Confirmed identification of *Mycolicibacterium smegmatis*, *Staphylococcus borealis* and *Paenibacillus xylanexedens* in Danish cows with high total bacterial count from the same farm

Lærke Astrup [1] & Desiree Lassen [1]

Background

A Danish farm with high-level management and generally high-level udder-health had suffered abrupt high total bacterial counts (TBC) on Bulk tank milk for a period of approximately 3 months (January through March 2024). Several cows had gone through individual TBC-analysis on composite samples (four quarters pooled) through the DIH-testing and the 10 cows with the highest TBC had also gone through PCR (DNA-diagnostics TBC4-kit, EUROFINS, Dk) and four of the same animals had additionally gone through a broader PCR-test (DNA-diagnostics TBC16-kit, EUROFINS, Dk). One of the cows was slaughtered while the remaining 9 cows had quarter-milk samples analyzed with extended bacteriological Culture.

Materials & Methods

Twentytwo quarter milk samples selected as they were culture-positive at the herd veterinarian laboratory, while the remaining quarter samples were culture-negative. The samples were kept and shipped frozen between labs.

Before MALDI-TOF MS-analysis each milk sample was cultured on blood agar (5% calf blood, SSI Diagnostica A/S, Hillerød, Dk). All plates were inspected at both 24 and 48 h of incubation. All bacterial colonies were sub-cultured on blood-agar and incubated for 24 h to obtain pure-cultures of all culturable bacteria present in the milk samples irrespective of definitions on contamination. All resulting pure sub-cultures were analyzed with MALDI-TOF MS. All unusual mastitis-causing bacteria with a MALDI-score of ≥ 2.0 was whole genome sequenced (WGS) (Novogene (UK), Co., Ltd., Cambridge, UK) to confirm their MALDI-TOF MS taxonomy. The WGS-based taxonomy identification was performed using KmerFinder (Center for Genomic Epidemiology). For the *S. borealis* genomes, an Average Nucleotide Identity (ANI) analysis was further performed to verify species taxonomy including the currently available 21 *S. borealis* reference genomes in the NCBI genome database. The species boundary cut-off >95% was applied. Finally, ResFinder (Center for Genomic Epidemiology) was used to investigate the presence of ac-quired antibiotic resistance genes (ARGs) of the two *M. smegmatis* genomes.

Results

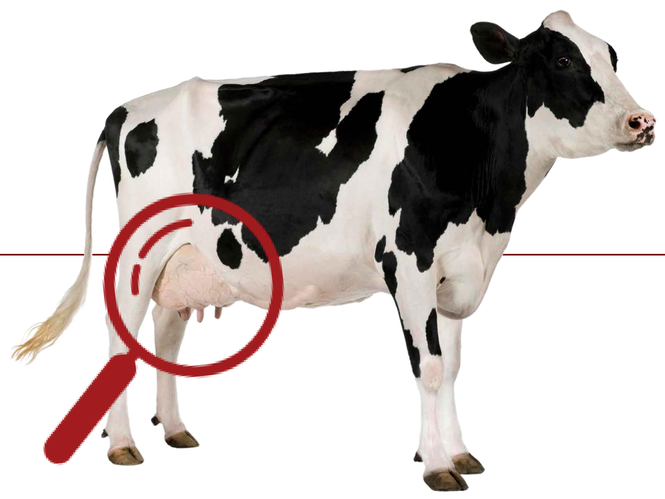
One of the nine cows had two glands that were culture-positive for *M. smegmatis*. One gland was a pure-culture and the other gland had a mixed culture (2 bacterial species) with *S. borealis*. WGS-analysis showed that the isolates were genetically confirmed as *M. smegmatis* and both isolates were suggested multi-drug resistant as each were harboring seven ARGs encoding resistance to a series of different antibiotics, e.g. penicillin, erythromycin, tetracycline and fosfomycin. Notably, both isolates also harbored a mecA gene associated with methicillin resistance. Three of the nine cows had one or two quarters each that were culture-positive for *S. borealis*.
According to NMC-guidelines two of these quarter-samples were pure-cultures while the third quarter-sample displayed *S. borealis* in mixed culture with *M. smegmatis* as described earlier. The fourth quarter sample was positive for *S. borealis* but at CFU below NMC-definitions of culture-positive samples and was found together with another predominant bacterium (Enterococcus sp.). All four *S. borealis*-isolates had their taxonomy genetically confirmed in ANI analysis, with percentage identities ranging from 97-99%. One of the nine cows had a gland that was culture-positive for a pure-culture, but at CFU below NMC-definitions of culture-positive samples. This culture was identified as *Paenibacillus amylolyticus* by MALDI-TOF MS, but WGS-based taxonomy identification using KmerFinder suggested reclassification as *Paenibacillus xylanexedens*

Conclusion

M. smegmatis is a rare mastitis-pathogen that has also been associated with pyrogranolomatous mastitis. A recent retrospective study suggested that *S. borealis* is a more common non-aureus Staphylococci than hitherto acknowledged. But to the best of our knowledge, *Paenibacillus xylanexedens* has never been associated with the bovine udder before. Also, the bacterium was not classified until 2009, which might explain the flawed species allocated by MALDI-TOF MS. Possibly, our finding of *P. xylanexedens* reflects low-grade contamination of a milk sample. Yet, the finding of all three rare pathogens in pure or mixed culture from cows with high total bacterial counting, and from the same farm, calls for more attention towards the possible complex nature behind the TBC relative to classical udder-health microbiology

Take home messages

- Out of 9 cows with high TBC 14 quarter milk samples were culture-negative (9x4 -22).
- Out of 22 culture-positive quarters no major pathogens were found.
- Out of 22 culture-positive quarters 3 pathogens were found that are not frequently/never described in bovine mastitis.
- The lack of traditional mastitis-pathogens in the high TBC cows calls for attention towards a possibly complex nature behind the TBC relative to classical udder-health microbiology.



1. SEGES Livestock Innovation, DK-8200 Aarhus, Denmark.

Present at Conference:
Lærke Astrup
lbas@seges.ku.dk

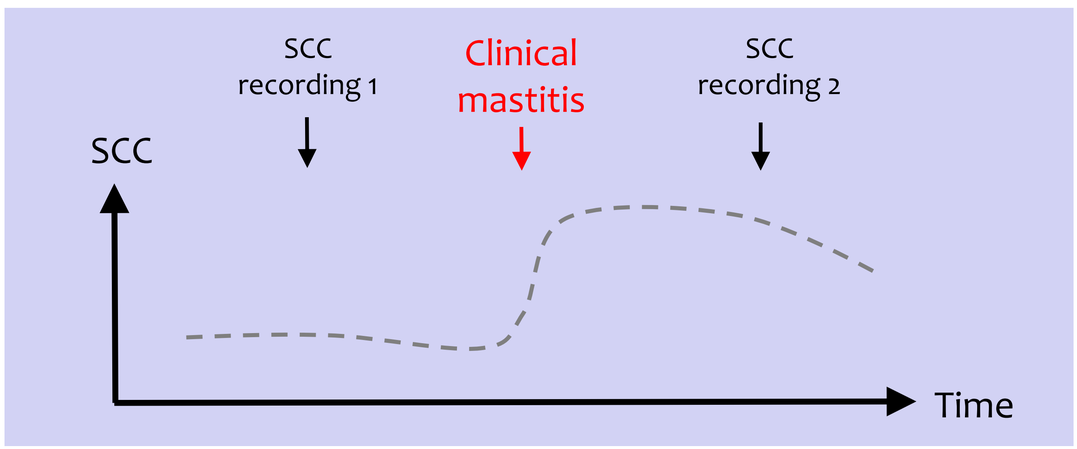


Figure 1. Theoretical response of SCC to a clinical infection occurring between two SCC measurements.

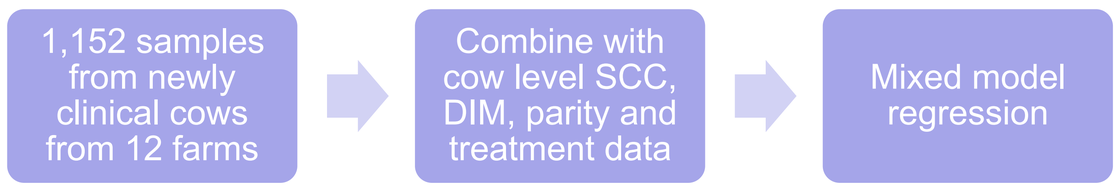


Table 1. Number of samples used in the study, specified per pathogen or pathogen combination, and treatment status.

Pathogen	No treatment	Treatment
No bacterial growth	333	105
Streptococcus uberis	52	203
Contaminated sample	43	10
Staphylococcus aureus	33	32
Escherichia coli	26	16
Staphylococcus epidermidis	20	6
Enterococcus faecium	15	12
Staphylococcus chromogenes	15	8
Staphylococcus haemolyticus	15	8
Staphylococcus sp.	15	16
Streptococcus dysgalactiae	14	28
Staphylococcus aureus + Staphylococcus aureus	13	4
Aerococcus viridans	12	0
Micrococcus luteus	11	2
Staphylococcus equorum	9	3
Staphylococcus sciuri	8	3
Escherichia coli + Streptococcus uberis	5	9
Streptococcus uberis + Streptococcus uberis	5	12
Staphylococcus sp. + Streptococcus uberis	2	16
Streptococcus uberis + Staphylococcus epidermidis	2	11

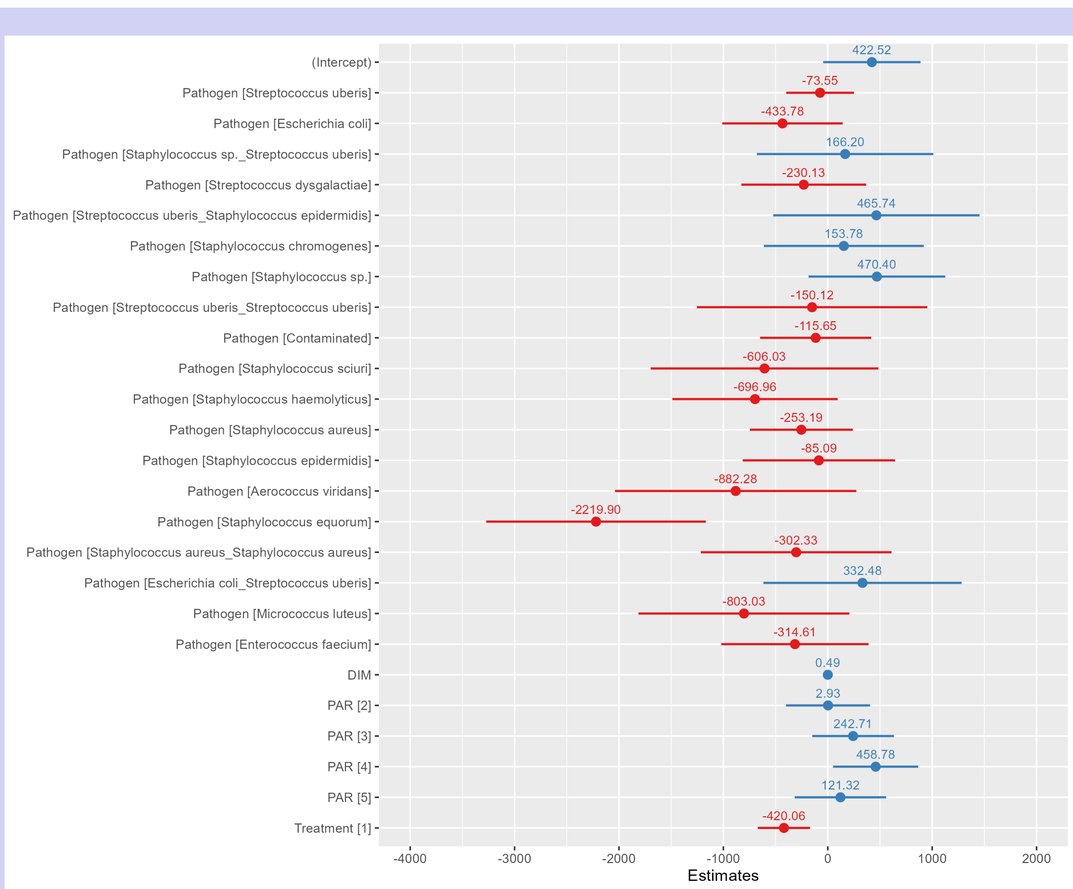


Figure 2. The estimated association between explanatory variables and the difference in SCC (x 1,000) before and after clinical mastitis detection. The reference is samples from untreated cows in first parity with no bacterial growth.