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Antibiotic resistance profiles of coagulase-negative staphylococci in livestock environments

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ABSTRACT

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) have globally emerged in animal husbandry. In addition to methicillin resistance, LA-MRSA may carry a variety of novel and uncommon antimicrobial resistance genes. Occurrence of the same resistance genes in coagulase-negative staphylococci (CoNS) and *S. aureus* suggests an ongoing genetic exchange between LA-MRSA and other staphylococci whose driving forces in the ecological niche of the farm environment are, however, still poorly understood. To assess the potential of CoNS as putative reservoirs for antibiotic resistance genes, we analysed the antimicrobial susceptibility of CoNS from dust and manure samples obtained in 41 pig farms in Germany, most of them (36 of 41) with a proven LA-MRSA/MSSA history. Among the 344 isolates analysed, 18 different CoNS species were identified and *S. sciuri* represented the most prevalent species (46%). High resistance rates were detected for tetracycline (71%), penicillin (65%) and oxacillin (64%) as well as fusidic acid (50%), which was mainly due to reduced susceptibility among *S. sciuri* isolates. *S. sciuri* exhibited pronounced multiresistance, and many isolates were characterised by the carriage of a number of uncommon (multi)resistance genes (*e.g. cfr, apmA, fexA*) and decreased susceptibility towards last resort antibiotics such as linezolid and daptomycin. The combined data suggest that *S. sciuri* harbours a significant resistance gene pool that requires further attention. We hypothesise that members of this species, due to their flexible lifestyle, might contribute to the spread of such genes in livestock environments.

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1. Introduction

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) of the CC398 clonal complex are highly prevalent among pig, cattle and poultry in Europe (Köck et al., 2014). They were previously demonstrated to carry a broad range of antibiotic resistance genes which confer, in addition to the SCC*mec*-borne *mecA*-mediated methicillin resistance, elevated minimal inhibitory concentrations (MICs) against numerous antibiotics, some of them being also relevant in human medicine (Kadlec et al., 2012b; Wendlandt et al., 2015b). Many of these resistance determinants, which are often located on plasmids and other mobile genetic elements, are novel and uncommon and their origin is widely unknown (Kadlec et al., 2012b). Recent research suggested that coagulase-negative staphylococci (CoNS) and methicillin susceptible *S. aureus* (MSSA) from farm environments represent a significant reservoir fuelling resistance gene acquisition by LA-MRSA through horizontal gene transfer (HGT) (Wendlandt et al., 2015b). However, the prevalence of antimicrobial resistant CoNS in farm environments is not very well established. Recent epidemiological studies mainly concentrate on LA-MRSA (and CoNS) directly isolated from animals or persons with occupational farm contact. In this study, we analysed CoNS in environmental samples (*i.e.* dust, liquid manure) from farms in Germany to assess the potential of CoNS as reservoirs of antimicrobial resistance genes. We determined the phenotypic susceptibility of environmental CoNS isolates against 21 antibiotics commonly used in veterinary and human medicine by agar disk diffusion and broth

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microdilution assays. We found high phenotypic resistance rates among the environmental CoNS against a number of commonly used antibiotics (*e.g.* beta-lactams, tetracyclines, florfenicol, macrolides) as well as alarmingly decreased susceptibilities, specifically among the *S. sciuri* isolates against last resort antibiotics, such as linezolid and daptomycin. PCR screening for the presence of selected antibiotic resistance determinants identified a highly diverse resistance gene pool among the CoNS consisting of both widespread and uncommon resistance genes, the latter being in part discovered for the first time in CoNS.

2. Material and methods

2.1. Bacterial strains and culture

Between February 2013 and September 2013 the prevalence of extended-spectrum betalactamase (ESBL)-producing enterobacteria was assessed in dust and manure samples from pig holdings in the German federal states of North Rhine-Westphalia and Lower Saxony (Garcia-Cobos et al., 2015). In addition to the samples used for ESBL-producing enterobacteria detection, six additional dust and manure samples were obtained from each farm and were tested for the presence of CoNS, MSSA and MRSA. Each dust and manure sample was enriched in Mueller-Hinton broth with 6.5% NaCl for 24 h at 35 ± 1 °C. Then 10 μl of this sample was streaked onto a colistin-aztreonam medium (CAP, Oxoid, Germany) which was incubated again for 24 h at 35 ± 1 °C. In addition, 1 ml of the NaCl enrichment broth was inoculated in MRSA-selective broth (Phenol Red Mannitol Broth with 5 mg/L ceftizoxime, 75 mg/L aztreonam) and incubated for 24 h at 35 ± 1 °C. Thereafter, 10 μl of the sample from the phenolred broth was streaked onto a chromogenic medium for the detection of MRSA (bioMérieux, Marcy l'Etoile, France) and incubated for 24 h at 35 ± 1 °C. Presumptive CoNS and *S. aureus* colonies were subcultured on Columbia blood agar. Species identification was done by 16S rDNA locus sequencing after PCR amplification using the following primers:

16S_rDNA_F (5′-GAGTTTGATCCTGGCTCA-3′) and 16S_rDNA_R (5′-TACGGCTA CCTTGTTACGACTT-3′). From 41 farms tested, a total of 344CoNS isolates were obtained and subjected to further analysis.

2.2. Antimicrobial susceptibility testing

MICs for benzylpenicillin (PEN), oxacillin (OXA), moxifloxacin (MOX), ciprofloxacin (CIP), trimethoprim/sulfamethoxazole (TMP-SMX), rifampicin (RIF), fusidic acid (FUS), tetracycline (TET), tigecycline (TIG), gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), fosfomycin (FOS), linezolid (LNZ), teicoplanin (TEC) and vancomycin (VAN) were determined using the VITEK® 2Compact System (bioMérieux Deutschland GmbH, Nürtingen) according to standard procedures provided by the manufacturer. MIC results were evaluated through the Advanced Expert System (AES™) according to EUCAST guidelines for CoNS. Antibiotic susceptibilities for apramycin (APR), spectinomycin (SPC), florfenicol (FFC) and chloramphenicol (CM) were performed by agar disk diffusion assays using disks with 15, 100, 30, and 30 μg of the respective antimicrobial agent according to EUCAST guidelines (http://www.eucast.org). As neither clinical breakpoints nor epidemiological cut-off values applicable to staphylococci are available for these antibiotics, inhibition zone distributions were determined (Suppl. Fig. S1). Isolates displaying reduced zone diameters were further tested by MIC determination (APR, SPC, FFC, CM) and molecular analysis for the presence of the respective resistance genes. Despite the lack of clinical breakpoints, isolates which displayed an elevated MIC and showed the presence of a respective resistance gene were considered as resistant.

2.3. Molecular analysis of resistance

CoNS displaying reduced susceptibilities towards oxacillin, apramycin, spectinomycin, florfenicol, linezolid and chloramphenicol were tested by PCR for presence of the respective resistance genes using the primers and conditions listed in Table 1. Primers used for 16S rDNA amplification were included in each PCR reaction as a control for DNA template integrity.

3. Results and discussion

3.1. CoNS species detected in dust and manure samples

From the 41 holdings tested a total of 344 CoNS isolates were obtained. On average, eight isolates were recovered per farm, and in 36 of the 41 farms LA-MRSA and/or MSSA were detected

Table 1
List of oligonucleotide sequences and conditions used for amplification of selected antimicrobial resistance genes.

Gene	Resistance phenotype	Oligonucleotide primer sequence	Annealing temp. Amplicon size	Reference
apmA	Apramycin	apmA-fw (5′-CGTTTGCTTCGTGC ATTAAA-3′)	60 °C	Feßler et al. (2011)
		apmA-rev (5′-TTGACACGAAGGAGGGTTTC-3′)	656 bp	
catpC194	Chloramphenicol	catpC194-F (5′-CGACTTTTAGTATAACCACAGA-3′)	55 °C	Schnellmann et al. (2006)
		catpC194-R (5′-GCCAGTCATTAGGCCTAT-3′)	570 bp	
catpC221	Chloramphenicol	catpC221-F (5′-ATTTATGCAATTATGGAAGTTG-3′)	50 °C	
		catpC221-R (5′-TGAAGCATGGTAACCATCAC-3′)	435 bp	
catpC223	Chloramphenicol	catpC223-F1 (5′-GAATCAAATGCTAGTTTTAACTC-3′)	50 °C	
		catpC223-R (5′-ACATGGTAACCATCACATAC-3′)	284 bp	
cfr	Phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A	cfr_for (5′-TGAAGTATAAAGCAGGTTGGGAGTCA-3′)	55 °C	Kehrenberg and Schwarz (2006)
		cfr_rev (5′-ACCATATAATTGACCACAAGCAGC-3′)	746 bp	
fexA	All phenicols	fexA_for (5′-GTACTTGTAGGTGCAATTACGGCTGA-3′)	60 °C	Kehrenberg and Schwarz (2006)
		fexA_rev (5′-CGCATCTGAGTAGGACATAGCGTC-3′)	1272 bp	
$mecA_{SCC}$	Oxacillin	mecA P4 (5′-TCCAGATTACAACTTCACCAGG-3′)	55 °C	Milheirico et al. (2007)
		mecA Re (5′-GTTCTGCAGTACCGGATTTGC-3′)	624 bp	This study
spc	Spectinomycin	aad9_for (5′-TGGAAGTTCAATAGTTGGAGTATATC-3′)	55 °C	Murphy (1985)
		aad9_rev (5′-CATCTTTCGAGGTAATTTCACCAG-3′)	544 bp	
spd	Spectinomycin	spd_for (5′-CATGAAAATGAAAATTGGTCTTATCC-3′)	52 °C	Jamrozy et al. (2014)
		spd_rev (5′-CCTGTTTCATAAGTTACGATC-3′)	317 bp	
spw	Spectinomycin	spw_for (5′-ACCATATAATTGACCACAAGCAGC-3′)	55 °C	Wendlandt et al. (2013)
		spw_rev (5′-CAGCCACCTCAGATTCCATT-3′)	630 bp	

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