Tiergesundheit, Mastitis **Antibiotische Kontrolle, Phylogenetisch**

Analysis of bovine intramammary resistome and of the bacterial transmission within dairy herds

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Key words
Mastitis, non-*aureus* Staphylococci (NAS), antimicrobial resistance, whole genome sequencing, phylogenetic.

Aim of the study

Mastitis is the most important and costly disease in dairy cows worldwide. Bovine intramammary infection (IMI) caused by pathogenic bacteria is common and well understood but very little is known about the bacteria and their resistome present in the mammary gland (= intramammary resistome, IR) of healthy and untreated cows whose milk is regularly delivered for human consumption. The aim that our research project wanted to: i) describe the bacteriome of healthy cows means all the bacteria that we can isolate from the milk of healthy cow, ii) describe the resistome means the antibiotic resistance correlated with the bacteriome isolated with the use of phenotypic and genomic methods iii) comparison of the bacteriome and resistome with the environmental isolates.

Material and methods

Healthy, untreated cows of nine dairy herds from the Swiss Canton Tessin were analyzed three times within one year to identify the most abundant species of the intramammary bacteriome of healthy animals. Aseptically collected milk samples were cultured and bacteria identified using MALDI-TOF mass spectrometry. To describe the intramammary resistome, 350 strains of the predominant species were selected and subjected to short-read based whole genome sequencing (WGS) combined with phenotypic analyses and antibiotic resistance gene profiling. Both chromosomes and mobile genetic elements were examined for antibiotic resistance genes (ARGs) using in-house and online software tools. ARGs were then associated with phenotypic antibiotic resistance profiling data from minimum inhibitory concentration assays. Furthermore, the phylogeny of the two main species isolated *Staphylococcus xylosus* (*S. xylosus*) and *Mammaliicoccus sciuri* (*M. sciuri*) was assessed using 70 housekeeping genes and the maximum likelihood approach (PhyML). Additionally statistical analyses were carried out.

Results and significance

Of 256 cows (1024 single quarters) analyzed, 96% were bacteriologically positive and 80% of the quarters were positive for at least one bacterial species. The 1288 isolates were assigned to 104 different bacterial species including 24 predominant species. *Staphylococcaceae* were most prevalent (14 different species; 73.5% bacteria-positive quarters) with *S. xylosus* and *M. sciuri* accounting for 41.5% of the strains. Our study demonstrates that *Staphylococcaceae* could be consider part of the healthy milk bacteriome. Furthermore, the different farm-specific patterns of the bacteriome are associated with the use of different bedding in the herd. The non-*aureus* Staphylococci (NAS) have been regarded as minor mastitis pathogens being the most abundantly observed bacteria in mastitis milk samples. Based on our study, however, their function needs to be reconsidered. We could assume that different subtypes of NAS may colonize the mammary gland whereas other subtypes may cause minor mastitis.

Phenotypic and genomic antimicrobial resistance (AMR) was bacterium-specific whereby resistance to clindamycin and oxacillin was most frequently observed (40% and 30%). In contrast, AMR to penicillin, although massively used for mastitis treatment during last decades, was rarely observed. The phenotypic findings, not in all the cases, could be linked to chromosomal or plasmid-borne ARGs, demonstrating a lack in understanding the mechanisms that lead to the observed phenotypic AMRs in the isolated bacteria. For some species/AMR, the observed phenotypic AMR could be explained. This is true for *M. sciuri* and its resistance to clindamycin (*salA* gene) as well as *S. xylosus*, *M. sciuri* and a few other bacteria in the case of tetracycline. For all of them, a small plasmid was found carrying the *tetK* or another tetracycline ARG. The presence of tetracycline AMR was herd dependent and was observed in various isolates of the same farm indicating a possible horizontal gene transfer among the different bacteria on the same farm, particularly among *S. xylosus* and *M. sciuri*.

The phylogenetic studies involved *S. xylosus* and *M. sciuri;* these bacteria were the most abundant isolated and were collected from both milk and environmental samples. *M. sciuri* was found predominantly in environmental samples (particularly straw bedding and teat liners before milking). Differently, the *S. xylosus* strains were mostly isolated from milk samples. Based on these results we could conclude that *S. xylosus* is mainly udder adapted whereas the habitat of *M. sciuri* is more environmentally related. Detailed phylogenetic analyses for *M. sciuri* revealed two main clades whereby the smaller one included the *M. sciuri* type strain. Typically, the strains forming this clade were almost exclusively isolated from bedding, milk, and teats, whereas those of the other clade were isolated from milk, teats, and liners, but hardly from bedding. These results suggest that *M. sciuri* circulates clade dependently more among environment and mammary gland (type strain clade) whereas *M. sciuri* of the other clade circulates more among mammary gland and milking equipment. These findings are confirmed by genotype analysis, inferring MLST, demonstrating that the distribution of the sequence types (STs) between milk and environment revealed only a few common STs. Interestingly, *M. sciuri* showed a remarkable variability at the STs level with the consequence that a number of different STs were observed on the same farm. In the case of *S. xylosus*, three main clades were observed. As in *M. sciuri*, the phylogenetic distances within each clade were very small showing that the taxa/strains evolved minimally and that they are genetically very similar. For all three clades, the taxa were largely milk associated whereas environmental taxa were hardly observed. These findings indicate that *S. xylosus* circulates primarily among mammary glands and the taxa found in the environment are more the result of a contamination by milk.

As a conclusion, the research project demonstrates that bacteria, particularly NAS, are very common in the mammary gland of healthy cows, a fact that needs to be considered when interpreting bacteriological results obtained from clinical milk samples. Furthermore, AMR in NAS is uncommon, also against penicillin, although it has been massively used for mastitis treatment during last decades. Unfortunately, AMR in these bacteria remains largely unexplained by genomic analyses. Furthermore, AMR against antibiotics used in human medicine is rare. Finally, phylogenetic studies demonstrate that *M. sciuri* and *S. xylosus* formed only a few major clades and within each clade the strains were genetically very similar; for *M. sciuri* the habitat was clade dependent.

Publications, posters and presentations

Short presentation: Analysis of the bovine intramammary resistome, the horizontal transfer of antibiotics resistance genes, and of the bacterial transmission during herd sanitation of *Staphylococcus aureus* GTB, Agroscope PhD/PostDoc symposium, Reckenholz, Switzerland, 13th September, 2018.

Poster: The intramammary bacteriome of herds positive and negative for *Staph. aureus* genotype B, IDF Mastitis Conference, Copenhagen, Denmark, 15-16th May 2019.

Short presentation: Analysis of bovine intramammary resistome and of the bacterial transmission within dairy herds, GCB symposium, University of Bern, 28 January 2021.

Poster: Analysis of Bovine Intramammary Resistome and of Bacterial Transmission Within Dairy Herds, World Microbe Forum, ASM and FEMS Collaboration (Online Worldwide), 20-24th June 2021.

Poster: Analysis of Bovine Intramammary Resistome and of Bacterial Transmission Within Dairy Herds, National Mastitis Council Annual Meeting, San Diego, California, USA, 1-3rd February 2022.

Presentation: Analysis of the bovine intramammary bacteriome and resistome, Scientific Minisymposium "University Wageningen meets Agroscope", Agroscope, 2nd June 2022

Poster: Analysis of bovine intramammary bacteriome and resistome, ASM MICROBE, Washington, USA, 9-13th, June 2022

Presentation: Analysis of the bovine intramammary bacteriome and resistome, Agroscope PhD/Postdoc symposium, Changis, Switzerland, 13th October 2022

Presentation: Elucidation of the Bovine Intramammary bacteriome and resistome from healthy cows of Swiss dairy farms in the Canton Tessin, TVL webinair, online, 1st December 2022

Publication: Elucidation of the Bovine Intramammary Bacteriome and Resistome from healthy cows of Swiss dairy farms in the Canton Tessin, draft ready for submission

Publication: Genomic, Phenotypic antibiotic resistance and Phylogenetic analyses of *Staphylococcus xylosus* and *Mammaliicoccus sciuri* isolated from quarter milk and environmental samples in Swiss dairy herds, in preparation.

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