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Penicillin resistance in bovine *Staphylococcus aureus*: Genomic evaluation of the discrepancy between phenotypic and molecular test methods

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ABSTRACT

Staphylococcus aureus is a major pathogen in humans and animals. In cattle, it is one of the most important agents of mastitis, causing serious costs in the dairy industry. Early diagnosis and adequate therapy are therefore 2 key factors to deal with the problems caused by this bacterium, and benzylpenicillin (penicillin) is usually the first choice to treat these infections. Unfortunately, penicillin resistance testing in bovine *S. aureus* strains shows discrepant results depending on the test used; consequently, the best method for assessing penicillin resistance is still unknown. The aim of this study was therefore to find a method that assesses penicillin resistance in *S. aureus* and to elucidate the mechanisms leading to the observed discrepancies. A total of 146 methicillin-sensitive *S. aureus* strains isolated from bovine mastitis were tested for penicillin resistance using a broth microdilution [minimum inhibitory concentration (MIC)] and 2 different disk diffusion protocols. Furthermore, the strains were analyzed for the presence of the *bla* operon genes (*blaI*, *blaR1*, *blaZ*) by PCR, and a subset of 45 strains was also subjected to whole genome sequencing (WGS). Discrepant results were obtained when penicillin resistance of bovine *S. aureus* was evaluated by disk diffusion, MIC, and PCR methods. The discrepancies, however, could be fully explained by WGS analysis. In fact, it turned out that penicillin resistance is highly dependent on the completeness of the *bla* operon promotor: when the *bla* operon was complete based on WGS analysis, all strains showed MIC $\geq$1 μg/mL, whereas when the *bla* operon was mutated (31-nucleotide deletion), they were penicillin sensitive except in those strains where an additional, *bla* operon-independent resistance mechanism was observed. Further, WGS analyses showed that penicillin resistance is truly assessed by the MIC assay. In contrast, caution is required when interpreting disk diffusion and PCR results.

Key words: *Staphylococcus aureus*, penicillin resistance, genomics, diagnostics, cattle

INTRODUCTION

The problem of antibiotic resistance in bacteria has increased at an alarming rate over recent decades, causing difficult-to-treat or even untreatable infections associated with high mortality rates (World Health Organization, 2014; CDC, 2019). To prevent the development of new resistances, the inappropriate use of antibiotics (**AB**) should be avoided. To achieve this, accurate diagnostics are crucial.

Antibiotics are used worldwide in both humans and animals for treating and preventing infectious diseases. Use and misuse of AB in either can cause the development of resistant bacteria strains.

Resistance to AB can be transmitted between strains and even species of bacteria, which in turn can be transferred to humans, animals, and the environment and circulate between them (World Health Organization, 2014; EFSA, 2015). Infections with resistant bacteria are a serious problem in health care settings, causing life-threatening infections such as bacteremia, pneumonia, and wound infections. In the United States alone, antimicrobial resistance (**AMR**) causes more than 23,000 deaths every year (CDC, 2019).

In veterinary medicine, mastitis is the leading cause of economic loss in dairy herds due to reduction in yield and quality of milk, treatment costs, and culling of animals because of treatment failure (Halasa et al., 2009; Peton and Le, 2014; Ruegg, 2017). In Switzerland, the disease results in total costs of about US$131 million per year (Heiniger et al., 2014). *Staphylococcus aureus*, together with *Escherichia coli* and *Streptococcus uberis*,

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is 1 of the 3 most important mastitis pathogens (Peton and Le, 2014). *Staphylococcus aureus* normally causes subclinical chronic mastitis in cows (Sears and McCarthy, 2003). In some cases, only a few cows are infected; in other cases, the majority of the herd is affected (Leuenberger et al., 2019). Genotyping of *S. aureus* by ribosomal spacer PCR (**RS-PCR**) as developed by Fournier et al. (2008) demonstrated that the rate of infected cows in a herd is highly dependent on the genotype. When *S. aureus* genotype B was isolated, up to 87% of cows in a herd were infected (Fournier et al., 2008; Graber et al., 2009; Cremonesi et al., 2015; van den Borne et al., 2017). In contrast, infections by genotype C, genotype S, or other genotypes were restricted to at most a few cows in a herd (Fournier et al., 2008; Graber et al., 2009; Cremonesi et al., 2015; Luini et al., 2015). The *S. aureus* genotype B was almost exclusively associated with clonal complex (**CC**) 8 when the strains were subtyped by multilocus sequence typing (**MLST**), whereas *spa* typing typically revealed t2953 (Boss et al., 2016). *Staphylococcus aureus* genotype C, however, was always t529, and in most cases it was CC705 (Boss et al., 2016). For all the other genotypes, the link between RS-PCR, MLST, and *spa* typing was less obvious, which is largely because the typing methods rely on different genetic information (Boss et al., 2016). Ribosomal spacer PCR is particularly suited for clinical application, as it is a high-throughput method that is cheap and comes with an analytical resolution for bovine strains that is at least as good as *spa* typing (Cremonesi et al., 2015; Boss et al., 2016). For subtyping at the biological level, however, MLST is more appropriate because it represents an *S. aureus* clone (Feil et al., 2003) and, as a consequence, its evolutionary identity (e.g., Kläui et al., 2019).

In Switzerland (Swiss Administration, 2018), Finland (Pyorala, 2009), and many other countries (EFSA, 2019), penicillin G (penicillin), also known as benzylpenicillin, is the most commonly used AB for treating IMI of cows caused by *S. aureus* and other gram-positive mastitis pathogens. In *S. aureus*, AMR against penicillin and all other β-lactamase-sensitive penicillin is encoded by the *bla* operon that can be located as a transposon on plasmids or on the chromosome (Lowy, 2003; Llarrull et al., 2011). The *bla* operon contains 3 genes (Clarke and Dyke, 2001; Llarrull et al., 2011): *blaI* encodes the repressor of the *bla* promoter inhibiting transcription of *blaR1* and *blaZ* in the absence of β-lactam AB; *blaR1* encodes the sensor for penicillin and other β-lactam AB and inactivates BlaI after AB binding; and *blaZ* encodes the β-lactamase that cleaves β-lactam AB by breaking down the β-lactam ring.

Previous studies showed marked discrepancies between the results of phenotypic resistance testing for penicillin and PCR results for the presence of *blaZ* gene in *S. aureus* isolates obtained from bovine mastitis: indeed, 40% of isolates carrying the *blaZ* gene were phenotypically susceptible to penicillin using the nitrocefin test (Haveri et al., 2005); furthermore, in the study by Russi et al. (2015), concordance between *blaZ*-PCR and phenotypic tests ranged between 87.5% (acidimetric test) and 93.0% [disk diffusion (**DD**)]. Although these concordance rates in the second study are better than in the first one, the discrepancy is still considerable. Even worse, the outcome is highly dependent on the method used. Taken together, penicillin resistance testing in bovine *S. aureus* strains appears to be highly unreliable, and the best method for assessing penicillin resistance is still unknown. As a consequence, this may lead to ineffective AB therapies and an increased risk of AMR.

The aim of this study was, therefore, to find the method that best assesses penicillin resistance in *S. aureus* and to elucidate the mechanisms that lead to the observed discrepancies. To this end, phenotypic and molecular methods including whole genome sequencing (**WGS**) were applied to a large number of *S. aureus* strains isolated from dairy cattle.

MATERIALS AND METHODS

Strains

Initially, 108 bovine strains of *S. aureus* were selected from our European strain collection, which includes 456 strains. The selection was made in a way that the distribution of CC types among strains was the same as observed for IMI in our European survey study (Boss et al., 2016). Accordingly, 37 strains of CC8, 33 of CC705, 18 of CC97, and 20 strains of other CC were selected. Within each CC, they were randomly chosen. Subsequently, an additional 41 CC8 strains were randomly selected for more detailed investigation, as the preliminary results frequently showed discrepancies among the different methods used to assess penicillin sensitivity.

These 149 strains all originated from different herds, located throughout Europe: Austria, Belgium, France, Germany, Ireland, Italy, Norway, Sweden, and Switzerland. They had all been isolated from aseptically collected milk samples of cows with IMI, having been sent in for diagnostic purposes. The strains were stored in skim milk at −20°C. Previous characterization included PCR for the *nuc* gene (highly specific for *S. aureus*; Brakstad et al., 1992; Graber et al., 2007), RS-PCR

Table 1. Primers used for amplification of the 3 *bla* genes by melting curve PCR[1]

Gene[2]	Primer	Sequence 5′–3′	Amplicon size, bp
blaI	GblaI-S	TCTATGGCTGAATGGGATGTTA	261
	GblaI-AS	CATGTCCCCTCCATACAGTTTA	
blaR1	GblaR1-S	TATCCATAAGTTTAATTGGGATTC	461
	GblaR1-AS	TATCGGCTTCTACTTCATTGT	
blaZ	GblaZ-S	AATTCAGATAAGAGATTTGCCTATG	374
	GblaZ-AS	CCGAAAGCAGCAGGTGTTGAAGT	

[1]Primers were designed based on the target sequence of the *Staphylococcus aureus* pLUH02 plasmid (FR714929).
[2]*blaI* = gene for repressor of *bla* promotor; *blaR1* = gene for sensor of penicillin and other β-lactam antibiotics; *blaZ* = β-lactamase gene.

for genotype and genotypic cluster (**CL**) attribution (Fournier et al., 2008; Cosandey et al., 2016), *spa* type, and MLST (CC attribution) as described (Boss et al., 2016). Genotypes and their variants of *S. aureus* were combined to genotypic CL. For genotype B and its variants, the resulting cluster was named CLB. Accordingly, other clusters such as CLC or CLR were obtained.

Bacterial Lysate Preparation

Strains were spread on sheep blood agar (Biomérieux Suisse SA) and aerobically incubated at 37°C for 18 h. DNA was obtained by the boiling method (Boss et al., 2016). In brief, a single colony was resuspended in 100 μL of 10 m*M* Tris-HCl (Merck) and 10 m*M* EDTA (pH = 8.5; Merck), incubated at 95°C for 10 min, and immediately placed on ice. Lysates were stored at −20°C. To be used as a PCR template, lysates were thawed and diluted 1:100 in H_2O.

Testing of Methicillin Susceptibility by PCR

All strains were tested for methicillin sensitivity by PCR using the commercial SureFast MRSA 4plex kit (Congen Biotechnologie GmbH). The assay is a real-time PCR for the direct, qualitative detection of *S. aureus*, of the *mecA* and *mecC* genes, and of the junction between the *orfX* gene and the staphylococcal cassette chromosome (SCCmec) carrying *mecA*/*mecC*. These genes code both for the penicillin binding protein (**PBP**) 2a, providing phenotypic resistance to β-lactam AB (Fergestad et al., 2020). The assay was performed according to the instructions of the manufacturer, using for each strain 5 μL of diluted lysate plus 20 μL of master mix (Congen). Real-time PCR was performed in a Mic qPCR Cycler (Bio Molecular Systems) using an initial denaturation of 95°C for 60 s, followed by 45 cycles with a profile of 95°C for 15 s and 60°C for 30 s. A strain giving positive quantitative PCR (**qPCR**) signals for *S. aureus*, the *orfX*/SCCmec junction, and the *mecA*/*mecC* gene was considered a methicillin-resistant *S. aureus*.

PCR Analysis of the bla Operon Genes

All strains were analyzed for the presence of *blaI*, *blaR1*, and *blaZ* of the *bla* operon using singleplex melting curve PCR (**mPCR**). Primers (Table 1) were designed based on the target sequence of the *S. aureus* pLUH02 plasmid (FR714929) using the OLIGO 6.53 software (Molecular Biology Insights Inc.). Primer synthesis was performed by Microsynth (Microsynth AG). The reactions were carried out in a total volume of 20 μL, containing KAPA SYBR Fast 2x (Merck), 300 n*M* of both primers, and 2.5 μL of diluted lysate as a template. The PCR amplifications were performed in a Rotor-Gene 6000 real-time thermal cycler (Corbett Life Science). For *blaI* as well as for *blaR1*, the following cycling protocol was used: an initial step of 95°C for 3 min, 35 cycles of 95°C for 3 s, 55°C for 30 s, 72°C for 2 s, and a final elongation of 55°C for 5 min. Afterward, melting of amplicons was performed from 55°C to 94°C with rising steps of 1°C and a 5-s waiting time at each step. For *blaZ*, amplification included an initial step of 95°C for 3 min, 35 cycles of 95°C for 3 s, 60°C for 30 s, and a final elongation of 60°C for 5 min. Melting of amplicons was performed from 60°C to 94°C with rising steps of 1°C and a 5-s waiting time at each step. Amplicons with a single melting peak identical to the positive control were considered as specific amplification: under our conditions, they were 76.2°C for *blaI*, 76.9°C for *blaR1*, and 77.4°C for *blaZ*. The DNA of *S. aureus* strains known to be positive or negative for all 3 *bla* genes were used as positive and negative controls, respectively.

MIC Assay

For the MIC assay, the broth microdilution method was used according to the protocols of the Clinical and Laboratory Standards Institute (**CLSI**; CLSI, 2018b, 2020). Specifically, in 96-well microtiter plates, crystalline penicillin (Merck) was diluted in a geometric 1:2 dilution series (from 8 μg/mL penicillin to 0.063 μg/mL) using freshly prepared, commercially avail-

able Ca^{++}- and Mg^{++}-adjusted Mueller-Hinton broth (Thermo Fisher Diagnostics AG). Preparation of the inoculum, inoculation, incubation (35°C, 18 ± 2 h), and visual determination of the MIC were performed according to CLSI protocols (CLSI, 2018b, 2020). As a reference, the strain *S. aureus* ATCC 29213 was used. The MIC method was chosen a priori as the reference for all phenotypic and mPCR methods because it is the standard method used in most reference laboratories in many countries (Tenover, 2019).

A MIC value greater than or equal to 0.25 μg/mL is considered resistant to penicillin, whereas strains with values less than or equal to 0.125 μg/mL (CLSI, 2020) require a negative induced β-lactamase test (see below) to confirm penicillin susceptibility (CLSI, 2020).

DD Assay

The DD was performed according to CLSI (**DDC**; CLSI, 2018a, 2020) and the European Committee on Antimicrobial Susceptibility Testing (**DDE**; EUCAST, 2022a,b) applying commercially available Mueller-Hinton agar plates (Thermo Fisher). The conditions for inoculum preparation and incubation (35 ± 1°C, 18 ± 2 h, aerobic) were the same for both methods. However, there was a difference in the penicillin content of the disks: the DDC protocol requires disks containing 10 IU of penicillin (Thermo Fisher), whereas 1 IU of penicillin is necessary for the DDE protocol. As reference strains, *S. aureus* ATCC 25923 was used for the DDC protocol, and *S. aureus* ATCC 29213 was used for the DDE protocol.

For both protocols, penicillin evaluations can be transferred to all β-lactamase-labile penicillins. According to the EUCAST guidelines (EUCAST, 2022b), plate reading includes evaluation of the zone diameter and of the zone edge: a fuzzy zone edge and a zone diameter greater than or equal to 26 mm is considered susceptible, whereas a sharp zone edge and a zone diameter greater than or equal to 26 mm or a diameter less than 26 mm is considered resistant. For the DDC, a zone diameter less than or equal to 28 mm is considered resistant to penicillin; for diameters greater than or equal to 29 mm, a negative induced β-lactamase test (see below) is required to confirm penicillin susceptibility (CLSI, 2018a, 2020).

Nitrocefin Test (Induced β-Lactamase)

The CLSI protocols (CLSI, 2018a, 2020) require a negative induced β-lactamase result to confirm sensitive MIC and DDC results. Therefore, a nitrocefin test was performed. Nitrocefin is a β-lactam molecule that changes color when hydrolyzed by a β-lactamase. Although only sensitive MIC and DDC results need to be confirmed, all strains were subjected to this test. For this purpose, the inoculum was spread on Mueller-Hinton agar, a cefoxitin disk (30 μg; Thermo Fisher) was added, and the plate was incubated aerobically for 17 ± 1 h at 35 ± 1°C. Using colonies from the zone margin surrounding the disk, a commercial nitrocefin test (Beta-Lactamase sticks, Thermo Fisher) was performed following the manufacturer's protocols. The strain *S. aureus* ATCC 29213 served as a positive control, and strain ATCC 25923 served as a negative control.

Whole Genome Sequencing

Strain Selection. Forty-five strains were selected for WGS. Twenty-nine of these strains were positive for CC8 and showed a positive result using *blaZ* mPCR. To rule out regional effects, they were selected from different herds that were spread all over Europe. The high number of CC8 was selected to better understand the considerable discrepancy between the molecular and phenotypic results (see below). In addition, all non-CC8 strains showing penicillin resistance by the MIC assay were included (n = 10). The CC of these strains were CC9, CC29 (each n = 1), CC97 (n = 6), and CC133 (n = 2). Additionally, 6 strains (1 CC20, 1 CC133, and 4 CC705) were selected as negative controls that were all penicillin sensitive and negative for *blaZ* by mPCR.

DNA Extraction. Three to 4 colonies picked from blood agar were resuspended in 4.5 mL TSB (Tryptic Soy Broth; Thermo Fisher) and incubated aerobically at 37 ± 1°C for 18 ± 2 h at 140 rpm. One mL of this culture was subsequently added to 500 mL TSB in a centrifuge bottle and incubated using the previous conditions. Then, the culture was centrifuged (4,600 × *g*, 4°C, 25 min), the supernatant discarded, and the pellet resuspended in 15 mL of 10 m*M* Tris/HCl (pH 7.8) buffer and transferred to a 50-mL tube. After recentrifugation (18,000 × *g*, 4°C, 5 min), the supernatant was discarded, and the pellet resuspended in 2 mL RES buffer of the NucleoBond Xtra Maxi Kit (Macherey-Nagel AG). The suspension (1.5 mL) was transferred to a 2-mL Eppendorf tube containing 350 mg of glass beads (bead size 212 to 300 μm; Merck). The cells were subsequently lysed using a Bead Ruptor Elite (Omni International) at intensity level 6 for 45 s. After centrifugation (13,500 × *g*, 4°C, 5 min), the supernatant was transferred to a 100-mL glass bottle containing 22 mL of RES and 24 mL of LYS buffer (Macherey-Nagel). Then DNA extraction was performed using the NucleoBond Xtra Maxi Kit (Macherey-Nagel) according to the manufacturer's protocol. The resulting pellet was dissolved in 200 μL of H_2O and the DNA repurified for maximum purity using the High Pure PCR Template

Preparation Kit (Roche). Elution was performed using 200 μL of elution buffer. Quality and total amount of extracted DNA were evaluated by spectroscopy using optical density (**OD**) ratio of OD_{260}/OD_{280} (QuickDrop; Molecular Devices) and by Qubit assay (Thermo Fisher), respectively.

This extraction procedure resulted in enrichment of plasmid DNA whereby, as desired, substantial amounts of chromosomal DNA were copurified. The molecular ratio between plasmid DNA and chromosomal DNA was approximately 1.5 for *S. aureus* genotype B when chromosomal DNA was quantified by lukEB qPCR and plasmid DNA by qPCR for *sed* (Boss et al., 2011).

WGS and Assembly of the Sequence Reads. The DNA samples were sent to Eurofins Genomics GmbH for WGS using the HiSeq sequencing platform (Illumina), guaranteeing at least 1.5 gigabytes of reads for each sequenced strain. A high number of reads was wanted to verify the nucleotide at each position of the genome as consistently as possible (high site coverage) and to avoid assembly interruption because of missing reads. To enrich *bla* operon-specific reads of each strain in silico, total reads were first assembled against the chromosome of *S. aureus* NCTC 8325 (NC_007795; devoid of *bla* operon containing transposons) using the SeqMan NGen 16 software (default settings) included in the DNASTAR Lasergene 16 software package (DNASTAR Inc.). The remaining unassembled reads of the query strain (containing plasmids, transposons) were then de novo assembled with the SeqMan NGen 16 software (DNASTAR), deactivating the "repeat handling" option in the software settings, selecting a minimum match for overlapping read segments of 93%, and selecting contigs with lengths greater than 1,000 nucleotides. The contigs were then screened for the presence of the *bla* operon using a blast-like algorithm of the Clone Manager 9.51 software (CM9; Sci Ed Software) and the *bla* operon on plasmid SAP047A as a reference.

In most cases, the *bla* operon was found on only 1 contig. In 5 CC8 strains, however, hits for the *bla* operon were found on 2 different contigs. For 1 of these contigs, alignments against the reference *bla* operon (SAP047A) always resulted in a similarity greater than 98%, whereas the similarity for the other contig was always less than or equal to 93%. Blasting one of these new, lower matching contigs against the NCBI nr database (https://blast.ncbi.nlm.nih.gov; Altschul et al., 1990) resulted in a 95% match with a transposon (Tn13616) and its *bla* operon present on the chromosome of the *S. aureus* strain NCTC 13616 (NZ_LR134193). The Tn13616 transposon was subsequently used as an assembly reference to obtain the complete transposon for these 5 CC8 strains using the SeqMan NGen 16 software (DNASTAR) and a minimum match for overlapping read segments set to 99%. Using this software setting, only those reads were selected that were highly specific for the new transposon.

In Silico Analysis of Plasmids

All contigs were tested for representing a plasmid using PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder; Carattoli et al., 2014). For naming, the new plasmids were blasted (https://blast.ncbi.nlm.nih.gov) against the NCBI nr (https://ncbi.nlm.nih.gov) database to look for the *bla* operon with which the unknown operon fits best in terms of highest query coverage and similarity. If there were several plasmids with identical coverage and similarity values, the best annotated one was selected (= reference plasmid) and the name was kept for all new plasmids, if coverage and similarity values were closest to the reference plasmid. The newly detected plasmids were further annotated using the Rapid Annotation using Subsystem Technology Server (https://rast.nmpdr.org; Overbeek et al., 2014).

Previous studies had shown that plasmid-based enterotoxin genes *sed*, *sej*, and *ser* (Benkerroum, 2018) can be present in bovine *S. aureus* strains (Fournier et al., 2008; Graber et al., 2009; Hummerjohann et al., 2014; Cosandey et al., 2016). As Rapid Annotation using Subsystem Technology is inconclusive for enterotoxin genes, the plasmids were further compared with our reference library for the staphylococcal enterotoxin genes *sea-sex* and *tst* using the Needleman-Wunsch algorithm (CM9, Sci Ed). The library was created based on data published by Merda et al. (2020).

In Silico Analysis of the bla Operon

The *bla* operon of plasmids and transposons were subsequently precisely located by nucleotide sequence alignment using the Needleman-Wunsch algorithm (CM9, Sci Ed) and the *bla* operon of the SAP047A plasmid as a reference. Afterward, within each operon, the 3 genes *blaI*, *blaR1*, and *blaZ* were translated in silico into their corresponding proteins using the standard genetic code and the CM9 software (Sci Ed). The translated AA sequences were checked for full length by aligning them to the Uniprot reference sequences for BlaI (P0A042), BlaR1 (P18357), and BlaZ (P00807), respectively. The BlaI protein was then checked for the presence of the AA Asn at position 101 (Asn101) and Phe102, which form the proteolytic cleavage site for BlaR1 (Zhang et al., 2001). BlaR1 was tested for the presence of Ser389 and Lys392, which are critical for sensing β-lactam AB (Zhang et al., 2001), as well as for

the presence of His201 and Glu202, which are key in activating the proteolytic domain of the protein (Zhang et al., 2001). BlaZ was checked for Ser70, which forms the active site for cleaving β-lactam AB (Chen et al., 1996). Furthermore, based on preceding analyses using the Uniprot reference sequences (P0A042, P18357, and P00807), the newly detected BlaI had to best match with the functional family (**FunFam**) 266241 of the CATH/Gene3D v4.3 database (http://www.cathdb.info). In the case of BlaR1 and BlaZ, the fit had to be best with FunFam 21251 and 12260, respectively (http://www.cathdb.info). Only if a protein fulfilled all the mentioned criteria (full length, presence of all key AA, FunFam) was it considered to be functional. According to Dawson et al. (2017), proteins with the same FunFam—that is, with the same 3-dimensional protein domain structure together with its specific AA sequence forming this domain—share the same biochemical function with high probability.

All in silico analyses were performed a priori—that is, without being aware of the phenotypic and mPCR tests results.

Statistics

Statistical analyses were performed using the Systat 13.1 software (Systat Software). Data were expressed as frequencies, percentage, or median, minimum, and maximum. To investigate the agreement between MIC (reference) and DDE, DDC, and mPCR, respectively, the following parameter were calculated: sensitivity (= fraction of penicillin-resistant or *blaZ*-positive strains), specificity (= fraction of penicillin-susceptible or *blaZ*-negative strains), and Cohen's kappa. The 95% confidence interval (**CI95**) for proportions was estimated by the Wilson score interval (Wilson, 1927) using R 4.0.3 (R Core Team, 2020) together with the "binom" library. Differences in penicillin resistance among CC or CL were calculated using Fisher's exact test for 2 × 2 contingency tables or a generalized version of the test for k × m tables as implemented in Systat 13.1 (Systat). For comparison of 2 methods (paired samples), McNemar's test for symmetry was applied (Systat).

RESULTS

Analysis of Methicillin-Sensitive S. aureus Strains

Out of the initial 149 selected *S. aureus* strains, 146 turned out to be methicillin-sensitive *S. aureus* (**MSSA**); 1 CC97 and 2 CC398 strains were resistant (methicillin-resistant *S. aureus*) and were excluded from the present study.

MIC Assay

In total, 59 (40%) out of the 146 MSSA were penicillin resistant, 87 (60%) were penicillin sensitive (MIC values ≤ 0.125 μg/mL) (Table 2 and Supplemental Table S1, https://data.mendeley.com/datasets/9hvs8s76gy/1, Ivanovic et al., 2022). The MIC50 value was 0.063 μg/mL (50% of the strains showed a MIC value less than or equal to 0.063 μg/mL), and the MIC90 value was 4 μg/mL. For the major CC (CC8, CC97, CC705), penicillin resistance was highly CC dependent ($P < 0.001$; Table 3). In fact, all CC705 strains (n = 33) were penicillin susceptible, whereas 41 of 78 (53%) CC8 strains and 10 of 16 (63%) CC97 strains were penicillin resistant. For all the remaining, low-abundance CC, there were both penicillin-sensitive and penicillin-resistant strains (Table 2 and Supplemental Table S1).

Further analyses revealed that penicillin resistance of the major CL was also highly CL dependent ($P < 0.001$; Table 3): for CLB (n = 75), CLC (n = 33), CLF (n = 7), CLI (n = 7), and CLR (n = 11), 53%, 0%, 57%, 100%, and 36% of the strains were penicillin resistant, respectively. For the 13 remaining strains of other CL, they were both penicillin sensitive and resistant (Table 3).

Disk Diffusion Assays and PCR Analysis

Disk diffusion analysis using the DDC protocol revealed 40 out of 146 (27%) strains that were penicillin resistant (Table 2 and Supplemental Table S1). For the DDE protocol, 82 strains (56%) were penicillin resistant, whereas for PCR, 87 (60%) *S. aureus* strains were positive for *blaZ* (Table 2 and Supplemental Table S1). Importantly, all strains were either positive for all 3 *bla* operon genes (*blaI*, *blaR1*, and *blaZ*) or negative for all of them (Table 2 and Supplemental Table S1). The DDC results clearly differed from those obtained by DDE or PCR (for each $P < 0.001$). In addition, a minor difference was observed between the DDE and the PCR assays ($P = 0.025$). For all 3 methods (DDC, DDE, PCR for *blaZ*), the results were highly CC and CL dependent (for each method $P < 0.001$). In fact, all CC705/CLC strains were always penicillin susceptible and always negative by PCR for *blaZ*, respectively. Regarding the other CC and CL, the results were variable (Table 2 and Supplemental Table S1).

Agreement Among Different AMR Testing Methods

Using the MIC method as the reference, the sensitivity (= fraction of penicillin-resistant/*blaZ*-positive strains) for the DDC protocol including all analyzed strains was

Table 2. Penicillin resistance testing of 146 methicillin-sensitive *Staphylococcus aureus* isolated from bovine IMI in 9 European countries[1]

n	Clonal complex (CC)	Genotypic cluster[2] (CL)	*blaI*, *blaR1*, *blaZ* mPCR[3]	DDC[4]	DDE[4]	MIC assay	MIC, μg/mL	Nitrocefin test
13	CC8	CLB	pos.	s	r	s	<0.063	neg.
8	CC8	CLB	neg.	s	s	s	<0.063	neg.
5	CC8	CLB	pos.	s	s	s	<0.063	neg.
1	CC8	Other	neg.	s	s	s	<0.063	neg.
1	CC8	Other	pos.	s	r	s	<0.063	neg.
1	CC8	CLB	neg.	s	s	s	0.063	neg.
5	CC8	CLB	pos.	s	r	s	0.125	neg.
3	CC8	CLB	pos.	s	r	s	0.125	neg.
17	CC8	CLB	pos.	s	r	r	0.250	neg.
1	CC8	Other	pos.	s	r	r	0.250	neg.
1	CC8	CLB	pos.	r	r	r	0.250	neg.
5	CC8	CLB	pos.	r	r	r	1.000	pos.
1	CC8	CLB	pos.	s	r	r	1.000	neg.
2	CC8	CLB	pos.	r	r	r	2.000	pos.
10	CC8	CLB	pos.	r	r	r	4.000	pos.
4	CC8	CLB	pos.	r	r	r	8.000	pos.
32	CC705	CLC	neg.	s	s	s	<0.063	neg.
1	CC705	CLC	neg.	s	s	s	0.125	neg.
3	CC479	Other	neg.	s	s	s	≤0.125	neg.
1	CC133	CLR	neg.	s	s	s	≤0.125	neg.
3	CC133	Other	neg.	s	s	s	≤0.125	neg.
2	CC133	CLR	pos.	r	r	r	≥4.000	pos.
5	CC97	CLR	neg.	s	s	s	≤0.125	neg.
1	CC97	Other	neg.	s	s	s	≤0.125	neg.
6	CC97	CLI	pos.	r	r	r	≥1.000	pos.
2	CC97	CLR	pos.	r	r	r	≥1.000	pos.
2	CC97	Other	pos.	r	r	r	≥1.000	pos.
1	CC20	CLF	neg.	s	s	s	0.125	neg.
1	CC20	CLF	pos.	s	r	s	0.125	neg.
1	CC20	CLF	pos.	r	r	r	0.500	pos.
1	CC9	CLF	neg.	s	s	s	<0.063	neg.
2	CC9	CLF	pos.	r	r	r	≥1.000	pos.
1	CC71	CLR	neg.	s	s	s	<0.063	neg.
3	div	div	pos.	r	r	r	≥4.000	pos.

[1]Comparison of PCR for 3 *bla* operon genes (*blaI*, *blaR1*, and *blaZ*), disk diffusion test according to Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST), and broth microdilution according to CLSI in order to assess MIC. The nitrocefin test was required by CLSI to confirm penicillin susceptibility. Full table is added as Supplemental Table S1 (https://data.mendeley.com/datasets/9hvs8s76gy/1; Ivanovic et al., 2022).

[2]Genotypes and their variants of *S. aureus* were combined to CL, resulting in the clusters CLB, CLC, CLF, CLI, and CLR.

[3]mPCR = melting curve PCR for *blaI* gene (repressor for promotor), *blaR1* gene (penicillin sensor), and *blaZ* gene (β-lactamase); pos. = positive, neg. = negative. PCR was always either positive for all 3 *bla* genes or negative for all of them.

[4]DDC = disk diffusion test for penicillin according to the CLSI protocol; DDE = disk diffusion test for penicillin according to the EUCAST protocol; r = resistant to penicillin, s = susceptible to penicillin.

68% (CI95 = 55% to 78%), the specificity (= fraction of penicillin-susceptible/*blaZ*-negative strains) was 100% (CI95 = 96% to 100%), and the kappa value was 0.715 ($P < 0.001$; Table 4). For the DDE protocol, the sensitivity was 100% (CI95 = 94% to 100%), the specificity was 74% (CI95 = 64% to 82%), and the kappa value was 0.692 ($P < 0.001$). Comparing the MIC with the mPCR method (n = 146), the overall sensitivity was 100% (CI95 = 94% to 100%), the specificity was 68% (CI95 = 57% to 77%), and the kappa value was 0.630 ($P < 0.001$) (Table 4).

If the CC8 strains were excluded, the following values were obtained when the MIC method served again as the reference (Table 4): for the DDC protocol, the sensitivity, specificity, and kappa value for the 68 strains were 100% (CI95 = 82% to 100%), 100% (CI95 = 93% to 100%), and 1.0 ($P < 0.001$), respectively. For the DDE protocol, the sensitivity was 100% (CI95 = 82% to 100%), the specificity was 98% (CI95 = 90% to 100%), and the kappa value was 0.963 ($P < 0.001$). For the mPCR method (CC8 excluded, n = 68), the sensitivity, specificity, and kappa value were 100% (CI95 = 85% to 100%), 98% (CI95 = 90% to 100%), and 0.963 ($P < 0.001$), respectively (Table 4).

Including all strains, negative mPCR results (n = 59) for all *bla* operon genes were linked to a MIC value less than or equal to 0.125 μg/mL, indicating penicillin susceptibility, with 53 isolates having a MIC less than 0.063 μg/mL, 1 isolate 0.063 μg/mL, and 5 isolates 0.125 μg/mL. If the CC8 strains were excluded, all the

Table 3. Distribution of MIC for penicillin among clonal complexes (by multilocus sequence typing) and genotypic clusters (by ribosomal spacer PCR) of 146 bovine *Staphylococcus aureus*[1]

		MIC assay, n (%)	
Classification	n of strains	Resistant	Susceptible
Clonal complex (CC)			
CC8	78	41 (53)	37 (47)
CC705	33	0 (0)	33 (100)
CC97	16	10 (63)	6 (38)
CC133	6	2 (33)	4 (67)
CC479	3	0 (0)	3 (100)
CC20	3	1 (33)	2 (67)
CC9	3	2 (67)	1 (33)
Others	4	3	1
Genotypic cluster (CL)			
CLB	75	40 (53)	35 (47)
CLC	33	0 (0)	33 (100)
CLF	7	4 (57)	3 (43)
CLI	7	7 (100)	0 (0)
CLR	11	4 (36)	7 (64)
Others	13	4	9

[1]MIC was assessed by broth microdilution susceptibility testing according to the Clinical and Laboratory Standards Institute protocol. Genotypes and their variants of *S. aureus* were combined to CL, resulting in the clusters CLB, CLC, CLF, CLI, and CLR.

remaining 19 PCR-positive strains except 1 showed MIC values from 0.5 to 8 μg/mL, indicating penicillin resistance (Table 2 and Supplemental Table S1).

Plasmid Analyses

The sequencing details and assembly statistics of strains analyzed by WGS are listed in Supplemental Table S2 (https://data.mendeley.com/datasets/9hvs8s76gy/1, Ivanovic et al., 2022).

Using WGS, a total of 28 plasmids were detected. Out of these, 27 originated from *S. aureus* CC8 and 1 from CC97 (Table 5). All plasmids isolated from CC8 and CC97 matched best with the pSK67 plasmid (NC_019010) with a query coverage ranging between 99% and 100% and similarities between 99.85% and 100%. The plasmid length ranged between 27,266 bp and 28,827 bp with a median of 27,735 bp being close to the length of pSK67 (27,439 bp). All the pSK67 plasmids carried the *repA* gene, 5 genes coding for plasmid replication proteins, together with the *acuI*, *cadC*, and *cadD* genes, as well as the enterotoxin genes *sed*, *sej*, and *ser*. Rapid Annotation using Subsystem Technology of the plasmids further revealed 3 genes with assigned FIG numbers (Figure 01109056, Figure 01109057, Figure 01109060) and 19 genes coding for hypothetical proteins.

Analysis of the bla Operon

A total of 45 strains were studied by WGS for *bla* operon analysis. In 23 strains, the operon was located on a plasmid, in 11 strains on the chromosome, and in 5 strains there was an operon on both, the chromosome and a plasmid. For the 6 randomly selected penicillin-sensitive strains, all negative for *blaI*, *blaR1*, and *blaZ* by mPCR, no *bla* operon could be detected. The operon structure was always the same (5′-3′): *blaI* (antisense) – *blaR1* (antisense) – promoter – *blaZ* (sense); see Figure 1.

All 45 BlaI proteins appeared complete and functional (Table 5). The same was true for 42 BlaR1 and 43 BlaZ proteins. Putative nonfunctionality was caused by frame shifts due to a deleted adenine nucleotide (**A**) within a poly A repeat, leading to a premature translational stop (Table 5). The overall AA similarity of BlaI compared with the appropriate reference protein ranged between 99.2% and 100% (Table 5). For functional BlaR1 (n = 42), the similarity was between

Table 4. Sensitivity and specificity of analytical techniques used in comparison to MIC analysis as the reference method to evaluation penicillin resistance in bovine *Staphylococcus aureus*[1]

	Sensitivity		Specificity		Cohen's kappa
Method	%	CI_{95}, %	%	CI_{95}, %	$P < 0.001$
All strains included (n = 146)					
DDC	68	55–78	100	96–100	0.715
DDE	100	94–100	74	64–82	0.692
mPCR	100	94–100	68	57–77	0.630
CC8 strains excluded (n = 68)					
DDC	100	82–100	100	93–100	1.000
DDE	100	82–100	98	90–100	0.963
mPCR	100	85–100	98	90–100	0.963

[1]DDC = disk diffusion test for penicillin according to the Clinical and Laboratory Standards Institute protocol; DDE = disk diffusion test for penicillin according to the European Committee on Antimicrobial Susceptibility Testing protocol; mPCR = melting curve PCR for *blaI* gene (repressor for promotor), *blaR1* gene (penicillin sensor), and *blaZ* gene (β-lactamase); CC8 = *S. aureus* strain of clonal cluster CC8 (multilocus sequence typing); CI_{95} = confidence interval 95%.

Table 5. Methicillin-sensitive strains of *Staphylococcus aureus*: genomic and phenotypic characterization of their penicillin resistance by whole genome sequencing

n	Clonal complex (CC)	Location	*bla* operon type	*bla* promoter structure	Similarity,[1] %			MIC,[2] μg/mL
					BlaI	BlaR1	BlaZ	
10	CC8	Plasmid	pSK67 (short)	Mutated	100.0	100.0	100.0	<0.063
1	CC8	Plasmid	pSK67 (short)	Mutated	100.0	Premature stop	100.0	<0.063
6	CC8	Plasmid	pSK67 (short)	Mutated	100.0	100.0	100.0	0.125
4	CC8	Plasmid	pSK67 (short)	Mutated	100.0	100.0	100.0	0.250
1	CC8	Plasmid	pSK67 (short)	Mutated	100.0	Premature stop	100.0	
		Chromosome	TnO217	Complete	100.0	99.7	99.6	1.000
1	CC8	Plasmid	pSK67 (short)	Mutated	100.0	100.0	100.0	
		Chromosome	TnO217	Complete	100.0	99.3	99.6	1.000
1	CC8	Plasmid	pSK67 (short)	Mutated	100.0	100.0	100.0	
		Chromosome	TnO217	Complete	100.0	100.0	100.0	2.000
1	CC8	Plasmid	pSK67 (short)	Mutated	100.0	100.0	100.0	
		Chromosome	TnO217	Complete	99.2	99.5	99.6	8.000
1	CC8	Plasmid	pSK67 (short)	Mutated	100.0	100.0	Premature stop	
		Chromosome	TnO217	Complete	100.0	99.5	99.6	1.000
2	CC8	Chromosome	TnO217	Complete	100.0	100.0	99.6	4.000
1	CC8	Plasmid	pSK67 (WT)	Complete	100.0	100.0	100.0	4.000
1	CC97	Chromosome	TnO217	Complete	100.0	99.8	100.0	1.000
2	CC97	Chromosome	TnO217	Complete	100.0	99.8	99.6	1.000
1	CC97	Chromosome	TnO217	Complete	100.0	99.8	99.6	2.000
1	CC97	Chromosome	TnO217	Complete	100.0	100.0	100.0	2.000
1	CC97	Plasmid	pSK67 (WT)	Complete	100.0	100.0	100.0	2.000
1	CC9	Chromosome	TnO217	Complete	99.2	98.1	98.9	1.000
1	CC29	Chromosome	TnO217	Complete	100.0	100.0	100.0	4.000
2	CC133	Chromosome	TnO217	Complete	100.0	100.0	100.0	4.000
1[2]	CC133	—	None	—	—	—	—	<0.063
3[2]	CC705	—	None	—	—	—	—	<0.063
1[2]	CC705	—	None	—	—	—	—	0.125
1[2]	CC20	—	None	—	—	—	—	0.125

[1]Sequence similarity compared with corresponding protein of the indicated *bla* operon type. Premature stop = abortion of translation because of a frame shift, leading to a nonfunctional protein.

[2]Strain that served as control, as it was known not to host a *bla* operon.

98.1% and 100%; for functional BlaZ (n = 43) it was between 98.9% and 100%. For all the analyzed proteins with a similarity less than 100%, the observed mutations never affected the key AA that were required for correct protein functioning.

The plasmid-located operons always belonged to 1 of 2 forms: the wild-type form as present on the original pSK67 plasmid with a length of 3,080 bp and a shorter version with a length of 3,049 bp (Table 5). All short operons lacked a 31-nucleotide fragment that was in its entirety located in the promoter region (Figure 1). The deletion that started at bp 2,175 and ended at bp 2,205 of the pSK67 *bla* operon wild-type sequence resulted in a mutated *bla* promoter. Indeed, it lacked almost the complete *blaR1* dyad, the complete Pribnow box for *blaZ*, the transcription starting point for the *blaZ* mRNA, and the beginning of the *blaZ* dyad (Figure 1). The mutated form of the *bla* promoter was exclusively observed in *S. aureus* CC8 strains. In all other CC, the operon, if present, showed the wild-type form (Table 5). The chromosomal *bla* operons were all located on a transposon identical to the one of *S. aureus* O217 (CP038461; TnO217) (Table 5). Comparing pSK67 and TnO217, the nucleotide similarity for the *bla* operon was 93.3%; the AA similarity for BlaI, BlaR1, and BlaZ was 96.1%, 88.1%, and 91.1%, respectively.

If at least 1 operon of a strain was complete, the corresponding MIC values for penicillin were always greater than or equal to 1.0 μg/mL, independent of whether the operon was located on the pSK67 plasmid or on the chromosomal TnO217 transposon (Table 5). If only a mutated *bla* promoter was present, the MIC values were always less than or equal to 0.25 μg/mL, independent of the presence of functional Bla proteins or the genomic location of the *bla* operon (Table 5).

Nitrocefin Test

To confirm the DDC results, strains were subjected to a nitrocefin test (Table 2 and Supplemental Table S1). All 106 strains that tested sensitive for penicillin by DDC showed a negative nitrocefin test, indicating that no β-lactamase was induced. All except 1 of the resistant strains showed a positive nitrocefin test.

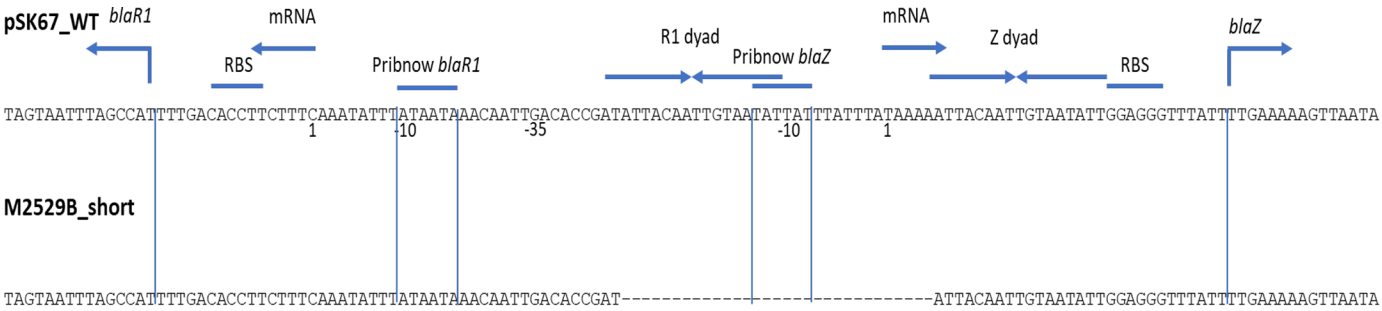

Figure 1. The sequence of the *bla* operon between the start sites for translation of BlaZ and BlaR1 is shown for a wild-type (WT) and a short, mutated *bla* promoter. The annotation of the WT form was performed according to Clarke and Dyke (2001). The transcription starting sites for *blaR1* and *blaZ* begin at position 1 and are labeled "mRNA." The start of each coding sequence is presented as an angled arrow. The Pribnow boxes at positions −10 and the ribosomal binding sites (RBS) for both genes are indicated. The R1 dyad and Z dyad with their inverted repeats are marked by 2 opposed arrows. The dyads serve as the binding sites for BlaI, which blocks the transcription of *blaR1* and *blaZ* (Clarke and Dyke, 2001).

DISCUSSION

The present study shows that discrepancies among different phenotypic and PCR methods for testing penicillin resistance in bovine MSSA can be fully explained by the combination of phenotypic, PCR, and WGS analyses. In particular, these examinations demonstrated that the genetic structure of the *bla* operon was highly associated with the level of the MIC values and with penicillin resistance. Compared with the MIC assay, however, DDC, DDE, or mPCR for the 3 *bla* genes revealed either inappropriate sensitivities or specificities.

Technical Aspects

The success of phenotypic penicillin resistance prediction by genomic methods was based on the following steps: (1) proven in vitro knowledge about the genes and their regulation in penicillin AMR; (2) reliable detection of the plasmid carrying penicillin resistance; (3) high number of WGS reads; and (4) bioinformatic protein and promoter analysis. As for (1), this knowledge is given by previous studies (Rowland and Dyke, 1989; Lewis et al., 1999; Clarke and Dyke, 2001; Lowy, 2003) and is key to link in silico data to a phenotypic outcome. As for (2), reliable detection of plasmids by WGS is essential to rule out false-negative results when compared with phenotypic methods. To do so, we used a kit dedicated to plasmid DNA extraction (Macherey-Nagel). As chromosomal DNA is only partially removed by these methods, it is copurified with the plasmid DNA, resulting in a molecular chromosome-to-plasmid ratio of approximately 1:2. As for (3), the present WGS approach using 1 flow cell per strain (Illumina) was selected because it generates a high number of reads so that reads of low abundance are also sufficiently represented. This procedure avoids, therefore, assembly interruptions in target regions that are only covered by a few or no reads if reads are not evenly distributed among the targets. A further advantage is that a high site coverage was obtained, enabling unambiguous statements about mutations and the genetic structure of the *bla* operon. And as for (4), phenotypic penicillin resistance in an *S. aureus* strain is only present if, in addition to the presence of all the *bla* operon genes, the proteins BlaI, BlaR1, and BlaZ are expressed and functional. To address these questions, the 3-dimensional structures of these proteins after in silico translation were analyzed using CATH/Gene3D v4.3 (http://www.cathdb.info) and their functionally relevant sites checked manually. Furthermore, the *bla* promoter was investigated by bioinformatic methods.

Importance of the bla Operon and bla Promoter on Phenotypic Penicillin Resistance

Bioinformatic promoter analysis showed a mutated promotor to be present in most CC8 strains (Table 5): it lacked almost the entire *blaR1* dyad, the entire Pribnow box for *blaZ*, the transcription starting point for the *blaZ* mRNA, and the beginning of the *blaZ* dyad (Figure 1). The mutated operon was always located on the pSK67 plasmid and never on the TnO217 transposon.

The present study demonstrates that the deletion within the *bla* promoter plays a crucial role in phenotypic penicillin resistance in *S. aureus*, as expected from current knowledge (Clarke and Dyke, 2001; Lowy, 2003; Llarrull et al., 2011): if the promoter was present and complete, the MIC values were always high (≥1.0 μg/mL; Table 5), whereas if the mutated promoter alone was present, the values were always low (≤0.25 μg/mL; Table 5). High MIC values were independent of

the promoter's localization (chromosome or plasmid) and were not affected by the minor operon changes as observed between complete pSK67 and TnO217. That the *bla* operon with its promoter is key for phenotypic penicillin resistance in *S. aureus* is also shown by the fact that, if the strains were negative for the *bla* genes, the MIC values were always less than or equal to 0.125 μg/mL.

The present study does not allow locating the precise elements within the promoter deletion that account for the low penicillin MIC values. Perhaps they are associated with the missing Pribnow box for *blaZ*, which is known to be vital for efficient transcription (Pribnow, 1975). To answer this question, a separate promoter study is needed that constructs transcriptional fusions between various *blaZ* and *blaR1* promoter regions and a reporter gene (Sadykov et al., 2019). These analyses will then allow a dynamic inspection of the fusion activities and discovery of the relevant promoter elements.

Bla Operon Diversity and Its Consequences

According to the present study, only 2 different genetic elements of the *bla* operon were observed in bovine *S. aureus* strains: the pSK67 plasmid and the transposon TnO217. Furthermore, with an overall similarity of 93.3% at the nucleotide level, the diversity of the *bla* operons was small. These results are in clear contrast to those by Olsen et al. (2006) suggesting a much higher diversity. The reason for this discrepancy may be that in the previous study, the Sanger method and overlapping amplicons were used to sequence part of *blaZ* and *blaR1*, whereas in the present study, the complete *bla* operon was sequenced by WGS, choosing a high site coverage. This allowed erroneous sequencing results to be reduced to a minimum. Other reasons such as restriction to only a few dairy herds or countries can be ruled out, as all our isolates analyzed by WGS were obtained from different herds that were located in various European countries.

The small number of *bla*-carrying genetic elements is unexpected. Apparently, bovine strains of *S. aureus* are very selective in their acquisition of these mobile elements, although a less restricted handling might have been beneficial for their survival. Even more striking is the fact that in most strains harboring the pSK67 plasmid, the *bla* promoter was mutated, resulting in penicillin susceptibility or resistance at low penicillin concentration (0.25 μg/mL). These findings indicate that there are more important mechanisms than penicillin resistance involved in enabling the survival of *S. aureus* in the udder. One mechanism might be the internalization of *S. aureus* into bovine mammary epithelial cells (Caldeira et al., 2019; Frutis-Murillo et al., 2019; Geng et al., 2020), which not only facilitates their replication (Wang et al., 2019) but may also protect them from being attacked by penicillin. Intracellular survival is enabled by the staphylococcal property to block the fusion of the phagosome with the lysosomes (Neumann et al., 2016; Geng et al., 2020), creating a safe environment for the pathogen.

Diagnostic Importance

For a patient infected by *S. aureus* and other bacteria, it is vital that an effective AB is used to curb the infection. It is, therefore, essential that the isolate is tested for AMR to select an appropriate drug. To do so, various methods are currently used, including MIC assay, DD, PCR, and in rare cases WGS. The present study shows, however, that for *S. aureus* and penicillin not all of these tests produce equally reliable results. The study also shows that these discrepancies can be fully explained by the combination of phenotypic, PCR, and genomics methods.

As WGS demonstrated (Table 5), all strains with at least 1 functional *bla* operon were resistant to penicillin, with MIC values always greater than or equal to 1 μg/mL, whereas all strains except 4 showing a mutated *bla* operon were penicillin sensitive. The 4 exceptional CC8 strains all exhibited MIC values of 0.25 μg/mL. On the other hand, all strains negative for the *bla* genes by mPCR showed MIC values of less than or equal to 0.125 μg/mL (Table 2 and Supplemental Table S1). Taken together, these observations indicate that MIC values equal to 0.25 μg/mL are most likely the result of an additional, *bla* operon- and PBP2a-independent, low-level mechanism for penicillin resistance. A possible candidate responsible for this kind of resistance is PBP4. In fact, this protein has recently been shown to provide resistance to the entire class of β-lactam AB (da Costa et al., 2018), particularly if its expression was increased (Basuino et al., 2018). Own genomic analyses using all 45 WGS strains (data not shown) and the *PBP4* gene of *S. aureus* N315 (NC_002745) demonstrated that the gene was present in all strains. With a median of 99.7% (minimum = 98.6%, maximum = 100%), the similarities at the nucleotide level were all very high. For CC8/CLB, all strains except 1 (5 mutations) showed 4 mutations, always at the same sites and not affecting the active and β-lactam binding sites of PBP4. Based on these observations, PBP4 hardly accounts for the low-level mechanism for penicillin resistance. Further in vitro and WGS studies, however, are required to elucidate the role of PBP4 and possibly other proteins.

Based on our analyses, the true percentage of penicillin-resistant strains is equal to the value obtained by the

MIC assay, which was 40%. Compared with the MIC assay, mPCR for *bla* genes detected penicillin-resistant strains with a 100% sensitivity but a low specificity of 68% (Table 4). The considerable number of false positives (32%) observed by mPCR can be explained by WGS analysis. In fact, all strains in which a *bla* operon was detected by WGS also showed a positive mPCR result. The mPCR method, however, only detected the presence of the specific gene and did not consider its regulation and functionality.

For DDC and DDE, the results were also substantially divergent. Compared with the MIC assay, the sensitivity and specificity for DDC were 68% and 100%, respectively; for DDE they were 100% and 74%. With kappa equaling 0.715 and 0.692, respectively, the values were just moderate. Normally DDC gave a positive result if the corresponding MIC values were greater than or equal to 1.0 μg/mL (Table 2 and Supplemental Table S1). In contrast, DDE, as the mPCR method, generated too many false-positive results. This shows that DDE and mPCR methods generated very comparable results, suggesting that *S. aureus* strains with positive PCR results for *blaZ* and negative results for *mecA* and *mecC* had possibly been selected to create the phenotypic DDE protocol. According to EUCAST (EUCAST, 2022a,b), a fuzzy zone edge together with a zone diameter greater than or equal to 26 mm is to be reported as penicillin susceptible, whereas a sharp zone edge and zone diameter greater than or equal to 26 mm is reported to be penicillin resistant (EUCAST, 2022b). Compared with the MIC assay results, however, the DDE protocol generated too many false-positive results, indicating that the criterion of the zone edge form should be reconsidered, at least for bovine strains of *S. aureus*. On the other hand, the DDC protocol should be reevaluated too, as its sensitivity is inappropriate. In fact, with a disk content of 10 IU (CLSI, 2018a, 2020), it uses a content that is 10 times higher than the DDE protocols (EUCAST, 2022a,b), obviously inflicting a high penicillin pressure on strains with MIC values between 0.125 and 1.0 μg/mL. Interestingly, among the DD methods given by CLSI and EUCAST, those for penicillin are among the most divergent ones, indicating that working out DD protocols for penicillin resistance in *S. aureus* is a difficult task. Applying additional WGS analyses as performed in the present study, however, will significantly contribute to establishing new DD protocols that can overcome the limitations of the current methods.

CONCLUSIONS

Penicillin resistance in *S. aureus* of bovine origin is highly dependent on the functionality of the *bla* operon promotor. When functional, all strains showed values of MIC ≥1 μg/mL and were penicillin resistant, whereas, when mutated, they were penicillin susceptible except in those rare cases with a putative, low-level, and *bla*-operon-independent mechanism for penicillin resistance. Our analyses also demonstrated that penicillin resistance in bovine *S. aureus* is truly assessed by the MIC assay. In contrast, the concordance between MIC assay, DD, and PCR for the *bla* genes was only moderate, demonstrating that DD and PCR analyses for clinical use need to be interpreted with great caution. In the present study about bovine *S. aureus*, a mutated promotor was exclusively found in (bovine) *S. aureus* of CC8 and was always plasmid based. A transfer of this discovery to *S. aureus* of human origin is possible and needs further investigation.

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