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

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Objectives:
The objectives were to confirm MALDI-TOF MS identification of *Mycolicibacterium smegmatis*, *Staphylococcus borealis* and *Paenibacillus amylolyticus*, respectively, and to evaluate antibiotic resistance profile of taxonomically confirmed *M. smegmatis*.

Material and methods:
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 a total of 22 aseptically obtained quarter milk samples shipped for bacteriological analysis by MALDI-TOF MS. These 22 quarters were selected as they were culture-positive at receival at the herd veterinarian, while the remaining quarter samples were culture-negative. The samples were kept and shipped frozen from the veterinary practitioner's lab to MALDI-TOF MS analysis. 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-cultures were analyzed with MALDI-TOF MS. (NB: Additional culture-protocols were carried out and other bacterial species identified, but these results are not included as the present abstract focuses on the identification of rare mastitis-pathogens and not on the full study-aspects related to high TBC). 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 acquired 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.