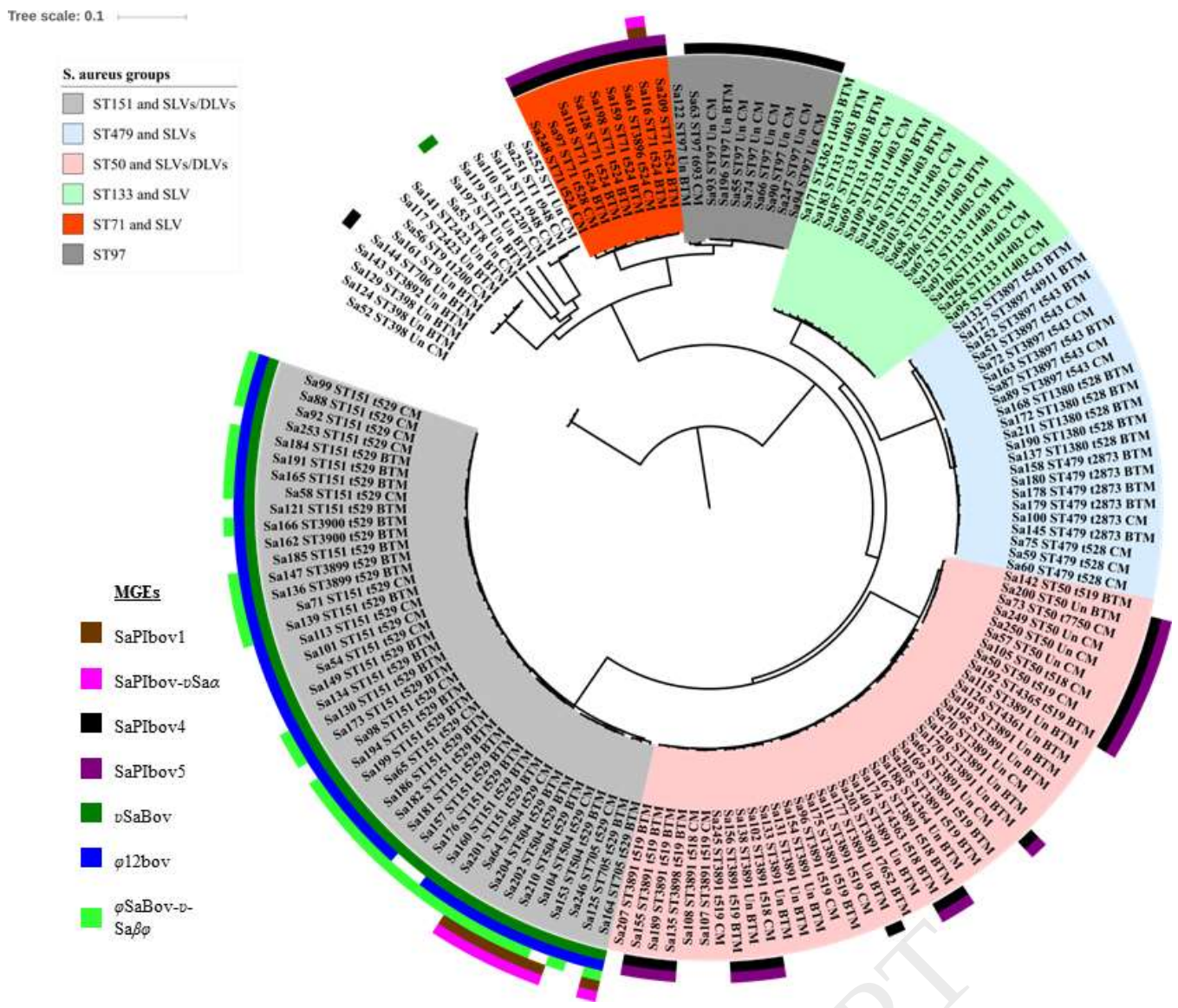
Fig1 Phylogenetic tree including the distribution of SaPIs



The phylogenetic tree is based on 38782 SNPs and *S. aureus* strain ED133 was used as reference chromosome. It shows that the 157 *S. aureus* isolates from bulk tank milk (BTM) and clinical mastitis (CM) cluster together into groups of closely related STs. Additionally, the distribution of seven different *S. aureus* pathogenicity Islands (SaPIs) is shown as colored strips. Three different SaPIs (*υ*SaBov and *φ*SaBov-*υ*-Sa*βφ*) were only found in a cluster that primarily consisted of ST151 isolates (marked in light gray). This cluster included single locus variants (SLVs) (ST3899, 3900 and 705) and double locus variants (DLVs) (ST504) of ST151. Only a single ST7 isolate outside this cluster carried *υ*SaBov. In contrast, SaPIbov4 and SaPIbov5 were found among various STs that were not all closely related.

Table 1 Prevalence of STs and *spa*-types among *S. aureus* isolates

ST (%)	CM (n=63)	BTM (n=94)	*P*-value	*spa*-type (%)	CM (n=63)	BTM (n=94)	*P*-value
151 (19)	11	19	0.6672	**t529** (27)	14	29	0.2347
3891* (17)	8	19	0. 2213	**t519** (10)	6	10	0.8210
133 (9)	9	5	0.0838	**t1403** (10)	9	7	0.1650
97 (6)	8	2	*0.0151*	**t528** (6)	4	5	1.0000
479 (6)	4	5	1.0000	**t524** (5)	2	6	0.4768
50 (5)	6	2	0.0608	**t543** (5)	4	3	0.4395
71 (5)	2	6	0.4768	t2873 (4)	1	5	0.4027
3897* (5)	4	4	0.7146	t518 (3)	2	3	1.0000
504 (4)	2	4	1.0000	t693 (1)	1	0	0.4013
1 (3)	4	0	*0.0245*	t948 (1)	2	0	0.1595
1380 (3)	0	5	0.0831	t1200 (1)	1	0	0.4013
398 (2)	1	2	1.0000	t2207 (1)	1	0	0.4013
705 (2)	1	2	1.0000	t4911 (1)	0	1	1.0000
7 (1)	0	1	1.0000	t7652 (1)	0	1	1.0000
8 (1)	1	0	0.4013	t7750 (1)	1	0	0.4013
9 (1)	1	1	1.0000	Unk (25)	15	24	0.8066
15 (1)	0	1	1.0000				
132 (1)	0	1	1.0000				
706 (1)	0	1	1.0000				
2423 (1)	0	2	0.5164				
3892* (1)	0	1	1.0000				
3896* (1)	1	0	0.4013				
3898* (1)	0	2	0.5164				
3899* (1)	0	2	0.5164				
3900* (1)	0	2	0.5164				
4361* (1)	0	1	1.0000				
4362* (1)	0	1	1.0000				
4363* (1)	0	1	1.0000				
4364* (1)	0	1	1.0000				
4365* (1)	0	1	1.0000				

The table shows the prevalence of 30 STs and 15 *spa*-types of *S. aureus* identified among 63 clinical mastitis (CM) isolates and 94 isolates from bulk tank milk (BTM). The eight most prevalent STs and six most prevalent *spa*-types are in bold whereas 12 new STs are marked with an asterisk (*). Statistical differences in distributions of STs and *spa*-types between CM and BTM isolates were investigated using statistical tests and significant *P*-values are in italic. Unk: Unknown.

Table 2 Prevalence of virulence and resistance genes among *S. aureus* isolates

Virulence genes (%)	CM (n=63)	BTM (n=94)	*P*-value	Resistance genes (%)	CM (n=63)	BTM (n=94)	*P*-value
aur (100)	63	94	1.0000	*norA* (99)	62	94	0.4013
hla (100)	63	94	1.0000	*blaZ* (17)	13	14	0.3501
hlb (99)	63	93	1.0000	*tetM* (3)	1	3	0.6495
hlgB (99)	63	93	1.0000	*dfrG* (2)	0	3	0.2746
hlgC (96)	61	89	0.7027	*ermB* (1)	1	0	0.4013
fib (96)	59	92	0.2196	*lnuA* (1)	0	1	1.0000
nuc (95)	61	88	0.4768	*lnuB* (1)	1	1	1.0000
icaD (94)	61	87	0.3162	*mecA* (1)	1	0	0.4013
hlgA (94)	58	89	0.5236	*tetK* (1)	1	0	0.4013
splA (92)	60	85	0.3638	*vgaA* (1)	0	1	1.0000
splB (92)	60	85	0.3638				
lukD (89)	55	85	0.7296				
lukE (81)	49	78	0.4165				
seu (69)	36	72	*0.0099*				
sem (68)	34	72	*0.0030*				
sen (68)	36	70	*0.0231*				
seo (66)	33	71	*0.0026*				
sei (66)	34	69	*0.0120*				
seg (45)	23	47	0.0955				
splE (16)	15	10	*0.0270*				
sec (5)	2	5	0.7027				
sel (5)	2	5	0.7027				
tst (5)	2	5	0.7027				
scn (3)	3	1	0.3029				
seh (3)	4	0	*0.0245*				
seq (2)	3	0	0.0628				
sak (2)	3	0	0.0628				
sek (2)	3	0	0.0628				
sea/sep (2)	3	0	0.0628				

The table shows the prevalence of 29 virulence genes and 10 resistance genes identified among 63 clinical mastitis (CM) isolates and 94 isolates from bulk tank milk (BTM). Virulence genes were divided into three groups according to prevalence among all isolates: Group 1 (genes found in ≥ 81%), Group 2 (genes found in 45-69%) and Group 3 (genes found in 2-16%). Statistical differences in distributions of virulence and resistance genes between CM and BTM isolates were investigated using statistical tests and significant *P*-values are in italics.

Table 3 Presence of SaPIs among *S. aureus* isolates

MGE	Strain/ CC	No. of isolates (CM)	No. of isolates (BTM)	ST	Reference
SaPIbov1	RF122/151	-	1	705	(Fitzgerald et al., 2001)
SaPIbov1	RF122/151	2	4	504	(Fitzgerald et al., 2001)
SaPIbov1	RF122/151	-	1	71	(Fitzgerald et al., 2001)
SaPIbov2	V329/126	-	-	-	(Cucarella et al., 2001)
SaPIbov-*υ*Sa*α*	RF122/151	-	1	705	(Herron-Olson et al., 2007)
SaPIbov-*υ*Sa*α*	RF122/151	2	4	504	(Herron-Olson et al., 2007)
SaPIbov-*υ*Sa*α*	RF122/151	-	1	71	(Herron-Olson et al., 2007)
SaPIbov4	BA4/97	-	1	4365*	(Viana et al., 2010)
SaPIbov4	BA4/97	-	1	4363*	(Viana et al., 2010)
SaPIbov4	BA4/97	-	1	3898*	(Viana et al., 2010)
SaPIbov4	BA4/97	1	-	3896*	(Viana et al., 2010)
SaPIbov4	BA4/97	1	7	3891*	(Viana et al., 2010)
SaPIbov4	BA4/97	-	1	706	(Viana et al., 2010)
SaPIbov4	BA4/97	8	1	97	(Viana et al., 2010)
SaPIbov4	BA4/97	2	6	71	(Viana et al., 2010)
SaPIbov4	BA4/97	6	1	50	(Viana et al., 2010)
SaPIbov5	JP5338/-	-	1	4365*	(Viana et al., 2010)
SaPIbov5	JP5338/-	-	1	4363*	(Viana et al., 2010)
SaPIbov5	JP5338/-	-	1	3898*	(Viana et al., 2010)
SaPIbov5	JP5338/-	1	-	3896*	(Viana et al., 2010)
SaPIbov5	JP5338/-	1	6	3891*	(Viana et al., 2010)
SaPIbov5	JP5338/-	2	6	71	(Viana et al., 2010)
SaPIbov5	JP5338/-	6	1	50	(Viana et al., 2010)
*υ*SaBov	RF122/151	-	2	3899*	(Herron-Olson et al., 2007)
*υ*SaBov	RF122/151	-	2	3900*	(Herron-Olson et al., 2007)
*υ*SaBov	RF122/151	1	2	705	(Herron-Olson et al., 2007)
*υ*SaBov	RF122/151	2	4	504	(Herron-Olson et al., 2007)
*υ*SaBov	RF122/151	11	19	151	(Herron-Olson et al., 2007)
*υ*SaBov	RF122/151	-	1	7	(Herron-Olson et al., 2007)
*φ*12bov	RF122/151	-	2	3899*	(Herron-Olson et al., 2007)
*φ*12bov	RF122/151	-	2	3900*	(Herron-Olson et al., 2007)
*φ*12bov	RF122/151	1	2	705	(Herron-Olson et al., 2007)
*φ*12bov	RF122/151	2	4	504	(Herron-Olson et al., 2007)
*φ*12bov	RF122/151	11	17	151	(Herron-Olson et al., 2007)
*φ*SaBov-*υ*-Sa*βφ*	RF122/151	-	2	3899*	(Herron-Olson et al., 2007)
*φ*SaBov-*υ*-Sa*βφ*	RF122/151	-	1	3900*	(Herron-Olson et al., 2007)
*φ*SaBov-*υ*-Sa*βφ*	RF122/151	1	1	705	(Herron-Olson et al., 2007)
*φ*SaBov-*υ*-Sa*βφ*	RF122/151	2	3	504	(Herron-Olson et al., 2007)
*φ*SaBov-*υ*-Sa*βφ*	RF122/151	6	15	151	(Herron-Olson et al., 2007)

The table shows seven *S. aureus* pathogenicity islands (SaPIs) identified among 94 isolates from bulk tank milk (BTM) and 63 isolates from clinical mastitis (CM) and the STs for these isolates are shown. SaPI bov2 was not identified in any of the isolates. The number of open reading frames (ORFs) associated with each of the SaPIs are shown together with the strains and their CCs, from where the SaPIs were initially found. References for each SaPI are shown and new STs are marked with an asterisk (*). Unk: Unknown