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Antimicrobial susceptibility of gram-positive udder pathogens from bovine mastitis milk in Switzerland

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Summary

We evaluated the susceptibility of the gram-positive mastitis pathogens *S. aureus*, *Str. uberis*, *Str. dysgalactiae*, *E. faecalis* and *L. garviae* to antibiotics that are of epidemiological interest or are critically important for mastitis therapy and human medicine. Penicillin resistance was found to be most frequent in *S. aureus*, and nearly 5 % of the *Str. uberis* strains displayed a decreased susceptibility to this antibiotic. Resistance to aminoglycosides and macrolides was also detected in the strains tested. The detection of methicillin-resistant *S. aureus* (MRSA) and of a ciprofloxacin-resistant *Str. dysgalactiae* isolate corroborated the emergence of mastitis pathogens resistant to critically important antibiotics and underscores the importance of susceptibility testing prior to antibiotic therapy. The monitoring of antibiotic susceptibility patterns and antibiogram analyses are strongly recommended for targeted antimicrobial treatment and to avoid the unnecessary use of the latest generation of antibiotics.

Keywords: Antimicrobial resistance, *Staphylococcus*, *Streptococcus, Enterococcus*, MRSA

Antibiotika-Empfindlichkeit von Gram-positiven, bovinen Mastitis-Erregern in der Schweiz

In dieser Studie wurde die Empfindlichkeit von gram-positiven Mastitis-Erregern wie *S. aureus, Str. uberis, Str. dysgalactiae, E. faecalis* und *L. garviae* gegenüber therapeutisch relevanten Antibiotika einerseits, wie auch epidemiologisch interessanten Antibiotika und Reserveantibiotika zur Anwendung in der Humanmedizin andererseits untersucht. Resistenzen gegenüber Penicillin wurden am häufigsten bei *S. aureus* nachgewiesen, aber auch rund 5 % der *Str. uberis* Stämme zeigten eine verminderte Empfindlichkeit gegen dieses Antibiotikum. Zudem wurden Resistenzen gegen Aminoglykoside und Makrolide festgestellt. Der Nachweis von Methicillin-resistenten *S. aureus* (MRSA) und eines Ciprofloxacin-resistenten *Str. dysgalactiae* Stammes beweisen, dass Mastitiserreger Resistenzen gegenüber wichtigen Reserveantibiotika entwickeln und unterstreichen die Bedeutung der Erstellung von Antibiogrammen vorgängig einer Antibiotika-Therapie. Eine Überwachung der Resistenzsituation sowie die Auswertung von Antibiogrammresultaten sind dringend notwendig, um eine gezielte antimikrobielle Behandlung durchzuführen und damit die unnötige Verwendung von Antibiotika der neuesten Generation zu vermeiden.

Schlüsselwörter: Antibiotikaresistenz, *Staphylococcus, Streptococcus, Enterococcus*, MRSA

Introduction

Because of the steady increase in milk production from cows, the importance of bovine mastitis is increasing worldwide. Mastitis is a painful inflammation of the udder that affects the quality and quantity of milk produced leading to high economic losses (Hogeveen et al., 2011; Heikkilä et al., 2012). Mastitis is usually treated with antibiotics, which are often prescribed without prior susceptibility testing. Given the recent establishment of molecular-based diagnostics of mastitis pathogens, the use of antibiotics without prior evaluation of an antibiogram is also expected to increase in the coming years (Hänni et al., 2011). To overcome treatment failures and prolonged treatments resulting from such blind therapy, highly potent antibiotics are often used, despite specific antibiotics such as penicillin, gentamicin,

neomycin and kanamycin being sufficient. Indeed, while mainly penicillin-based suspensions commonly used in combination with aminoglycosides have been frequently and successfully used in Switzerland for mastitis treatment, the use of cephalosporins, especially of the newer generations, has significantly increased in the past few years (Büttner et al., 2011). Consequently, the presence of extended-spectrum β-lactamase (ESBL)- and class C plasmid-mediated AmpC (pAmpC)- producing *E. coli* in mastitis samples have been reported recently (Endimiani et al., 2011; Geser et al., 2012; Dahmen et al., 2013). Moreover, methicillin-resistance, encoded by the *mecA* gene, which confers resistance to all ß-lactam antibiotics including cephalosporins, has been detected in *Staphylococcus aureus* (*S. aureus*) (MRSA) and coagulase-negative staphylococci *(S. epidermidis*, *S. fleurettii*, *S. haemolyticus* and *S. xylosus*) present in bovine mastitis milk samples in Switzerland (Monecke et al., 2007; Huber et al., 2010; Frey et al., 2013). Monitoring the pattern of antimicrobial susceptibility of mastitis pathogens is necessary to demonstrate the development of their resistance to various antimicrobial agents. In this study, we focused on examining the susceptibility patterns of the gram-positive mastitis pathogens *S. aureus*, *Streptococcus uberis* (*Str. uberis*), *Streptococcus dysgalactiae* subspecies *dysgalactiae* (*Str. dysgalactiae*), which are frequently detected in mastitis milk samples obtained from dairy cows in Switzerland. *Enterococcus faecalis* (*E. faecalis*) and *Lactococcus garviae* (*L. garviae*) were included as these pathogens are nowadays identifiable with matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI TOF MS). Studies on the coagulase-negative staphylococci, which are also relevant bovine udder pathogens, have been published separately (Frey et al., 2013; Moser et al., 2013).

Material and Methods

Sample collection

A total of 287 *S. aureus*, 208 *Str. uberis*, 46 *Str. dysgalactiae*, 13 *E. faecalis* and 12 *L. garviae* strains isolated from mastitis milk from cows sampled between 2010 and February 2012 were tested for antimicrobial susceptibility. Most strains were isolated at the Institute of Veterinary Bacteriology, Bern, except for 106 *Str. uberis* and 31 *Str. dysgalactiae* strains that came from the Institute of Food Safety and Hygiene, Zürich. The cows showed signs of acute, subclinical, or chronic mastitis. Milk samples were cultured according to standard bacterial culture methods (National Mastitis Council, 1999) and isolated strains were identified by MALDI TOF MS (Biotyper 3.0, Bruker Daltonics, Bremen, Germany or Axima Confidence, Shimadzu-Biotech Corp., Kyoto, Japan) and kept at –80 °C in tryptone soy medium containing 30 % glycerol.

Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) of the antibiotics was determined by broth microdilution in cation-adjusted Müller-Hinton broth using the Sensititre susceptibility plate EUST (Trek Diagnostics Systems, East Grinstead, England; MCS Diagnostics BV, Swalmen, The Netherlands). *S. aureus* ATCC 29213 was used as the control strain for MIC susceptibility testing. The list of antibiotics and the range of concentrations tested is presented in Tables 1 to 5. Erythromycin was used as class representative for macrolides, ciprofloxacin as class representative for fluoroquinolones, sulfamethoxazole as class representative for sulfonamides and clindamycin as class representative for lincosamides. Isolates were classified as susceptible or resistant according to clinical breakpoints issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines Version 3.0 (www.eucast.org). Breakpoints of *Streptococcus* group A, B, C, and G were used for *Str. dysgalactiae*. When EUCAST breakpoints were unavailable, clinical breakpoints from the Clinical and Laboratory Standards Institute (CLSI) documents M31-A3 (Clinical and Laboratory Standards Institute, 2008) and M100-S22 (Clinical and Laboratory Standards Institute, 2012) were used. For *L. garviae*, resistance breakpoints proposed for *L. lactis* by the European Food Safety Agency (EFSA) were used (EFSA, 2008). Breakpoints are indicated with vertical lines in Tables 1 to 5. Cefoxitin was used as the indicator antibiotic for methicillin-resistant *S. aureus* (MRSA). *S. aureus* strains exhibiting MIC of cefoxitin ≥ 4 µg/ml were tested for the presence of methicillin resistance genes *mecA* and *mecC* by PCR (Frey et al., 2013). MRSA was characterised by *spa* typing and multi locus sequence typing (MLST) as described previously (Overesch et al., 2011). Correlation of MIC values with strain resistance or susceptibility was only made for antibiotics for which clinical breakpoints for the tested species were available. Other antibiotics were measured for epidemiological purposes only and are not intended for clinical or therapeutic interventions. The resistance (calculated as a percentage) and 95 % confidence intervals were calculated using the Bayesian Calculator software (http://www.causascientia.org).

Results

Penicillin resistance was found in 16.4 % of the *S. aureus* isolates and was absent in the *Str. dysgalactiae* and *E. faecalis* strains. MIC50 and MIC90 of penicillin for *L. garviae* were 1.0 µg/ml each (Tab. 5). While the majority of *Str. uberis* strains showed a MIC of ≤ 0.12 µg/ml for penicillin, 10 isolates showed decreased susceptibility with a MIC of 0.25 µg/ml (Tab. 2). *Str. uberis* strains with higher MIC levels have been shown to have mutations in the penicillin-binding protein (PBP) (Haenni et al., 2010a). One *S. aureus* strain (M905-1) resistant to

Table 1: Minimal inhibitory concentration (MIC) in µg/ml for *Staphylococcus aureus* (n = 287).

Antimicrobial	MIC (µg/ml)																	MIC 50 % (µg/ml)	MIC 90 % (µg/ml)	Resistance (%)	95 % CI
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024				
Penicillin				240	4	3	19	13	8									≤ 0.12	1	16.4	12.6–21.1
Cefoxitin						1	1	135	149			1						4	4	0.3	0.0–1.9
Kanamycin									274	7		1		5				≤ 4	≤ 4	1.7	0.8–4.0
Gentamicin							282					5						≤ 1	≤ 1	1.7	0.8–4.0
Streptomycin									186	81	11		9					≤ 4	8	n. d.	n. d.
Erythromycin					256	23					8							≤ 0.25	0.5	2.8	1.4–5.4
Clindamycin				280	1					6								≤ 0.12	≤ 0.12	2.1	0.9–4.5
Ciprofloxacin					241	44	2											≤ 0.25	0.5	0	0.0–1.3
Tetracycline						272	5				1	9						≤ 0.5	≤ 0.5	3.5	1.9–6.3
Sulfamethoxazole													231	21	16	7	12	≤ 64	256	6.6	4.3–10.1
Trimethoprim								282					5					≤ 2	≤ 2	1.7	0.8–4.0
Chloramphenicol									21	237	29							8	16	10.1	7.1–14.1
Tiamulin						177	110											≤ 0.5	1	n. d.	n. d.
Fusidic acid						285	1		1									≤ 0.5	≤ 0.5	0.3	0.0–1.9
Rifampicin	285	1		1														≤ 0.016	≤ 0.016	0	0.0–1.3
Mupirocin						287												≤ 0.5	≤ 0.5	0	0.0–1.3
Vancomycin							286	1										≤ 1	≤ 1	0	0.0–1.3
Quinupristin/ Dalfopristin						271	16											≤ 0.5	≤ 0.5	0	0.0–1.3
Linezolid							20	180	87									2	4	0	0.0–1.3

Numbers indicate the number of isolates with corresponding MIC value. Light blue areas indicate range of dilutions tested for each antimicrobial agent; values above or below this range denote MIC values greater than the highest concentration tested and MIC values smaller than or equal to the lowest concentration tested, respectively. Vertical lines indicate clinical breakpoints according to EUCAST guidelines (version 3.0, 2013, *Staphylococcus* spp.). Vertical dotted lines show breakpoints according to CLSI (M100-S22, 2012 *Staphylococcus* spp).
MIC minimal inhibitory concentration, n. d. not determined; CI confidence interval

Table 2: Minimal inhibitory concentration (MIC) in µg/ml for *Streptococcus uberis* (n = 208).

Antimicrobial	MIC (µg/ml)																	MIC 50 % (µg/ml)	MIC 90 % (µg/ml)	Resistance (%)	95 %CI
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024				
Penicillin				198	10													≤ 0.12	≤ 0.12	n. d.	n. d.
Kanamycin									12	23	17	59	92	5				32	64	n. d.	n. d.
Gentamicin							13	28	12	46	107	2						16	16	n. d.	n. d.
Streptomycin										5	10	24	169					≥ 64	≥ 64	n. d.	n. d.
Erythromycin					184	2	2	2	1		17							≤ 0.25	≤ 0.25	10.6	7.1 – 15.5
Clindamycin				188	1				2	17								≤ 0.12	≤ 0.12	n. d.	n. d.
Ciprofloxacin					22	123	61	2										0.5	1	n. d.	n. d.
Tetracycline						144	4	1			1	58						≤ 0.5	≤ 0.5	28.4	22.7 – 34.9
Sulfamethoxazole															2	13	193	≥ 1024	≥ 1024	n. d.	n. d.
Trimethoprim								207	1									≤ 2	≤ 2	n. d.	n. d.
Chloramphenicol									204	3	1							≤ 4	≤ 4	0.4	0.1 – 2.6
Tiamulin						202	2	1		3								≤ 0.5	≤ 0.5	n. d.	n. d.
Fusidic acid*						1		10	78	119								≥ 8	≥ 8	n. d.	n. d.
Rifampicin		1	39	125	43													0.12	0.25	n. d.	n. d.
Vancomycin							208											≤ 1	≤ 1	0.0	0.0 – 1.7
Quinupristin/ Dalfopristin						205	2		1									≤ 0.5	≤ 0.5	n. d.	n. d.
Linezolid							123	85										≤ 1	≤ 1	n. d.	n. d.

Numbers indicate the number of isolates with corresponding MIC value. Light blue areas indicate the range of dilutions tested for each antimicrobial agent; values above or below this range denote MIC values greater than the highest concentration tested and MIC values smaller than or equal to the lowest concentration tested, respectively. Vertical dotted lines indicate breakpoints according to CLSI (M31-A3 (2008) recommended for streptococci.

MIC minimal inhibitory concentration, n. d. not determined; CI confidence interval

* intrinsic resistance

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Table 3: Minimal inhibitory concentration (MIC) in µg/ml for *Streptococcus dysgalactiae* subspecies *dysgalactiae* (n = 46).

Antimicrobial	MIC (µg/ml)																	MIC 50% (µg/ml)	MIC 90% (µg/ml)	Resistance (%)	95%CI
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024				
Penicillin				46														≤ 0.12	≤ 0.12	0.0	0.0–7.5
Kanamycin									31	11	1		1	2				≤ 4	8	n. d.	n. d.
Gentamicin							42	3			1							≤ 1	≤ 1	n. d.	n. d.
Streptomycin									39	3		1	3					≤ 4	8	n. d.	n. d.
Erythromycin					43						3							≤ 0.25	≤ 0.25	6.5	2.4–17.5
Clindamycin				43	1	1				1								≤ 0.12	≤ 0.12	2.2	0.5–11.3
Ciprofloxacin					13	31	1		1									0.5	0.5	n. d.	n. d.
Tetracycline						2	1	13	18	3	2	7						4	≥ 32	65.2	50.7–77.3
Sulfamethoxazole													27	7	2	4	6	≤ 64	≥ 1024	21.7	12.3–35.7
Trimethoprim								46										≤ 2	≤ 2	n. d.	n. d.
Chloramphenicol									46									≤ 4	≤ 4	0.0	0.0–7.5
Tiamulin						46												≤ 0.5	≤ 0.5	n. d.	n. d.
Fusidic acid*							20	24		2								2	2	n. d.	n. d.
Rifampicin	9	29	4	1	1	2												0.03	0.06	0.0	0.0–7.5
Vancomycin							46											≤ 1	≤ 1	0.0	0.0–7.5
Quinupristin/ Dalfopristin						46												≤ 0.5	≤ 0.5	n. d.	n. d.
Linezolid							44	2										≤ 1	≤ 1	0.0	0.0–7.5

Numbers indicate the number of isolates with corresponding MIC value. Light blue areas indicate the range of dilutions tested for each antimicrobial agent; values above or below this range denote MIC values greater than the highest concentration tested and MIC values smaller than or equal to the lowest concentration tested, respectively. Vertical lines indicate clinical breakpoints according EUCAST guidelines (version 3.0, 2013, *streptococcus* groups A, B, C and G).

MIC minimal inhibitory concentration, n. d. not determined; CI confidence interval

* intrinsic resistance

Table 4: Minimal inhibitory concentration (MIC) in µg/ml for *Enterococcus faecalis* (n = 13).

Antimicrobial	MIC (µg/ml)																	MIC 50 % (µg/ml)	MIC 90 % (µg/ml)	Resistance (%)	95 % CI
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024				
Penicillin									13									≥ 4	≥ 4	0.0	0.0–23.1
Kanamycin**												1	4	8				≥ 128	≥ 128	n. d.	n. d.
Gentamicin**									2	4	6	1						16	16	0.0	0.0–23.1
Streptomycin**													13					≥ 64	≥ 64	0.0	0.0–23.1
Erythromycin						2	2	4	1	4								2	8	30.8	12.8–58.1
Ciprofloxacin							8	5										1	2	0.0	0.0–23.1
Clindamycin*										13								≥ 8	≥ 8	n. d.	n. d.
Tetracycline						1						12						≥ 32	≥ 32	92.3	66.1–98.2
Sulfamethoxazole*																	13	≥ 1024	≥ 1024	n. d.	n. d.
Trimethoprim								11					2					≤ 2	≥ 64	n. d.	n. d.
Chloramphenicol										6	3	4						16	32	30.8	12.8–58.1
Tiamulin										13								≥ 8	≥ 8	n. d.	n. d.
Fusidic acid*								3	6	4								4	≥ 8	n. d.	n. d.
Rifampicin							13											≥ 1	≥ 1	0.0	0.0–23.1
Vancomycin							8	3	2									≤ 1	4	0.0	0.0–23.1
Quinupristin/ Dalfopristin*										13								≥ 8	≥ 8	n. d.	n. d.
Linezolid									13									4	4	0.0	0.0–23.1

Numbers indicate the number of isolates with corresponding MIC value. Light blue areas indicate the range of dilutions tested for each antimicrobial agent; values above or below this range denote MIC values greater than the highest concentration tested and MIC values smaller than or equal to the lowest concentration tested, respectively. Vertical lines indicate clinical breakpoints according EUCAST guidelines (version 3.0, 2013, *Enterococcus* spp.). Dotted lines indicate breakpoints according to CLSI (M100-S22, 2012) recommended for *Enterococcus* spp.
MIC minimal inhibitory concentration, n. d. not determined; CI confidence interval
* intrinsic resistance, ** low-level intrinsic resistance to aminoglycosides

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Table 5: Minimal inhibitory concentration (MIC) in µg/ml for *Lactococcus garviae* (n = 12).

Antimicrobial	MIC (µg/ml)																	MIC 50 % (µg/ml)	MIC 90 % (µg/ml)	Resistance (%)	95 % CI
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024				
Penicillin						4	8											1	1	n. d.	n. d.
Kanamycin									8	1	3							≤ 4	16	0.0	0.0–24.7
Gentamicin							9	2	1									≤ 1	2	0.0	0.0–24.7
Streptomycin									1	6	5							8	16	0.0	0.0–24.7
Erythromycin					12													≤ 0.25	≤ 0.25	0.0	0.0–24.7
Clindamycin*										12								8	8	n. d.	n. d.
Ciprofloxacin						1	7	4										1	2	n. d.	n. d.
Tetracycline						4	2					6						1	≥ 32	50.0	25.1–74.9
Sulfamethoxazole																12		512	512	100	75.3–99.8
Trimethoprim									7		4	1						4	16	n. d.	n. d.
Chloramphenicol									12									≤ 4	≤ 4	0.0	0.0–24.7
Tiamulin										12								≥ 8	≥ 8	n. d.	n. d.
Fusidic acid										12								≥ 8	≥ 8	n. d.	n. d.
Rifampicin							12											≥ 1	≥ 1	n. d.	n. d.
Vancomycin							12											≤ 1	≤ 1	0.0	0.0–24.7
Quinupristin/ Dalfopristin							1	4	7									4	4	0.0	0.0–24.7
Linezolid							3	9										2	2	n. d.	n. d.

Numbers indicate the number of isolates with corresponding MIC value. Light blue areas indicate the range of dilutions tested for each antimicrobial agent; values above or below this range denote MIC values greater than the highest concentration tested and MIC values smaller than or equal to the lowest concentration tested, respectively. Vertical lines indicate resistance breakpoints proposed by the EFSA for *L. lactis* (The EFSA Journal (2008) 732, 1–15).

MIC minimal inhibitory concentration, n. d. not determined; CI confidence interval

* intrinsic resistance

cefoxitin (MIC ≥ 32 µg/ml) was confirmed to contain the *mecA* gene and belong to ST398 and *spa* type t011. Nearly half of the *Str. uberis* isolates (46.7 %) exhibited elevated MIC to kanamycin (MIC50 = 64 µg/ml; MIC90 ≥ 128 µg/ml). In contrast, only 3 *Str. dysgalactiae* isolates (6.5 %) showed elevated MIC (MIC ≥ 64 µg/ml) for kanamycin, 1.7 % of the *S. aureus* isolates were resistant to kanamycin and all *L. garviae* were susceptible. Similar to kanamycin, elevated MIC to gentamicin (MIC50 = 32 µg/ml; MIC90 = 64 µg/ml) was found in 52.4 % of the *Str. uberis* isolates, in 2.2 % and 1.7 % of the *Str. dysgalactiae* and *S. aureus* isolates, respectively, and was absent in *L. garviae*. Elevated MICs to streptomycin (MIC ≥ 64 µg/ml) were observed for *Str. uberis* (81.3 %) and to a lesser extent for *Str. dysgalactiae* (6.5 %) and *S. aureus* (3.1 %). High-level resistance to aminoglycosides was not present among the *E. faecalis* isolates. An elevated MIC to ciprofloxacin (MIC = 4 µg/ml) was only detected in 1 *Str. dysgalactiae* isolate, while *Str. uberis* and *L. garviae* showed low ciprofloxacin MIC90 values of 1 µg/ml and 2 µg/ml, respectively. Resistance to erythromycin was found in *Str. uberis* (10.6 %), *Str. dysgalactiae* (6.5 %) and *S. aureus* (2.8 %) but not in *L. garviae* (0 %). All erythromycin-resistant *Str. uberis* isolates were also resistant to clindamycin (10.6 %), suggesting the presence of the *erm methylase gene*. The *S. aureus* and *Str. dysgalactiae* isolates showed low resistance clindamycin (2.1 % and 2.2 %, respectively). Resistance to tetracyclines was the most common among all tested bacterial species, with 92.3 % of the *E. faecalis* strains being resistant, followed by *Str. dysgalactiae* (65.2 %), *L. garviae* (50 %), *Str. uberis* (28.4 %), and *S. aureus* (3.5 %) (Tab. 1 to 5). Nearly all of the *Str. uberis* and *L. garviae* strains (99–100 %) and 21.7 % of the *Str. dysgalactiae* strains exhibited elevated MIC to sulfamethoxazole (MIC90 ≥ 1024 µg/ml for streptococci, MIC90 = 512 µg/ml for *L. garviae*), and 6.6 % of the *S. aureus* isolates were resistant to sulfonamides (Tab. 1 to 5). Low percentage of trimethoprim resistance was found in *S. aureus* (1.7 %), while *Str. dysgalactiae* and Str. uberis exhibited low MIC90 (≤ 2 µg/ml). In contrast, elevated trimethoprim MIC was detected for the *L. garviae* (MIC90 = 16 µg/ml) and *E. faecalis* (MIC90 ≥ 64 µg/ml) isolates. Resistance to chloramphenicol was detected in *E. faecalis* (30.8 %) and *S. aureus* (10.1 %) but not in *Str. dysgalactiae*, *L. garviae* and *Str. uberis*. The MIC90 of tiamulin for *S. aureus was* 1 µg/ml and ≤ 0.5 µg/ml for *Str. dysgalactiae* and *Str. uberis*. Three *Str. uberis* isolates showed decreased susceptibility to tiamulin with an MIC of 8 µg/ml, while the *E. faecalis* and *L. garviae* isolates showed a MIC90 ≥ 8 µg/ml. In our study, only 1 out of the 287 *S. aureus* isolates tested showed resistance to fusidic acid (0.3 %). Elevated MIC (MIC90 ≥ 8 µg/ml) to fusidic acid was detected in *Str. uberis*, *E. faecalis* and *L. garviae*, which is consistent with the observation that *E. faecalis* and *Str. uberis* have an intrinsic resistance to this compound (Leclercq et al., 2011). Resistance to mupirocin was tested only for *S. aureus*, and no isolates showed resistance to this antibiotic. All of the *S. aureus*, *Str. dysgalactiae* and *E. faecalis* isolates were susceptible to rifampicin; *Str. uberis* had a low MIC90 value (MIC90 ≤ 0.25 µg/ml), and *L. garviae* isolates displayed an MIC90 ≥ 1 µg/ml (Tab. 1 to 5). For antibiotics used as last resort in human medicine, such as vancomycin, quinupristin/dalfopristin and linezolid, all isolates except for 1 *Str. uberis* isolate (MIC 4 µg/ml for quinupristin/dalfopristin) were susceptible, with low MIC90 values (Tab. 1, 2, 3 and 5). Intrinsic resistance is indicated in Tables 1 to 5.

Discussion

In our study, resistance to penicillin was found to be the most prevalent resistance in *S. aureus* (16 %). Penicillin resistance of *S. aureus* isolates from mastitis samples has been described previously (Bennedsgaard et al., 2006; Sakwinska et al., 2009; Persson et al., 2011). Sakwinska et al. (2009) reported penicillin resistance in 26 % of *S. aureus* strains (n = 343) isolated from Swiss and French farms. In contrast, a study from Sweden reported that only 4 % of *S. aureus* strains isolated from subclinical mastitis samples were penicillin-resistant and rarely resistant to other antimicrobials (Persson et al., 2011). A study in Denmark reported a moderate prevalence (12 %) of penicillin-resistant *S. aureus* in cows with high somatic cell counts (Bennedsgaard et al., 2006). Our study also demonstrated the presence of MRSA in Swiss bovine mastitis milk. MRSA are resistant to all ß-lactam antibiotics, including cephalosporins, and are often resistant to other classes of drugs, such as aminoglycosides and macrolides. The MRSA strain detected in our study was identified as ST398-t011 type and was also found to be resistant to tetracyclines but not to aminoglycosides or macrolides. This livestock-associated clone is distributed worldwide and frequently found in pigs (Tavakol et al., 2012). MRSA in Swiss mastitis samples was first described by Monecke et al. (2007), and other reports of studies on bulk milk samples have been published more recently (Huber et al., 2010; Büttner and Overesch 2012). Based on our data, although the actual prevalence of MRSA in mastitis samples is very low (0.4 %), the isolation of this clone indicates that MRSA strains are present in mastitis milk, thereby highlighting the importance of antibiogram analysis and susceptibility monitoring. Most of the *S. aureus* strains were found to be susceptible to aminoglycosides, macrolides and fluoroquinolones, of which aminoglycosides and macrolides are antibiotics frequently used for mastitis treatment in Switzerland (Büttner et al., 2011). A significant proportion (4.8 %) of *Str. uberis* isolates showed decreased susceptibility (MIC 0.25 µg/ml) to penicillin. German, French and American studies have also reported decreased penicillin susceptibility of *Str. uberis* strains isolated from mastitis samples, interestingly, the latter 2 countries showed a much higher proportion of strains with decreased susceptibility with

44 % and 46 %, respectively (Guerin-Faublee et al., 2002; Rossitto et al., 2002; Minst et al., 2012). Alterations in the penicillin-binding protein gene of *Str. uberis* leading to decreased penicillin susceptibility can be achieved *in vitro* by repeated exposures to penicillin, and consistent with this hypothesis, field strains of *Str. uberis* exhibiting an MIC ≥ 0.25 µg/ml were found to have mutations in the penicillin-binding protein gene (Haenni et al., 2010a). This observation is alarming because *Str. uberis* has now emerged as the major mastitis pathogen strain worldwide (Rossitto et al., 2002; Bradley et al., 2007; Botrel et al., 2010; Haenni et al., 2010c). In addition, *Str. uberis* isolates showed decreased susceptibility to aminoglycosides, and 10.6 % of the *Str. uberis* isolates tested in this study were already resistant to macrolides. Resistance to macrolides and clindamycin has been shown to be associated with the presence of *erm*(B) in *Str. uberis* (Haenni et al., 2010b). Resistance to aminoglycosides and macrolides has already been reported in Swiss *S. uberis* isolates and in several international studies (Roesch et al., 2006; Minst et al., 2012; Rato et al., 2013). It is important to note that no clinical breakpoints exist currently for *Str. uberis*, and more representative MIC values of relevant veterinary pathogens such as *Str. uberis* are needed to determine epidemiological cut-offs and clinical breakpoints in the future. Drug resistance and decreased drug susceptibility limits therapeutic options for *Str. uberis*, which has been reported to represent 21 % of the intramammarian infections caused by streptococci (Guélat-Brechbuehl et al., 2010). Similarly, *Str. dysgalactiae* isolates were found to be resistant to macrolides and aminoglycosides. However, decreased susceptibility to penicillin was not observed in this *Streptococcus* species. In general, a lower number of *E. faecalis* and *L. garviae* are isolated from mastitis milk regularly, and the strains tested were generally susceptible to antibiotics. Although significantly high gentamycin and streptomycin resistance was not detected in *E. faecalis*, comparatively lower resistance to macrolides was observed in this strain. In our study, *L. garviae* did not show resistance to aminoglycosides and macrolides, although resistance of *L. garviae* to macrolides has previously been reported from Swiss mastitis milk (Walther et al., 2008). The pathogens tested did not harbour resistance to antibiotics that are not licensed for intra-mammary infections except for chloramphenicol, fusidic acid and clindamycin. Resistance to clindamycin is generally associated with the methylase gene *erm*, which confers cross-resistance to macrolides, lincosamides and streptogramins antibiotics (Roberts et al., 1999). The presence of chloramphenicol resistance is most likely due to co-selection during the use of other antibiotics due to the presence of several genes on the same element (Schwarz et al., 2001). Resistance to fusidic acid was found in only 1 *S. aureus* strain. While no resistance to fluoroquinolones was detected in *S. aureus* and *E. faecalis*, 1 strain of *Str. dysgalactiae* exhibited decreased susceptibility to ciprofloxacin (MIC 4 µg/ml), indicating that fluoroquinolone resistance is emerging in this mastitis pathogen. Fluoroquinolones are extremely important antibiotics for human medicine, and therefore, their restrictive use in veterinary medicine has been recommended (WHO, 1998). Resistance to antibiotics used in human treatments, such as mupirocin, vancomycin, rifampicin, quinupristin/dalfopristin and linezolid, was not detected. Of note, 2 out of 13 *E. faecalis* strains showed decreased susceptibility to vancomycin (MIC 4 µg/ml), and 1 *Str. uberis* isolate showed decreased susceptibility to quinupristin/dalfopristin (MIC 4 µg/ml). Vancomycin-resistant enterococci (VRE), which cause nosocomial infections in hospitals and intensive care facilities, are important pathogens in human health. Because animals are known to be reservoirs of VRE, the presence of VRE in milk should also be assessed (Nilsson, 2012). Therefore, it is important to evaluate the resistance of pathogens to antibiotics that are used for mastitis treatment and are critically important for the treatment of infections in humans.

Conclusions

Resistance to classes of antibiotics commonly used for mastitis treatment is present among mastitis pathogens. However, this study showed that penicillins and aminoglycosides still represent good therapeutical options for the treatment of infections caused by gram-positive bacteria. Particulary, due to the increase of methicillin-resistant staphylococci in bovine mastitis milk (Frey et al., 2013), combinations with aminoglycosides should be considered instead of using critically important antibiotics, like cephalosporins, macrolides and fluoroquinolones. The use of these antibiotics can be avoided if antibiograms are consulted. Because of the higher percentage of resistance to tetracyclines, these antibiotics should only be used for the treatment of specific bacteria and only after susceptibility testing.

Such approaches are crucial for the prudent use of antibiotics in dairy cows to avoid the application of critically important antibiotics. The use of these antibiotics has been shown to be important risk factors for the selection of multi-resistant bacteria (Faires et al., 2009). A nationwide monitoring program, based on antibiogram analysis and random sampling, is necessary to understand the evolution of resistance in mastitis pathogens. Such an effort would also contribute to better management of the disease and help limit the spread of multi-resistant bacteria within the herd, the dairy cow population, the environment and humans.

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Sensibilité aux antibiotiques des germes gram positifs causant des mammites en Suisse

Dans cette étude, on étudie la sensibilité des germes gram positifs causant des mammites, tels que *S. aureus*, *Str. uberis*, *Str. dysgalactiae*, *E. faecalis* und *L. garviae*, vis à vis d'une part d'antibiotiques à signification thérapeutique et, dautre part, vis à vis d'antibiotiques d'importance épidémiologique et d'antibiotiques de réserve en médecine humaine. On constate le plus souvent des résistances face à la pénicilline chez *S. aureus* mais environ 5 % des souches de *Str. uberis* présentaient également une sensibilité réduite à cet antibiotique. On a en outre constaté des résistances face aux aminoglycosides et aux macrolides. L'identification de *S.aureus* résistants à la méthicilline (MRSA) et d'un *Str. dysgalactiae* résistant à la ciprofloxacine démontre que les germes de mammites développent des résistances face aux antibiotiques de réserve importants ainsi que l'importance de la réalisation d'un antibiogramme avant un traitement antibiotique. Une surveillance de la situation de résistance et une interprétation des résultats des antibiogrammes sont indispensables pour effectuer un traitement antibactérien ciblé pour éviter l'utilisation inutile d'antibiotiques de nouvelle génération.

Sensibilità agli antibiotici degli agenti patogeni gram-positivi della mastite bovina in Svizzera

In questo studio si è analizzata la sensibilità degli agenti patogeni gram-positivi della mastite come *S. aureus*, *Str. uberis*, *Str. dysgalactiae*, *E. faecalis* e *L. garviae* contro, da una parte, gli antibiotici con rilevanza terapeutica e gli antibiotici di interesse epidemiologico, dall'altra, gli antibiotici di riserva in medicina umana. Per *S. aureus* è stata dimostrata una resistenza più frequente alla penicillina e ca. il 5 % di ceppi di *Str. uberis* hanno pure comprovato una diminuita sensibilità a questo antibiotico. Sono state ancora constatate resistenze contro aminoglicoside e macrolide. La rilevazione di *S. aureus* resistente alla meticillina (MRSA) e di ceppi di *Str. dysgalactiae* resistenti alla ciprofolcacina dimostrano che gli agenti patogeni della mastite sviluppano delle resistenze contro i principali antibiotici di riserva e sottolineano l'importanza della realizzazione di antibiogrammi prima della terapia antibiotica. Una sorveglianza della situazione di resistenza e una valutazione dei risultati di antibiogrammi sono urgentemente necessarie alfine di procedere a una terapia antimicrobica mirata per evitare l'inutile uso di antibiotici della nuova generazione.

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