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Function impairing mutations in *blaZ* and *blaR* genes of penicillin susceptible *Staphylococcus aureus* strains isolated from bovine mastitis

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Summary

Molecular based approaches have gained increasing importance in routine mastitis diagnostics for typing and antibiotic resistance testing of *Staphylococcus aureus*. Out of 78 *S. aureus* strains isolated from bovine mastitis milk 10 of them harbored *blaZ*, *blaI* and *blaR* genes. Although 5 strains were phenotypically resistant to penicillin, the other 5 (all belonging the clonal complex 8) were penicillin susceptible. PCR amplification confirmed the presence of the *blaZ*, *blaR* and *blaI* genes in all 5 strains. Sequencing of these genes uncovered a 29 base deletion within the *blaZ* gene in all these strains that causes a translational frame shift, which is predicted to induce abrogation of BlaZ expression. Additionally single nucleotide insertions and deletions were detected in *blaR* of 3 strains. These insertions cause translation reading frame shifts and premature stop codons that are predicted to induce expression of truncated BlaR proteins. Using the genetically altered *blaZ* genes detected as targets, a real-time PCR system for detecting CC8 associated *blaZ* positive *S. aureus* strains that still remain susceptible to penicillin was developed. Such strains are part of detection challenges that must be considered in routine application of genotypic resistance testing of bovine mastitis *S. aureus*.

Keywords: bovine mastitis, *Staphylococcus aureus*, beta-lactamase, penicillin resistance

Funktionell bedeutsame Mutationen in den *blaZ* und *blaR* Genen von Penicillin empfindlichen *S. aureus* Mastitisstämmen

Molekulare Methoden zur Typisierung von *S. aureus* und zur Testung solcher Stämme auf das Vorkommen von Antibiotikaresistenzgenen gewinnen in der Mastitisdiagnostik zunehmend an Bedeutung. Von 78 untersuchten *S. aureus* Stämmen, isoliert aus bovinen Mastitiden, wiesen 10 davon die Gene *blaZ*, *blaI* and *blaR* auf. Nur 5 dieser Stämme waren allerdings phänotypisch Penicillin resistent, die anderen 5 Stämme (alle gehörten zum klonalen Komplex 8) waren Penicillin empfindlich. Mittels PCR konnte in letzteren 5 Stämmen das Vorhandensein der Gene *blaI* and *blaR* bestätigt werden. Die Sequenzierung des *blaZ* Gens zeigte in allen Stämmen eine 29 bp grosse Deletion, mit der Folge, dass das BlaZ Protein nicht exprimiert wird. Zudem wurde in den *blaR* Genen von 3 Stämmen Insertionen und Deletionen von einzelnen Nucleotiden gefunden. Diese führen zu verfrühten Stopcodons und damit zur Expression von veränderten BlaR Proteinen. Gestützt auf den genetisch veränderten *blaZ* Genen wurden eine real-time PCR zum Nachweis von *balZ* tragenden *S. aureus* Stämmen des klonalen Komplexes 8, die phänotypisch Penicillin empfindlich sind, etabliert. Solche Stämme stellten bei der routinemässigen Anwendung von PCR Methoden in der Mastitisdiagnostik bis anhin eine diagnostische Herausforderung dar.

Schlüsselwörter: bovine Mastitis, *Staphylococcus aureus*, Beta-Lactamasen, Penicillinresistenz

Introduction

Staphylococcus aureus β-lactam antibiotic resistance is conferred via two primary mechanisms. These depend on expression of β-lactamase that degrades β-lactams or production of penicillin binding protein 2a (PBP2A), which is not susceptible to β-lactam inhibition (Fuda et al., 2005; McCallum et al., 2010). The production of β-lactamase is transcriptionally controlled through the *bla* regulatory system, which is comprised of *blaZ*, *blaI* and *blaR* genes (Fuda et al., 2005; McCallum et al., 2010). Beta-lactamase dependent penicillin resistance is common amongst ruminant mastitis *S. aureus* strains (Haveri et al., 2005; Johler et al., 2011; Kunz et al., 2011; Nam et al., 2011; Haran et al., 2012).

The use of PCR based techniques and DNA microarrays are gaining increasing importance for genotyping and antibiotic resistance testing of *S. aureus* in routine mastitis diagnostics. In a recently published study based on DNA microarray analysis 10 out of 78 *S. aureus* strains isolated from bovine mastitis milk harbored *blaZ*, *blaI* and *blaR* genes (Moser et al., 2013). Five strains were phenotypically resistant to penicillin in agreement with the genotypic test results. In contrast, the other 5 strains were phenotypically penicillin susceptible. All these strains belonged to the MSLT clonal complex 8 and harbored *entA*, *entD* and *entJ* genes. *S. aureus* strains harboring this combination of enterotoxin genes are classified as genotype B, and are exclusively associated with very high within-herd prevalence of mastitis (Graber et al., 2009). Data of some other studies have also shown that although widely adopted as a "golden standard" to determine penicillin resistant *S. aureus* in genotypic tests, *blaZ* gene presence in a significant number of strains is not associated with phenotypic penicillin resistance (Haveri et al., 2005; Pitkala et al., 2007). Nevertheless, possible causes for this loss of phenotype have not yet been extensively investigated. The aim of the current study was therefore to elucidate the molecular basis for penicillin susceptibility in these five CC8 *S. aureus* strains harboring the *blaZ*, *blaI* and *blaR* genes.

Methods

Conventional PCR detection of the *bla* genes

Primers used to amplify the *blaZ*, *blaI* and *blaR* genes as well as the entire *bla* genetic system are listed in Table 1. The *S. aureus* genomic DNA templates were extracted from stationary growth phase stage overnight cultures using the DNAeasy kit (Qiagen AG, Hombrechtikon, Switzerland). Nucleic acid concentrations were measured using the Nanodrop ND-1000 spectrophotometer (Nanodrop Instruments, Delaware, USA). PCR amplification was performed using the Phusion High-fidelity DNA polymerase (Thermo Scientific, Chicago, USA) in accordance with instructions provided by the supplier.

Sequence analysis

The entire DNA region in different *S. aureus* strains carrying the *blaZ*, *blaI* and *blaR* genes was amplified using primers listed in Table 1 and Phusion High-fidelity DNA polymerase (Thermo Scientific, Chicago, USA). PCR products were purified using the Qiagen MiniElute Purification Kit (QIAGEN GmbH, Germany). DNA sequencing was performed at Microsynth (Balgach, Switzerland). Resulting *blaZ*, *blaR* and *blaI* sequences were assembled and analyzed using CLC Genomics Workbench 5.7.1 (CLC Bio, Aargus, Denmark).

blaZ detection and genotype discrimination by LC-PCR assays

Real-time PCR was conducted in the LightCycler 2.0 instrument (Roche Molecular Diagnostics) in a total reaction volume of 20 µl. Primers listed in Table 2 were used. PCR reactions contained the LightCycler Faststart DNA master plus hybridization probes mix, 1000 nM of the primers and 200 nM of the LC probes. Fifty Nano grams of *S. aureus* DNA template in 2.5 µl was added to each reaction. PCR amplification comprised of an initial preincubation step at 95 °C for 10 min to activate the DNA polymerase, followed by 45 cycles of 95 °C for 10 s, 56 °C for 20 s, and 72 °C for 18 s. The fluorescence signals corresponding to *blaZ* amplification were monitored during the 56 °C annealing step in the LC Red 610 (*blaZ* detection) nm and LC Red 640 (*blaZ* genotype discrimination) detection channels of the LightCycler 2.0 instrument, respectively.

Results and Discussion

PCR was used to rule out the possibility that genotypic resistance observed in these 5 *S. aureus* strains might be due to false hybridization signals on the DNA microarray. Primers targeting defined regions within *blaZ*, *blaI* and *blaR* all gave amplicons that were of expected size in all 5 strains (Tab. 3). Using a set of primers that targeted the entire DNA region harboring the *blaZ*, *blaI*, *blaR* genes

Table 1: Conventional PCR primers used for detection of the *blaZ*, *blaR* and *blaI* genes and amplification of the entire *bla* gene region.

Target gene	Primer sequence (5'–3')	Reference
blaZ	CAAAGATGATATAGTTGCTTATTCTCC TGCTTGACCACTTTTATCAGC	Pitkala *et al*, 2007
blaR	TGCGGCTCAACTCAAG CCCAGACGGGTTTCCA	This study
blaI	ATGACCAATAAGCAAGTTGAAA CATATCCCCTCCATACAGT	This study
bla region	GTTTGTTAAAGAGAATTAAGAAAATAAATMTCGA GATTACRAGGTCTTTTRTGAACATAGAT	This study

Table 2: Real-time PCR primers and probes designed in this study and used for the detection of *blaZ* genotypes.

Primer or probe	Sequence (5'–3')	Purpose
blaZ F blaZ R blaZ 3'Fluo blaZ 5'LC Red 610	TCTTGGCGGTTTCACT GATGGTGTTYCAAAAGACT ATTCGTAAAAATGACTAAAACAATAGGTTCAGA GGCCCTTAGGATAAACAAAAGCAAC	*blaZ* detection
blaZ 2–30nt del F blaZ 2–30nt del R blaZ 2–30nt del 3'Fluo blaZ 2–30nt del 5'Red 640	GAAGTCGAAGCATAGGC GCCATTTTGACACCTTCT AATCATTTAACTCTTTGGCATGTGAACT TTGAATTACATGCACTTAAAACTAAAGCCA	Distinguish between intact and impaired *blaZ* genes

amplicons of expected size were also obtained in all 5 strains. This PCR analysis therefore confirmed that the whole *bla* resistance genetic system was present in all 5 *S. aureus* strains despite their penicillin susceptible phenotypes. Moreover it also excluded the possibility that these strains might have substantial genetic deletions in the *bla* genetic resistance system that may be located outside the regions targeted by DNA microarray hybridization.

Although the whole *bla* genetic system was detected in all the 5 penicillin susceptible *S. aureus* strains, it could not be ruled out that individual genes of this system might harbor function impairing smaller genetic changes. To determine this, entire DNA regions harboring *blaZ*, *blaI* and *blaR* genes in these 5 strains were sequenced. For comparison purposes, 2 other *blaZ*, *blaI*, *blaR* carrying but penicillin resistant bovine mastitis *S. aureus* strains (SA 833 and SA711) were included as positive controls (Tab. 3). No major genetic changes were detected between *blaI* sequences of the positive control strains and the 5 penicillin susceptible *S. aureus* strains. However, interesting genetic changes were detected in the *blaZ* and *blaR* genes of the 5 penicillin susceptible *S. aureus* strains. In comparison to the sequences in the 2 positive control *S. aureus* strains, a 29 base deletion was detected in the *blaZ* gene of all the 5 penicillin susceptible *S. aureus* strains (Fig. 1). This genetic change affected nucleotides positions 2 to 30 of the *blaZ* gene leading to the loss of the first ten codons. Moreover this genetic deletion causes a translation reading frame shift change in the remaining nucleotide sequence, which changes the methionine encoding translation-start codon to a codon for serine.

The *blaR* gene in 3 (SA1289, SA1772 and SA2055) of these *S. aureus* strains carried single nucleotide insertions that shifted the BlaR translation reading frame and induce premature stop codons. In two of the strains (SA1289 and SA1772) an adenosine insertion was detected at nucleotide position 726 (Fig. 1B). This causes a translation reading frame shift and induces a stop codon after translation of 254 of the 600 BlaR codons. Meanwhile in SA2055 a thiamine insertion was also detected at nucleotide position 771 of the *blaR* gene (Fig. 1C). This genetic change similarly induces a translation frame shift, which leads to BlaR truncation after translation of 256 codons. Thus *blaZ* and *blaR* genetic mutations detected in these 5 *S. aureus* strains can all lead to a penicillin susceptible phenotype despite the penicillin resistant genotypes detected using DNA microarray and PCR analysis.

Table 3: DNA microarray genotypes, genotypic and phenotypic susceptibility testing overview of bovine mastitis *S. aureus* strains examined in this study.

Strain	SA692	SA23	SA2055	SA1772	SA1289	SA883	SA711
Penicillin phenotype	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant
Diameter of zone of inhibition	35 mm	44 mm	38 mm	30 mm	40 mm	26 mm	16 mm
MIC (µg/mL)[1]	< 0.03	< 0.03	< 0.03	0.06	< 0.03	0.12	> 0.5
Production of beta-lactamase[2]	negative	negative	negative	negative	negative	weakly positive	positive
Clonal complex	CC8	CC8	CC8	CC8	CC8	C45	CC7
blaZ , *blaR* and *blaI*	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PCR analysis *blaZ*/*blaI*/*blaR* PCR	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Gene sequence analysis *blaZ* deletion	yes	yes	yes	yes	yes	no	no
blaR 726A insertion	no	no	no	yes	yes	no	no
blaR 771T insertion	no	no	yes	no	no	no	no

[1] tested by VITEC® 2; [2] tested induced (0.003 µg/mL) and not-induced by Beta Lactamase identification sticks (Oxoid)

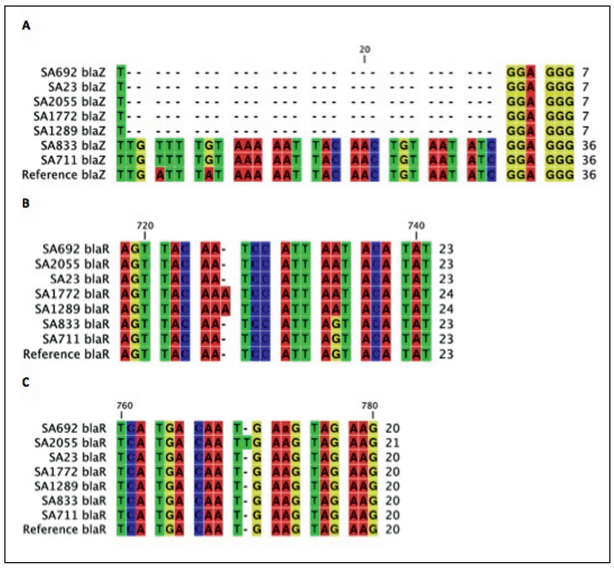

Figure 1: DNA sequence alignments showing genetic changes detected in *blaZ* and *blaR* genes of penicillin susceptible CC8 *S. aureus* strains. (A) A 29 base deletion found at the beginning of the *blaZ* gene in all the five strains (B) An adenine (A) insertion detected after 725 nucleotides in the *blaR* gene of SA1772 and SA1289 leading to a translation frame shift and a stop codon after translation of 254 BlaR codons (C) A thiamine (T) inserted detected after 770 nucleotides in the *blaR* gene of SA2055 leading to a translation reading frame shift and a stop codon after translation of 256 BlaR codons. Reference sequences are based on the complete sequence of the *S. aureus* strain 18809 plasmid (p18809-PO3).

Based on genotypic tests alone such CC8 *blaZ* carrying *S. aureus* strains are classified as penicillin resistant although they are penicillin susceptible. In such situations penicillin despite being still effective cannot be used as the first choice drug for bovine mastitis treatment. It is thus important that strains harboring functionally impaired *blaZ* genes are appropriately detected. Therefore, a real-time PCR system that can rapidly detect strains carrying *blaZ* and distinguish those harboring the non-functional gene has been developed. This comprises 2 sets of primers that can be used separately or combined in a duplex assay (Tab. 2). One set of primers targets the *blaZ* region found in both functional and non-functional alleles. The second set targets the 29-base region, which is deleted in those strains that are susceptible to penicillin. An amplification with this primer set thus indicates an intact *blaZ* gene, whilst no amplification indicates a non-functional *blaZ* allele lacking nucleotides 2–30 at its beginning. *S. aureus* strains carrying this form of *blaZ* are thus still amenable to penicillin treatment although they are classified as possessing genotypic resistance to this drug using genotyping tests. As a duplex this PCR system can thus be used to simultaneously detect and profile *blaZ* genes in CC8 *S. aureus* strains to determine if they carry the non-functional genotype. Alternatively the second primer set alone can be used to screen *blaZ* carrying CC8 *S. aureus* strains for those harboring the impaired genotype. As proof of principle, this PCR system was demonstrated to correctly detect the 5 CC8 *S. aureus* strains that carry the impaired *blaZ* gene from the two control strains carrying the intact gene (Tab. 4).

Table 4: Detection and discrimination of *blaZ* genotypes in *S. aureus* strains using real-time PCR.

Strain	*blaZ* presence	*blaZ* 2–30 nt deletion	Penicillin phenotype
SA692	+	+	susceptible
SA23	+	+	susceptible
SA2055	+	+	susceptible
SA1772	+	+	susceptible
SA1289	+	+	susceptible
SA833	+	–	resistant
SA711	+	–	resistant

Conclusions

Investigations performed in this study have identified genetic impairments in *blaZ* and *blaR* genes that are associated with penicillin susceptibility in *blaZ* carrying CC8 *S. aureus* strains isolated from bovine mastitis. A PCR based system to detect such strains has been developed. Its incorporation into available molecular resistance genotyping tests can be useful in enhancing their reliability.

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Mutations fonctionnelles significatives des gènes *blaZ* et *blaR* de *S. aureus* sensibles à la pénicilline

Les techniques moléculaires pour typiser les *S. aureus* et pour tester ces souches quant à la présence des gènes de résistance aux antibiotiques gagnent régulièrement

Mutazioni funzionali significative nei geni *blaZ* e *blaR* nei ceppi di *S. aureus* della mastite, sensibili alla penicillina

Metodi molecolari per la tipizzazione di *S. aureus* e per verificare la presenza di tali ceppi in geni resistenti agli antibiotici stanno diventando sempre più impor-

en importance dans le diagnostic des mammites. Sur 78 souches de *S. aureus*, isolées de mammites bovines, 10 présentaient les gènes *blaZ*, *blaI* et *blaR*. Toutefois seules 5 de ces souches présentaient une résistance à la pénicilline, les 5 autres (appartenant toutes au complexe clonal 8) étaient sensibles à la pénicilline. On a pu confirmer par PCR la présence des gènes *blaI* et *blaR* dans ces 5 dernières souches. Le séquençage du gène *blaZ* a montré dans toutes ces souches une délétion importante de 29 bp avec pour conséquence que la protéine BlaZ n'était pas exprimée. En outre on a trouvé sur les gènes *blaR* de 3 souches des insertions et des délétions de nucléotides isolés. Cela conduit à des stop codons prématurés et donc à l'expression de protéines BlaR modifiées. En se basant sur les gènes *blaZ* modifiés, on a établi une PCR en temps réel pour identifier les souches de *S. aureus* du complexe clonal 8 sensibles à la pénicilline. De telles souches représentaient jusqu'à maintenant un défi dans l'utilisation de routine de la PCR dans le diagnostic des mammites.

tanti nella diagnostica della mastite. Sono stati controllati 78 ceppi di *S. aureus*, isolati da mastite bovina, di cui 10 presentavano i geni *blaZ*, *blaI* e *blaR*. Solo 5 ceppi erano sotto il profilo fenotipico resistenti alla penicillina, gli altri 5 (tutti appartenenti al complesso clonale 8) erano sensibili a quest'ultima. Negli ultimi 5 ceppi è stata confermata, via PCR, la presenza dei geni *blaI* e *blaR*. Il sequenziamento del gene *blaZ* ha rivelato in tutti i ceppi una grande delezione 29 bp con conseguenza che la proteina *BlaZ* non veniva espressa. Inoltre nel gene *blaR* di 3 ceppi, si sono riscontrati inserimenti e delezioni di singoli nucleotidi. Questi hanno condotto a dei codoni di stop prematuri e quindi all'espressione delle proteine BlaR alterata. Sulla base dei geni *blaZ*, geneticamente modificati, è stato definito un PCR in tempo reale per dimostrare che i ceppi di *S. aureus*, portatori di *blaZ* nel complesso clonale 8, sono sensibili a livello fenotipico alla penicillina. Questi ceppi confermano che un'applicazione di routine dei metodi PCR nella diagnosi della mastite è stata finora una sfida diagnostica.

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