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Article

Recent Trends of Antibiotic Resistance in *Staphylococcus aureus* Causing Clinical Mastitis in Dairy Herds in Abruzzo and Molise Regions, Italy

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Abstract: This study was aimed to examine the recent trends of antibiotic resistance (AR) prevalence in *Staphylococcus aureus* isolated from milk of animals with clinical mastitis in areas of the Abruzzo and Molise regions in central Italy.

Fifty-four *S. aureus* isolates could be obtained from routine testing for clinical mastitis agents carried out in the author institution in years 2021 and 2022. These were analyzed for phenotypic resistance to eight antibiotics recommended for testing by European norms and belonging to the antibiotic classes used for mastitis treatment in milk producing animals. Moreover, the presence of 14 transferable genetic determinants encoding resistance to the same antibiotics was analyzed by qPCR tests developed in this study.

Phenotypic resistance to non-β-lactams was infrequent, with only one 2022 isolate resistant to clindamycin. However, low level resistance to the β-lactam cefoxitin was observed in 59.2% isolates in both years making these isolates classifiable as methicillin resistant.

The AR genotypes detected were *bla*Z gene (50% 2021 isolates and 44.4% 2022 isolates), *erm*C/T-*aph*A3-*bla*Z (one 2021 isolate), *ant*6-*erm*C/T-*aph*A3-*bla*Z (one 2021 isolate), *erm*B-*bla*Z (one 2022 isolate) and *mec*A-*mph* (one 2022 isolate).

An interview to the veterinarians who conferred the samples, regarding antimicrobials prescribed for mastitis treatment and criteria of usage, indicated a possible causal relation with the AR test results.

The low prevalence of AR genotypes, not increasing in time, most probably reflecting the reported management of antibiotic therapies in farms. However, the frequently observed cefoxitin resistance needs to be explained genotypically, further monitored and limited by modifying antibiotic usage practices. The identification of a *mec*A positive isolate in 2022 suggests to investigate further if this genotype is emerging locally.

Keywords: *Staphylococcus aureus*; clinical mastitis; antibiotic resistance (AR) prevalence; AR phenotype; AR genotype; recent trend

1. Introduction

Staphylococcus aureus is one of the main causative agents of mastitis in milk producing animals and the first for clinical bovine mastitis with ability to give origin to persistent intramammary infections [1]. This bacterial species is also a major human pathogen capable of causing food poisoning for the production of multiple heat-stable enterotoxins (SE), localized soft tissue or skin infections and systemic infections triggered by virulence factors comprising staphylococcal superantigens (SAgs) [2,3,4], cytotoxic proteins [5] and factors that favor colonization and immune evasion [6]. The emergence of antibiotic resistant (AR) *S. aureus* strains, among which the methicillin-resistant *Staphylococcus aureus* (MRSA) are listed by the World Health Organization among the pathogens of "high priority"

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against which new antibiotics are urgently needed, worsens the threat to human health posed by this bacterial species, since MRSA cause infections with high mortality rates [7]. The transferable genetic element conferring the MRSA phenotype is the chromosomal cassette *mec* (SCC*mec*), that most often carry the *mec*A or the *mec*C gene, and sometimes other rare homologues, encoding for additional penicillin-binding proteins (PBP2a) with reduced affinity for β-lactams plus genes for site-specific recombinases [8,9].

Use of antibiotics in the animal farming sector to treat conditions such as mastitis can select for MRSA transmissible to humans through raw milk and derived products [10]. Some risk factors have been identified for MRSA transmission in dairy farms, such as poor milking hygiene, while the role of antimicrobial usage has been little investigated, with the exception of one study reporting an increase in antibiotic minimum inhibitory concentration (MIC) values and in occurrence of AR genes *tet*K, *tet*M, and *bla*Z after enrofloxacin treatment of persistent mastitis in goats, underlining the role of antimicrobial usage on the emergence of AR *S. aureus* strains [11]. Phylogenetic analysis based on multi-locus sequence typing (MLST) put in evidence that some *S. aureus* lineages are found both in human and animal hosts, in particular strains from bovine mastitis, as a consequence of transfer from human to animal and *vice versa*. Moreover, it was demonstrated that *S. aureus* has the capacity to switch hosts [12] so that animal isolates *S. aureus* with resistance to antibiotics must be considered a threat to public health.

Investigating the trends of AR *S. aureus* prevalence can indicate if risk factors that favor their increase in farms are acting and allow to adopt measures to reduce the dissemination of the genetic determinants encoding resistance. Therefore, this study was undertaken to analyze the prevalence of AR *S. aureus* in farms by taking into account isolates from milk of animals affected by clinical mastitis requiring antibiotic treatment. The study was carried out in areas of the Abruzzo and Molise regions in Central Italy and aspects of mastitis management in the sampled farms were taken into account to explain the results of phenotypic and genotypic AR prevalence.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study were all isolates from mastitic milk samples analyzed upon request of veterinarians by the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM), Campobasso and Lanciano branches, for identification of the infectious agent and antibiogram execution. Strains phenotypically identified as *S. aureus* in routine analysis, 27 isolated in year 2021 and 27 in year 2022, were obtained after analysis of milk from 56 farms and 52 farms, respectively. These were propagated by streaking on blood agar (10/l g tryptose, 10 g/l meat extract, 5 g/l NaCl, 15 g/l agar, 100 ml of defibrinated sheep blood added aseptically after autoclaving and cooling of the base medium) incubated in aerobic conditions at 37°C for 24-48 h. Cell biomass from a colony isolated after two subsequent streaks on blood agar was used for each phenotypic or genotypic test. For long term storage the isolates were maintained in Microbank (Biolife Italiana, Milan, Italy) at -80°C.

2.2. Phenotypic AR testing

The antibiotics tested phenotypically were those of human usage belonging to the classes of antibiotics used for mastitis treatment and recommended for testing by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to deduce resistance to the same antibiotic classes [13], namely cefoxitin (FOX), clindamycin (CD), erythromycin (ERY), gentamicin (CN), kanamycin (KAN), norfloxacin (NOR), oxacillin (OXA) and tobramycin (TOB). The minimum inhibitory concentration (MIC) values were determined by using the Liofilchem® MIC Test Strips (Liofilchem, Roseto degli Abruzzi, TE, Italy) according to the instructions. The MIC values were assigned in accordance with EUCAST guidelines on antimicrobial suceptibility testing (AST) [14]. For norfloxacin resistance was defined by using discs with 10 µg of the antibiotic (Liofilchem) as recommended [13]. The reference to the epidemiological cut off (ECOFF) values [13] was used to define the position of the new isolates in the range of observed MIC values for the species *S. aureus*.

2.3. Quantitative PCR primer design

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New qPCR tests for the transferable AR genes encoding resistance to the antibiotics tested phenotypically were designed after and defining the most frequently occurring genes in *S. aureus* based on sequence database analysis. Oligonucleotides were designed by searching and aligning sequences in the NCBI databases (https://www.ncbi.nlm.nih.gov/, accessed on 1 October 2022) and in the National Database of Antibiotic Resistant Organisms (NDARO, https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/, accessed on 2 October 2022). For each gene a BLASTN analysis restricted to the *S. aureus* taxon was carried out in order to consider different variants to be aligned such to design oligonucleotides targeting all of them. The primer/probe systems designed in this study are listed in Table 1 with respective target genes and amplicon dimensions. These were synthetized by Eurofins Genomics (Ebersberg, Germany).

Table 1. Primers and probes designed in this study for the detection of AR genes, respective targets and dimensions of the amplification products.

Primer and probe labels and sequences (5′-3′)	Target gene	Amplicon size (bp)
AadA12f: CCTGGAGAGAGCGAGA AadA12p: FAM-TTTGGAGAATGGCAGCGCAATGAC-BHQ1 AadA12r: CTATGTTCTCTTGCTTTTGT	*aad*A12	197
AadA-aph2f: GGTAGTGGTTATGATAGTG AadA-aph2p: FAM-TAGAAACTAATGTAAAAATTCCTAA-MGBEQ AadA-aph2r: TTCTGGTGTTAAAAAAGTTCC	*aad*A-*aph*2	231
Aac6f: CCTTGCGATGCTCTATG Aac6p: Cy5-CCCGACACTTGCTGACGTACA-MGBEQ Aac6r: TCCCCGCTTCCAAGAG	*aac*6[b] (*aac*4)	204
Ant6f: GCGCAAATATTAATATACCTAAA Ant6P: Cy5-TGGGAATATAATAATGATG-MGBEQ Ant6r: GGGCAATAAGGTAAGATCA	*ant*6[b] (*aad*E)	157
Aph3f: TGGCTGGAAGGAAAGC Aph3p: FAM-TGATGGCTGGAGCAATCTGCT-BHQ1 Aph3r: TGTCGATGGAGTGAAAGA	*aph*3-III	184
BlaZf: AAGGTTGCTGATAAAAGTGG BlaZp: FAM-GTTTATCCTAAGGGCCAATCTGAACCT-BHQ1 BlaZr: AAATTCCTTCATTACACTCTTG	*blaZ*	182
Cfrf: AAAACCTAACTGTAGATGAGA Cfrp: Cy5-GATAGCATTTCTTTTATGGGAATGGG-BHQ1 Cfrr: TAAACGAATCAAGAGCATCA	*cfr*	138
ErmAf: GGTAAACCCCTCTGAGA ErmAp: Cy5-CATCAGTACGGATATTGTC-MGBEQ ErmAr: CCCTTCTCAACGATAAGA	*erm*A	177
ErmBf: TACTCGTGTCACTTTAATTCAC ErmBp: Cy5-CAGTTTCAATTCCCTAACAAACAGAGG-BHQ1 ErmBr: CCCTAGTGTTCGGTGAA	*erm*B	205
ErmCTf: AAATGGGTTAACAAAGAATACA ErmCTp: Cy5-GAATTGACGATTTAAACAATATTAGCTTTG-BHQ1 ErmCTr: TATTGAAAAGAGACAAGAATTG	*erm*C/T[a]	123
LnuBf: TAATTCTACCTTATCTAATCG lnuBp: FAM-GTTTAGCCAATTATCAGCAT-MGBEQ LnuBr: CGTTCATTAGAACTCTTATC	*lnu*B	113
MecAf: AGAAAAAGAAAAAAGATGGCAAA MecAp: FAM-CAACATGAAAAATGATTATGGCTCAG-BHQ1 MecAr: CTCATGCCATACATAAATGGA	*mec*A	184
mecA/Cf: ACWTCACCAGGTTCAAC mecA/Cp: Cy5-ATGGTAARGGTTGGCAAA-MGBEQ mecA/Cr: TCTGATGATTCTATTGCTTG	*mec*A/C[c]	194

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Mhpf: GGGACTTACATCCAGG Mphp: FAM-AAGCAAACGTCACAGGTCT-MGBEQ Mhpr: TCGTCGTCGAATACACG	*mhp*	134
MsrAF: CTTACCAATTTGAAAAAATAGCA MrsAp: Cy5-GGCAAAACCACATTACTAAATATGATTG-BHQ1 MsrAR: TTCACTCATTAAACTACCGT	*mrs*A	240

[a]this primer/probe system targets both *erm*C and *erm*T genes;
[b]these genes have alternative names in the sequence databases;
[c]this primer/probe system targets both *mec*A and *mec*C genes and, coupled with the *mec*A-specific test, can allow the detection of the *mec*C gene.

The gene regions comprised between each pair of oligonucleotides, ranging in size between 130 and 246 bp, were synthetized upon request by GenScript Biotech (Rijswijk, Netherlands) and delivered as pUC57 vector constructs to serve as positive controls in the qPCR runs.

2.4. DNA extraction

DNA was extracted from one loopful biomass resuspended in 200 µl of Macherey Nagel T1 buffer (Carlo Erba, Cornaredo, MI, Italy) containing 100 mg of sterile 200 µm diameter glass beads in safe lock Eppendorf tubes (Eppendorf). The suspension was bead beaten in a TissueLyser II (Qiagen) at 30 hz for 2 min). Then 200 µl of Macherey Nagel B3 buffer (Carlo Erba) were added and the extraction was continued according to the Macherey Nagel Nucleospin Tissue (Carlo Erba) protocol.

2.5. Quantitative PCR conditions

The qPCR reactions were carried out in a QuantStudio 5 thermal cycler (Thermo Fisher Scientific, Rodano, MI, Italy). Identification of isolates at the species level was carried out as described by Poli et al. [15]. For AR gene detection a unique program suitable for all the primer/probe systems designed was used. This comprised initial denaturation at 94°C for 5 min and 40 cycles of denaturation at 94°C for 15 s and annealing at 51°C for 30 s. The qPCR reaction of 20 µl volume comprised 10 µl of Takara Premix Ex Taq (Probe qPCR) (Diatech, Jesi, AN, Italy), 0.2 µM primers and probe, TaqMan Exogenous Internal Positive Control Reagents (Thermo Fisher Scientific) in the recommended concentration, 2 µl of DNA sample and Nuclease Free water (Thermo Fisher Scientific) to the final volume. Four nanograms of synthetic positive control construct was used in the positive control reaction.

2.6. Veterinarian questionnaire

The 18 veterinarians who requested the bacteriological examinations and antibiograms for mastitis diagnosis in years 2021 and 2022 were interviewed to identify the antibiotic classes prescribed, the criteria adopted for antibiotic usage and different aspects of mastitis management in farms by delivering a questionnaire with closed ended questions.

2.7. Statistical analyses

MIC values plots, Student t test evaluation of distinctness of MIC data series obtained in 2021 and 2022 and correlation analyses were carried out by using PAST 4.03 free statistical software downloaded from https://past.en.lo4d.com/windows (accessed on 23 December 2022). Data series were considered distinct for $P<0.05$.

3. Results and discussion

3.1. *Rate of mastitis caused by* S. aureus *in 2021 and 2022*

The number of farms with clinical mastitis caused by *S. aureus* were 16 among 56 analyzed in 2021 and 13 among 52 analyzed in 2022, accounting for percentages of 28.5% and 25% of mastitis outbreaks, respectively. More than one isolate was obtained from the same sample if colonies of different dimension, appearance and hemolysis halo aspect were observed on blood agar. This led to obtaining 27 isolates for each year. All the isolates were received already phenotypically identified and identification was confirmed by qPCR targeted on the *nuc*A gene [15]. The isolates are listed according to year and farm of isolation in Table 2, section 3.3, reporting also the variable AR phenotypes and genotypes observed.

3.2. *Phenotypic AR of* S. aureus *isolates*

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In this study isolates with cefoxitin MIC 6 – 8 µg/ml accounted for 59.2% isolates in both years. According to the EUCAST indications [13] these isolates are resistant to this antibiotic, though at low levels, and must be considered methicillin resistant. However, all of them were sensitive to oxacillin and only one isolate from 2022 showed a MIC equal to the ECOFF for this antibiotic. The same isolate was also resistant to clindamycin (*S. aureus* isolate 2022 6, Table 2). The percentages of isolates assigned to groups with different cefoxitin MIC values in years 2021 and 2022 is shown in Figure 1.

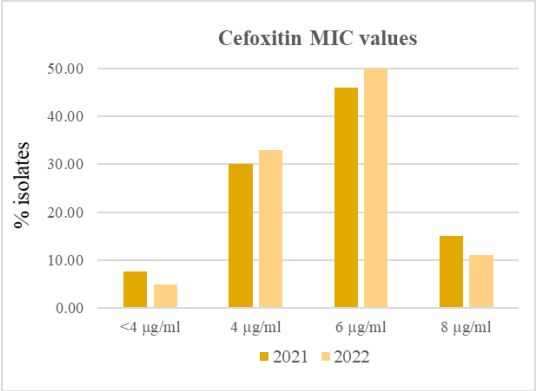

Figure 1. Percentages of isolates with different MIC values for cefoxitin in years 2021 and 2022.

An high correlation with (r=0.99) was found between the numerosity of groups with different MIC values for cefoxitin in the two years, thus indicating that there was very little variation in the distribution of the isolates among different cefoxitin resistance levels in the investigation period.

The distribution of MIC values for all antibiotics tested in the two years is shown in Figure 2.

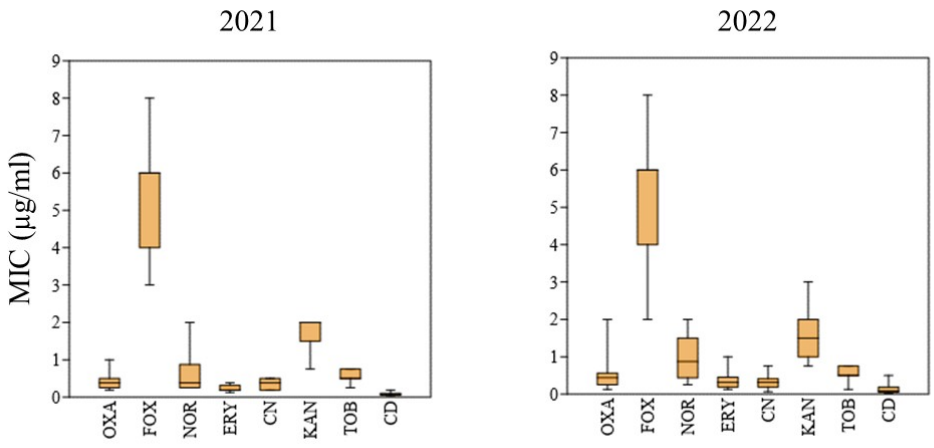

Figure 2. Distribution of MIC values for oxacillin (OXA), cefoxitin (FOX), norfloxacin (NOR), erythromycin (ERY), gentamicin (CN), kanamycin (KAN), tobramycin (TOB) and clindamycin (CD) in years 2021 and 2022.

The MIC distributions for *S. aureus* isolates between years 2021 and 2022 were not statistically distinct for any of the antibiotics considered. However, it can be noted that MICs for norfloxacin showed a shift to higher values in 2022, though resistance was not detected by the disc diffusion assay. Nevertheless, this trend could be indicative of an

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increase in resistance to quinolones that in *S. aureus* is mediated by the core gene *nor*A encoding different variants of an efflux pump, and other efflux systems [16]. These are increasingly expressed under antimicrobial pressure and can lead to the emergence of resistance phenotypes [17]. Accordin to the MIC values, all the isolates were susceptible to oxacillin, norfloxacin, erythomycin, gentamicin, kanamycin, tobramycin and clindamycin, except isolate *S. aureus* 2022 6 (Table 2).

The percentage of isolates resistant to cefoxitin observed in this study was among the highest reported for European countries [18]. On the other hand, an increase in AR to the other antimicrobials tested was not observed though it was reported to occur globally for *S. aureus* strains causing bovine mastitis or isolated from milk and dairy products, specifically to clindamycin, erythromycin, gentamycin and oxacillin [19,20].

3.3. Occurrence of AR genes in the S. aureus *isolates*

The AR genes sought in this study were those encoding AR to the antibiotics of interest and found most frequently in *S. aureus*, as deduced from consultation of the sequence databases and from a recent survey on identity and frequency of AR genes in 29,679 genomes of *S. aureus* isolated worldwide [21]. In addition, the *cfr* gene was sought in this study since it codes for a 23S rRNA methyltransferase that confers resistance to different antibiotic classes, among which lincosamides, beyond phenicols [22].

The AR gene most frequently detected in this study was *bla*Z, present in 59.2% 2021 isolates and in 48.1% 2022 isolates (Table 2).

Table 2. List of isolates in year 2021 or 2022 with respective AR phenotypes for the antibiotics for which resistance (R) was detected, i.e. cefoxitin (FOX) and clindamycin (CD), and genotypic AR profiles.

2021			2022		
Farm/ Isolate*	**AR phenotype**	**AR genotype**	**Farm/ Isolate***	**AR phenotype**	**AR genotype**
1			1 1	FOX R	*bla*Z
2	FOX R		1 2	FOX R	*bla*Z
3	FOX R		2 1		
4 1	FOX R	*bla*Z	2 2		
4 3	FOX R		3 1	FOX R	*erm*B, *bla*Z
5	FOX R	*erm*CT, *aph*3, *bla*Z	3 2	FOX R	
6 1	FOX R		4	FOX R	*bla*Z
6 2	FOX R		5	FOX R	
7	FOX R	*ant*6, *erm*CT, *aph*3, *bla*Z	6	FOX R, CD R	*mec*A, *mph*
8 1	FOX R	*bla*Z	7		*bla*Z
8 2	FOX R	*bla*Z	8 1		
8 3	FOX R	*bla*Z	8 2		*bla*Z
9	FOX R		8 3		
10	FOX R		8 4		
11			9		
12	FOX R		10 1	FOX R	*bla*Z
13		*bla*Z	10 2	FOX R	*bla*Z
14 1	FOX R	*bla*Z	10 3	FOX R	*bla*Z
14 2	FOX R	*bla*Z	11 1		*bla*Z
15 1		*bla*Z	11 2		*bla*Z

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15 2	*bla*Z	11 3		*bla*Z
15 3		12 1	FOX R	
16 1	*bla*Z	12 2	FOX R	*bla*Z
16 2	*bla*Z	12 3	FOX R	
16 3	*bla*Z	13 1	FOX R	
16 4	*bla*Z	13 2	FOX R	
16 5	*bla*Z	13 3	FOX R	

*the first one or two numbers indicate the farm, while the last number preceded by a space is the isolate number for cases in which more that one isolate was obtained from the same milk sample.

Differences in the occurrence of this gene between the two years were not statistically significant according to the Student's t test. In investigations carried out in different countries this gene was found at high frequencies with a maximum of 95.7% [23].

Only a few other genetic determinants were identified in the isolates studied here. In particular, the *mec*A gene was found only in one 2022 isolate, indicating a frequency lower than reported in other studies [24,25,26,27] but similar to that reported in Southern Italy for bulk tank milk of small ruminants [28] indicating that its prevalence can vary on a local basis. The *mec*A positive strain was not resistant to oxacillin, showing an MIC equal to the *S. aureus* ECOFF for this antibiotic. The occurrence of *mec*A positive and oxacillin sensitive strains was reported recently [29].

According to the EUCAST AST guidelines *S. aureus* strains resistant to cefoxitin have an MIC>4 μg/ml, a value that coincides with the ECOFF of the species for cefoxitin, and in most cases harbor a *mec*A or *mec*C gene [12]. However, in this study cefoxitin resistance was not associated to the presence of the *mec*A or the *mec*C gene, resulting in an example of *mec*-independent β-lactam resistance phenotype. The occurrence of cefoxitin resistant isolates without the *mec* genetic determinants was described previously [30,31] and different genetic features were found to determine the cefoxitin resistance phenotype [32,33,34] so further investigations should be devoted to definig the genetic basis of cefoxitin resistance in the isolates obtained in this study.

Other AR genes occurring in the *S. aureus* isolates examined, namely *aph*3'-III, *ant*6-Ia, *erm*B, *erm*C/T and *mph*, with the exception of *erm*B found in a 2022 isolate harboring only this gene, were found mostly in association with other AR determinants (Table 2). In particular, MDR genotypes *ant*6-Ia-*aph*3-III-*bla*Z-*erm*C/T and *aph*3-III-*bla*Z-*erm*C/T were found each in one 2021 isolate. The occurrence of multiresistance encoding mobile genetic elements should be investigated in these isolates.

The gene *mph* for resistance to macrolides was found in the sole *mec*A positive strain. This strain was also resistant to clindamycin, possibly for the presence of a genetic determinant different from the genes *lnu*B and *cfr*, tested in this study. The finding that isolates harboring AR genes were susceptible to the antibiotics for which resistance was encoded suggests to carry out experiments to elucidate if those genes can be induced upon gradual exposure to antimicrobials.

A high prevalence of the *bla*Z gene was observed in this study but none of the isolates overexpressed *bla*Z to levels determining a borderline oxacillin resistant *S. aureus* (BORSA) phenotype [12].

3.4. Evaluation of antibiotic management by veterinarian interview

In order to understand if the results of AR screenings might be linked with a causal relation with the antibiotic usage practices adopted locally, the 18 veterinarians providing medical care to the sampled farms were interviewed by a questionnaire regarding antibiotics used, farm hygiene and the criteria adopted for antibiotic use decision in clinical mastitis. The results of the interviews are presented in Table 3.

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Table 3. Answers (%) given by veterinarians providing medical care to the farms considered in this study to a questionnaire with closed ended questions on antibiotic usage and farm hygiene.

Question	% answers*
1. Antibiotic classes prescribed	
Aminoglycosides (gentamicin, neomicin, kanamycin)	11
Penicillins (ampicillin, amoxicillin/clavulanic acid, penicillin)	50
Cephalosporins (cefalexin, cefoperazone)	28
Lincosamides (lincomycin-spectinomycin)	11
Fluoroquinolones (enrofloxacin)	50
Macrolides (spiramycin, tylosin)	11
2. Hygiene conditions in farms	
Excellent	0
Good	39
Acceptable	50
Inadequate	11
3. Milking hygiene	
Excellent	0
Good	50
Acceptable	50
Inadequate	0
4. Mastitis prevention measures	
Excellent	0
Good	39
Acceptable	39
Inadequate	28
5. Reason for bacteriological examination and antibiogram request for mastitis cases	
Always	0
In most cases	11
For severe infections	0
For recidivating mastitis	78
After treatment failure	11
6. Protocol of antibiotic usage adopted	
Always	0
In most cases	39
Frequent	39
Rare	11
None	11
7. Evidences of AR	
Frequent	11
Rare	89
None	0
8. Measures adopted for AR management	
Infectious disease expert consultation	0
Therapy against specific infectious agents	100
Reduction of antibiotic usage	50

*some professionals gave multiple responses to questions 1, 5 and 8.

It is possible to observe that all the antibiotic classes allowed for mastitis treatment were used but β-lactams and fluoroquinolones prevailed. This could explain the high prevalence of strains harboring *blaZ* genes and the increase in norfloxacin MIC values. A further continuation of the antibiotic usage stated could enhance the trends observed.

Answers to the other aspects considered in the interview might indicate low usage of antibiotics since hygiene in the farm was considered good or acceptable in most cases and milking hygiene was found to be adequate in all instances. These conditions reduce the occurrence of infections and the need for antibiotic treatment. In addition, most farms

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were reported to adopt adequate mastitis prevention measures and protocols for antibiotic usage. Notably, half of the interviewed veterinarians declared to be committed to the reduction of antibiotic usage and all of them declared to use antibiotics based on the antibiogram outcomes for the specific pathogens.

However, according to the statements, strains causing mastitis were isolated and tested by antibiogram only in case of recidivating mastitis or in case of treatment failure and this could imply that initial treatments carried out without antibiogram execution can select for antibiotic resistant strains that become difficult to eradicate. Changes in this practice, together with improvements of mastitis management, could reduce prevalence of AR *S. aureus* in farms.

4. Conclusions

This study showed that the prevalence of both genotypic and phenotypic AR is currently low for non-β-lactam antibiotics and with no increasing trend in *S. aureus* isolates from the areas of Abruzzo and Molise considered. This is probably the consequence of overall good farm and milking hygiene, as reported by veterinarian professionals interviewed. However, strains harboring β-lactam resistance *blaZ* genes, already known to be widespread in the species *S. aureus*, occurred frequently, probably for the preferential use of β-lactams in mastitis therapy. Phenotypic resistance to cefoxitin in *mec*A/C negative isolates was frequent and its genetic basis needs to be identified. Moreover, the occurrence of one MRSA and two genotypically MDR isolates suggested to continue monitoring the presence of these AR profiles in dairy herds to understand if these genotypes tend to disseminate.

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