Antimicrobial resistance of *Staphylococcus aureus* isolated from bovine milk in Italy from 2005 to 2011

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SUMMARY

Introduction - Bovine mastitis is a major cause of economic losses in the dairy sector and *Staphylococcus aureus* represents the most prevalent and contagious aetiologic agent, resulting responsible for about one-third of clinical and subclinical mastitis worldwide. β-lactams, lincosamides and macrolides are among the most recommended antimicrobial agents for treating mastitis and increasing level of acquired resistance to penicillin G, lincomycin, erythromycin, gentamycin and streptomycin has been reported. Therefore, antimicrobial susceptibility tests are important for the selection of the most appropriate antimicrobial agent for treatment of bovine mastitis caused by *S. aureus*.

Aim - The aim of this retrospective study was to evaluate the antimicrobial resistance rates and the trend in resistance of *S. aureus* strains isolated from bovine with clinical or sub-clinical mastitis in Italy from 2005 to 2011.

Materials and methods - A total of 1,200 *S. aureus* isolates recovered from milk samples from bovine with clinical or sub-clinical mastitis were collected from 2005 to 2011 at the Diagnostic Sections of Piacenza of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna and tested for antimicrobial susceptibility by the disk diffusion method.

Results and discussion - High resistance rates were observed mostly for penicillin G, erythromycin and lincomycin, while a high *in vitro* activity was showed by trimethoprim-sulfamethoxazole and cefquinome, confirming these antimicrobial agents as an alternative choice for the treatment of bovine mastitis. Resistance showed a significant increasing trend for cephalotin, cefquinome, enrofloxacin, lincomycin, nafcillin, oxacillin and tetracycline, while resistance to amoxicillin/clavulanic acid, cefoperazone, erythromycin, penicillin G and trimethoprim-sulfamethoxazole did not change significantly over the study period.

Conclusions - Periodic surveillance for antimicrobial resistance of *S. aureus* isolated from dairy cows with mastitis is strongly recommended as an important component of prudent antimicrobial use practices.

KEY WORDS

Staphylococcus aureus / bovine mastitis / antimicrobial resistance / trend / Italy.

INTRODUCTION

Bovine mastitis is the most prevalent disease in dairy herds worldwide, with average incidence rates of 30-50% reported in many countries, and decrease in milk production due to clinical and subclinical mastitis is widely recognized as the main cause of the economic losses in the dairy sector[1]. Among the several different causes of intra-mammary infections in dairy cattle, *Staphylococcus aureus* represents the most prevalent and contagious aetiologic agent, resulting responsible for about one-third of clinical and subclinical mastitis worldwide[2]. In addition to the causative infective agent, the environment is another major factor involved in mastitis representing a considerable reservoir of pathogens, so that "environmental mastitis" is more prevalent than contagious transmission between animals in some countries[3].

Although the environmental factors may be controlled through appropriate management practices, such as the use of proper milking procedures and culling of chronically infected animals, antimicrobial therapy continues to be a primary tool to control staphylococcal mastitis. β-lactams, particularly penicillin G, are considered as first choice for treating mastitis due to penicillin-susceptible *S. aureus*, while lincosamides and macrolides represent an alternative in case of β-lactamase-producing isolates[4]. Although *S. aureus* isolated from bovine mastitis is generally reported as susceptible to antimicrobials commonly used in animal husbandry, including β-lactams, macrolides, lincosamides and aminoglycosides[5], increasing level of acquired resistance to penicillin G, lincomycin, erythromycin, gentamycin and streptomycin has been reported[6]. Moreover, multi-resistant strains, defined as strains resistant to at least three different antimicrobial classes[7], have recently emerged, mainly due to the rapid spread of methicillin-resistant *S. aureus* (MRSA) and the detection of methicillin- and multi-resistant *S. aureus* in milk or dairy cattle have recently expanded in many countries[8]. Therefore, antimicrobial susceptibility tests are important for the selection of the most appropriate antimicrobial agent for treatment of bovine mastitis caused by *S. aureus*.

The aim of the present study was to retrospectively investigate the trends in antimicrobial resistance of *S. aureus* strains isolated from bovine with clinical or sub-clinical mastitis in Italy from 2005 to 2011.

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MATERIALS AND METHODS

Bacterial strains

A total of 1,200 *S. aureus* isolates, recovered from milk samples from bovine with clinical or sub-clinical mastitis were included in this study. The isolates were collected from 2005 to 2011 at the Diagnostic Sections of Piacenza of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, which routinely receives more than 40,000 milk samples a year from bovine farms located in central and northern Italy. No more than one isolate of *S. aureus* from the same herd per year was included in the study. Animals have not been treated with antimicrobial agents in the 3 weeks prior to sample collection. Bacterial strains were isolated on Aesculin Blood Agar (Oxoid, Milano, Italy) at 37°C for 24 h and routinely identified on the basis of colony morphology, Gram staining, and free coagulase test (Coagulase Plasma EDTA, Biolife Italiana, Milano, Italy). Identification was confirmed by using the API ID32STAPH system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. All isolates were immediately subcultured on Brain Heart Infusion broth and subjected to antimicrobial susceptibility testing.

Antimicrobial susceptibility testing

S. aureus isolates were routinely tested for their susceptibility to a panel of antimicrobials by the disk diffusion method[9] on Mueller Hinton Agar (Oxoid, Milano, Italy). The following antimicrobial agents were tested: amoxicillin/clavulanic acid (20 μg/10 μg), cephalotin (30 μg), cefoperazone (75 μg), cefquinome (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), lincomycin (15 μg), penicillin G (10 IU, International Units), oxacillin (1 μg), nafcillin (1 μg), tetracycline (30 μg), and trimethoprim/sulfamethoxazole (1.25/23.75 μg). The choice of antimicrobials to be tested over the seven-year period was based on the requests of submitting veterinarians, as well as on the basis of specific fields requirements. This resulted in a "core number" of 703 out of 1,200 *S. aureus* isolates which were tested for susceptibility against all the considered antimicrobials, while the remaining 497 isolates were tested with a variable number of the above mentioned antimicrobials. Regular quality assurance by using the American Type Culture Collection reference strain of *S. aureus* (ATCC 25923, Oxoid, Milano, Italy) was performed. Isolates were classified as resistant or susceptible towards the tested antimicrobials in accordance with breakpoints proposed by the Clinical and Laboratory Standards Institute[10,11,12], except for enrofloxacin and lincomycin for which standards and criteria of the Comité de l'Antibiogramme de la Société Française de Microbiologie were applied[13]. Intermediate isolates were grouped with the resistant ones.

Data and statistical analyses

Antimicrobial resistance rate of *S. aureus* was calculated for each year as the number of resistant/intermediate isolates divided by the total number of tested isolates for a given antimicrobial. The χ^2 test to determine the trend of resistance of *S. aureus* towards the selected antimicrobials over the seven-year period was performed. A trend was considered statistically significant for $P<0.05$. The regression coefficient, or slope, was provided as well. The slope represented the mean change in the antimicrobial susceptibility rate for every 1-year increase in time. The direction of the trend was indicated by the sign of the regression coefficient, a negative slope indicating a decrease in susceptibility over time, while a positive slope indicating an increase in susceptibility over time. Statistical analyses were performed using the GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

The resistance rates and the trends in resistance of *S. aureus* isolates towards individual antimicrobials are shown in Table 1. In 2011, the tested isolates resulted resistant mostly to lincomycin (92.6%) and penicillin G (63.1%), as well as to erythromycin (41.7%), tetracycline (37.5%), enrofloxacin (36.9%) and cefoperazone (36.1%), while only 3.3% showed resistance to trimethoprim-sulfamethoxazole. Isolates showed a statistically significant increasing trend of resistance over the whole study period to cephalotin (from 2.4 to 16.4%), cefquinome (from 1.2 to 12.3%), enrofloxacin (from 5.9 to 36.9%), lincomycin (from 90.5 to 92.6%), nafcillin (from 1.2 to 16.4%), oxacillin (from 2.9 to 18.9%) and tetracycline (from 15.3 to 37.5%). No significant changes in resistance rates ($P\geq0.05$) from 2005 to 2011 for amoxicillin/clavulanic acid, cefoperazone, erythromycin, penicillin G and trimethoprim-sulfamethoxazole were found.

The antimicrobial resistance and multi-resistance patterns of the "core number" of 703 out of 1,200 *S. aureus* isolates are summarized in Tables 2 and 3, respectively. Forty-six isolates (6.5%) showed no resistance towards all the tested antimicrobial agents. Sixty-seven different resistance patterns were observed: 21.8%, 26.3%, 24% and 11.5% of the isolates resulted resistant to one, two, three and four antimicrobials, respectively, while 9.8% showed resistance from five to twelve antimicrobial agents (Table 2). Multi-resistance was detected in 216 out of 703 *S. aureus* strains of the "core number" (30.7%) (Table 3). The large majority of multi-resistant isolates showed resistance towards three (18.8%) and four (7.9%) antimicrobial classes, respectively, while less than 4% showed resistance from five to seven antimicrobial classes. The most prevalent multi-resistance pattern, shown by 73 isolates, included resistance towards β-lactams, lincosamides and macrolides.

DISCUSSION

This study reports on the antimicrobial resistance rates and the trend in resistance of *S. aureus* isolates collected from bovine with clinical or sub-clinical mastitis in Italy between 2005 and 2011. Antimicrobial agents are frequently used in cattle for the therapy of many disease conditions, included mastitis that represents the most common reason for antimicrobial treatment of dairy cows[4]. As a result, the extensive use of antimicrobials in cattle and the consequent selective pressure have intensified the risk of the emergence of resistant bacteria. In the present study, the antimicrobial agents most commonly used for the treatment of *S. aureus*-caused bovine mastitis were taken into account, including those suggested as first (β-lactams) and alternative choice (macrolides, lincosamides) according with the guidelines of prudent use of antimicrobials[4].

Table 1 - Antimicrobial resistance rates of 1,200 S. aureus strains isolated from bovine mastitis.

Antimicrobials	% of resistant isolates[a] (no. of tested isolates)								Statistical analysis	
	2005	2006	2007	2008	2009	2010	2011	total	*P*[b]	R[c]
Penicillins										
amoxicillin/clav. ac.	5.3 (170)	9.9 (232)	8.8 (194)	10.8 (194)	11.6 (121)	8.7 (46)	12.3 (81)	9.4 (1,038)	0.08	0.6
nafcillin	1.2 (170)	1.7 (234)	7.8 (206)	5.6 (197)	12.1 (140)	13.3 (128)	16.4 (122)	7.3 (1,197)	<0.0001	0.96
oxacillin	2.9 (170)	5.1 (235)	10.2 (206)	17.3 (197)	27.1 (140)	18.8 (128)	18.9 (122)	13.1 (1,198)	<0.0001	0.83
penicillin G	61.8 (170)	72.6 (234)	71.4 (206)	56.3 (197)	61.4 (140)	59.4 (128)	63.1 (122)	64.5 (1,197)	0.052	-0.41
Cephalosporins										
cephalotin	2.4 (170)	1.7 (233)	9.3 (204)	8.2 (196)	10.7 (140)	14.1 (128)	16.4 (122)	8.0 (1,193)	<0.0001	0.96
cefoperazone	28.2 (170)	50.2 (235)	50.0 (206)	40.6 (197)	37.7 (138)	28.1 (128)	36.1 (122)	40.2 (1,196)	0.08	-0.28
cefquinome	1.2 (170)	0.9 (235)	5.3 (206)	7.2 (194)	8.6 (139)	2.3 (128)	12.3 (122)	4.9 (1,194)	<0.0001	0.71
Tetracyclines										
tetracycline	15.3 (170)	21.3 (235)	16.7 (204)	27.4 (197)	37.1 (140)	30.5 (128)	37.5 (120)	25.1 (1,194)	<0.0001	0.89
Fluoroquinolones										
enrofloxacin	5.9 (170)	9.0 (234)	8.7 (206)	16.2 (197)	35.7 (140)	26.6 (124)	36.9 (122)	17.5 (1,193)	<0.0001	0.91
Macrolides										
erythromycin	50.6 (170)	40.5 (232)	39.2 (204)	39.1 (197)	45.0 (140)	47.7 (128)	41.7 (120)	42.9 (1,191)	0.76	-0.11
Lincosamides										
lincomycin	90.5 (169)	84.3 (235)	77.5 (204)	82.7 (197)	95.7 (140)	89.8 (128)	92.6 (122)	86.5 (1,195)	0.018	0.43
Potentiated sulfa drugs										
trimethoprim/ sulfamethoxazole	3.5 (170)	2.6 (234)	2.9 (206)	5.6 (195)	1.4 (139)	4.7 (128)	3.3 (122)	3.4 (1,194)	0.7	0.12

[a] Intermediate isolates were grouped with the resistant ones.
[b] χ^2 for the trend (a trend was considered statistically significant for $P<0.05$).
[c] Regression coefficient.

At present, β-lactams are among the most widely used antimicrobial classes for treatment and prevention of bovine mastitis[14]. Although the efficacy of systemic administration of penicillins has been shown in several clinical trials[15,16], the massive use of these antimicrobials has been proposed to exert a selection pressure favoring the emergence and the spread of penicillin-resistant *S. aureus* strains. As a consequence, high rates of mastitis-causing *S. aureus* that are resistant to penicillin G have been repeatedly reported worldwide[17,18] and changes in prevalence of resistance with time have been identified. After a rapid trend of increase, the percentage of resistant strains has not changed over time or slightly decreased[17]. Accordingly, more than 60% of *S. aureus* isolates included in our study were penicillin G-resistant, although no statistically significant changes over time were observed. On the contrary, the combination of amoxicillin with clavulanic acid, a β-lactamase inhibitor, revealed a high *in vitro* activity over time, with less than 10% of overall resistant isolates. Although information on the susceptibility of *S. aureus* from bovine milk to amoxicillin/clavulanic acid is rare in the literature, our results are similar to those already reported[5]. Low level of resistance was observed also for nafcillin and oxacillin, two β-lactamase-resistant penicillins frequently used to treat infections caused by penicillin G-resistant staphylococci. Considering that oxacillin disk diffusion represents a *marker* of methicillin-resistance in staphylococci[12], the significant increase of oxacillin-resistant isolates observed in the considered seven-year period, which reached 19% in 2011, suggests that MRSA could be an emerging problem in dairy herds[5,19]. Detection of MRSA is of key importance as methicillin-resistance is associated with resistance towards all the β-lactams, often in addition to a large number of non-β-lactam antimicrobials, therefore making infections extremely difficult to treat[20]. However, it must be emphasized that the present study is limited in terms of its methodology for the detec-

Table 2 - Antimicrobial resistance patterns of 703 *S. aureus* strains isolated from bovine milk.

No. of antimicrobial agents	Antimicrobial resistance patterns (number of isolates)	Resistant isolates	
		n	%
0	No resistance	46	6.5
1	L (103); P (44); E (3); Z (2); C (1); F (1)	153	21.8
2	LP (94); EL (60); PZ (13); LT (6); FL (4); EP (2); CL (1); CP (1); ET (1); FP (1); IP (1); LZ (1)	185	26.3
3	LPZ (96); ELT (23); ELP (21); EFL (11); LPT (6); PTZ (3); ELZ (2); CLP (1); EPZ (1); FLP (1); FLT (1); FPT (1); ILP (1); LQZ (1)	169	24
4	ELPZ (48); LPTZ (9); ELPT (7); EFLT (6); FLPZ (3); ELTZ (2); CELT (1); CLPZ (1); EFLP (1); EFTZ (1); EPTZ (1); FLPT (1)	81	11.5
5	ELPTZ (21); EFLPZ (6); EFLPT (4); CELPZ (2); EILPZ (2); CEFLT (1); EFLQT (1); EFLTZ (1); ILNPZ (1)	39	5.5
6	EFLPTZ (5); CELPTZ (2); CEFLPZ (1); EFILPZ (1); EILPTZ (1); EFLPQZ (1); ELOPTZ (1)	12	1.7
7	CEFLPTZ (4); EFILPTZ (2)	6	0.8
9	ACEFLNOPZ (1); AILNOPQTZ (1)	2	0.3
10	AEILNOPQTZ (4)	4	0.6
11	AEFILNOPQTZ (3)	3	0.4
12	ACEFILNOPQTZ (3)	3	0.4

A: amoxicillin/clavulanic acid; I: cephalotin; Z: cefoperazone; Q: cefquinome; C: cotrimoxazole; F: enrofloxacin; E: erythromycin; L: lincomycin; P: penicillin G; O: oxacillin; N: nafcillin; T: tetracycline.

Table 3 - Multi-resistance of 703 *S. aureus* strains isolated from bovine milk.

No. of antimicrobial agents	Antimicrobial resistance patterns (number of isolates)	Resistant isolates	
		n	%
0-2	No multi-resistance	487	69.3
3	LA-LI-MA (73); LI-MA-TE (23); LA-LI-TE (16); FQ-LI-MA (11); FQ-LA-LI (4); LA-LI-PS (2); FQ-LA-TE (1); FQ-LI-TE (1); LA-MA-TE (1)	132	18.8
4	LA-LI-MA-TE (36); FQ-LA-LI-MA (9); FQ-LI-MA-TE (6); LA-LI-MA-PS (2); FQ-LA-LI-TE (1); FQ-LA-MA-TE (1); LI-MA-PS-TE (1)	56	7.9
5	FQ-LA-LI-MA-TE (16); FQ-LA-LI-MA-PS (2); LA-LI-MA-PS-TE (2); FQ-LI-MA-PS-TE (1)	21	2.9
6	FQ-LA-LI-MA-PS-TE (7)	7	0.9

LA: β-lactams; FQ: fluoroquinolones; LI: lincosamides; TE: tetracyclines; MA: macrolides; PS: potentiated sulfonamides.

tion of MRSA. The use of genotypic methods to detect the presence of *mecA* gene is considered to be the *gold standard* while performing a single phenotypic test could lead to false-negative or false-positive results[21].

First-generation cephalosporins usually demonstrate good to excellent activity against Gram-positive bacteria[22]. Similar to previous research[19], low resistance rates to cephalotin were observed in our study. Not surprisingly, high level of resistance was observed towards cefoperazone, confirming the moderate activity of third generation cephalosporins against Gram-positive bacteria[22]. Cefquinome, a fourth generation cephalosporin approved only for veterinary use[23], resulted the most active β-lactam among those tested in the present study, although a significant increasing trend of resistance was observed in the seven-year period. Our results are in agreement with those of previous reports that already suggested that cefquinome might be of value in the treatment of *S. aureus* mastitis[24]. Nevertheless, according to the EU position on the prudent use of antimicrobials, fourth generation cephalosporins are recognized as critically important antimicrobials in human medicine and their veterinary use should be reserved for the treatment of clinical conditions which have responded poorly, or are expected to respond poorly, to other classes of antimicrobials[25].

Macrolides and lincosamides are considered as second-line antimicrobial agents in bovine mastitis treatment and cross-resistance between the two antimicrobial classes has been reported due to a similar mechanism of action against the 50S subunit of the bacterial ribosome[26]. The resistance rates observed in our study for erythromycin and lincomycin were considerably higher than those reported in other countries[18], with 35% of the "core number" of isolates showing cross-resistance between lincosamides and macrolides. Considering that 57% of the macrolides/lincosamides-resistant isolates resulted also resistant to penicillin G, this finding may represent a potential risk of therapy failure of bovine mastitis caused by penicillin-resistant *S. aureus*[4].

Tetracyclines have been used extensively to treat animal infections because of their relative safety and broad-spectrum activity[27]. Nevertheless, increasing resistance rates in *S. aureus* strains from bovine mastitis have been reported[6]. Accordingly, a relatively high level of resistance and a significant increasing trend of resistance in the considered seven-year period were found for tetracycline in our study. The low le-

vel of tetracycline *in vitro* activity against *S. aureus*, together with its low bioavailability due to the irreversible binding with milk components, make the use of this antimicrobial of limited effectiveness in the control of bovine mastitis[27].
Although not specifically recommended for bovine mastitis treatment[4], potentiated sulphonamides are among the most used antimicrobials in bovine husbandry. In accordance with previous studies, which already reported low resistance rates to trimethoprim-sulphametoxazole for *S. aureus*[7], a relatively stable high degree of the *in vitro* activity of this antimicrobial combination was found in the present study.
High *in vitro* activity of enrofloxacin, a second generation fluoroquinolone, has been reported for *S. aureus*[18], nevertheless enrofloxacin-resistant isolates have been observed[5] maybe due to the frequent extra-label use of this drug for bovine mastitis treatment. Accordingly, in the present study resistance to enrofloxacin resulted particularly high in 2011 and a significant increasing trend was observed throughout the study period. However, as already stated for fourth generation cephalosporins, fluoroquinolones should be limited for use in cattle do to their importance in the treatment of severe and invasive infections in humans and, whenever possible, the use of this antimicrobial class should be based on *in vitro* susceptibility test results[28].

CONCLUSIONS

High resistance rates and a significant increasing trend in resistance between 2005-2011 towards antimicrobial agents commonly used for the treatment of mastitis caused by *S. aureus* were observed in the present study, as well as a relatively high rate of multi-resistant isolates. Although a successful control program should consider overall management practices, the use of antimicrobials continues to be a prerequisite for treatment of bovine mastitis. For this reason, periodic surveillance for antimicrobial resistance of *S. aureus* isolated from dairy cows with mastitis is strongly recommended as an important component of prudent antimicrobial use practices.

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