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

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ABSTRACT

Staphylococcus aureus is one of the most common pathogens that cause mastitis in dairy cows. Various subtypes, virulence genes and mobile genetic elements 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 mobile genetic elements 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.

1. 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 adaptation. Most of these genes are found within specific types of MGEs such as *S. aureus* pathogenicity islands (SaPIs), phages and genomic islands (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/FM/S*, *etA/B*) and biofilm formation (*icaD, fnbB*) (Fueyo et al., 2005; Bardiau et al., 2016; Xu et al., 2015; Fournier et al., 2008; Yamada et al., 2005). 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).

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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; Larsen et al., 2000a, 2002, 2000b) 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.

2. Materials and methods

2.1. 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 to 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.

2.2. 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 × 151 bp. The Illumina sequence reads have been deposited in NCBI's short read archive with the study accession no. SRP119902.

2.3. 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).

2.4. Identification of genomic content

The isolates were investigated for all resistance and virulence genes in *de novo* assembled contigs using ResFinder v2.1 (Zankari et al., 2012) and VirulenceFinder v1.5 (Joensen et al., 2014), respectively. Furthermore, the prevalence of four specific virulence genes (*fib*, *hla*, *icaD* and *nuc*) from strain Sa52 (Ronco et al., 2017) was investigated. 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 MGEs (Tables S1–S8) was investigated using CLC bio's GW.

2.5. 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 $P < 0.05$.

2.6. 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).

3. Results

3.1. 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).

3.2. 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)