ANTIMICROBIAL RESISTANCE OF *STREPTOCOCCUS AGALACTIAE* AND *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM BOVINE MILK OVER THE PERIOD 2009-2011 IN THE AOSTA VALLEY REGION (ITALY) *

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SUMMARY

As part of the bovine mastitis control program conducted in the Aosta Valley Region (Italy) in the period 2009-2011, 574 strains of *Streptococcus agalactiae* and 933 strains of *Staphylococcus aureus* were isolated. All strains were analyzed for antimicrobial susceptibility by the Kirby-Bauer method, based on the evaluation of the inhibition zones around the following compounds: amoxicillin, amoxicillin and clavulanic acid, ampicillin, cephalexin, cephalothin, cefoperazone, cefuroxime, cloxacillin, enrofloxacin, neomycin, cloxacillin, penicillin, spiramycin, streptomycin, tetracycline and thiamphenicol. For *Streptococcus agalactiae* the most significant resistance (> 10 % of not-sensitive strains) concerned cloxacillin (54.6 %), neomycin (81.4 %) and tetracycline (25.5 %), while for *Staphylococcus aureus* it concerned amoxicillin (41.7 %), amoxicillin and clavulanic acid (40.2 %), ampicillin (41.8 %), penicillin (41.4 %), cloxacillin (42.6 %), tetracycline (13.9 %) and cefoperazone (15.2 %). Given the high levels of resistance against beta-lactam antibiotics and - through these - especially natural and semi-synthetic penicillins (or rather the molecules most frequently indicated for the pharmacological treatment of mammary infections), regular monitoring of the susceptibility of udder pathogens bacteria to this category of antimicrobials appears essential for success of a mastitis control program in the geographical area considered by our study.

Keyword: *Streptococcus agalactiae*, *Staphylococcus aureus*, Bovine mastitis, Aosta Valley Region (Italy).

RÉSUMÉ

Dans le cadre du programme de lutte contre les mammites bovines menée dans la Région Vallée d'Aoste (Italie) dans la période 2009-2011, ont été isolées 574 souches de *Streptococcus agalactiae* et 933 souches de *Staphylococcus aureus*. On a mesuré par la méthode de Kirby-Bauer, la sensibilité de toutes ces souches aux antimicrobiens suivants : amoxicilline, amoxicilline et acide clavulanique, ampicilline, céphalexine, céphalothine, cefoperazone, céfuroxime, cloxacilline, enrofloxacine, néomycine, cloxacilline, pénicilline, spiramycine, streptomycine, tétracyclines et thiamphénicol. Pour *Streptococcus agalactiae* les résistances les plus importantes (> 10 % des souches insensibles) concernaient la cloxacilline (54,6 %), la néomycine (81,4 %) et la tétracycline (25,5 %), tandis que, pour *Staphylococcus aureus*, elles concernaient l'amoxilline (41,7 %), l'amoxicilline /acide clavulanique (40,2 %), l'ampicilline (41,8 %), la pénicilline (41,4 %), la cloxacilline (42,6 %), la tétracycline (13,9 %) et le cefoperazone (15,2 %).

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Compte tenu du niveau élevé des résistances aux antibiotiques bêta-lactamines et aux pénicillines naturelles et semi-synthétiques (ou plutôt les molécules les plus fréquemment prescrites dans le traitement des infections mammaires), un suivi régulier de la sensibilité des bactéries pathogènes de la mamelle à cette catégorie d'agents antimicrobiens est essentiel pour assurer le succès du traitement des mammites dans la zone géographique considérée par notre étude.

Mots-clés : *Streptococcus agalactiae*, *Stahylococcus aureus*, mammite bovine, région Vallée d'Aoste (Italie).

I - INTRODUCTION

Mastitis is one of the burden of greatest health and economic impact on the dairy cow. Each case of sub-clinical mastitis costs to the farmer approximately $ 110/year [Seegers *et al*., 2002], a significant economic loss due to various factors such as reduction in milk production, abnormal milk composition (decrease of fat and protein resulting in difficult caseification), loss (for wasting) of milk contaminated by antibiotics used for therapy, premature culling (or death, in the event of severe mastitis) of animals in production and especially the cost of veterinary interventions and drugs. The use of antibiotics, which constitute the elective therapy for the treatment and prophylaxis of bovine intramammary infections, is definitely the most expensive item for the farm budget.

However, beyond this purely financial aspect, pharmacological treatments may result in a damage even more serious, namely the selection of antibiotic-resistant strains with potential risk of transmission to humans of bacteria not-sensitive to antimicrobial molecules Such eventuality occurs mostly when, for reasons of time or cost saving, the antibiotic is used in an empirical way, without prior testing for the definition of antibiotic sensitivity of the involved bacterial strain. Although the phenomenon is more connected to the poultry and pig farming, where the use of antibiotics is more massive, some studies report a relative spread of antibiotic resistance among bacteria responsible for mastitis. Given that many of the 130 species of bacteria recognized as potential cause of mastitis [Watts, 1988] can sometimes give rise to minor zoonoses, the development of resistant strains determines not only a risk for public health - by transmission to humans through raw milk - but also, in many cases, the failure of mastitis' therapy. In view of these aspects, in Valle d'Aosta region (Northeast Italy) for about a decade has been active a plan to combat bovine mastitis by seeking the udder pathogens in the milk of the cows in lactation. With particular regard to contagious bacteria, the most recent results reveal that in this territory the prevalence of farms infected by *Streptococcus agalactiae* has decreased from 28.2 % in 2009 to 18.2 % in 2011 while that of *Staphylococcus aureus* has remained almost unchanged with a mean prevalence of 38 % in the period of the study [Domenis *et al.*, 2012]. The purpose of this paper is to present the data on the resistance of strains of *Streptococcus agalactiae* and *Staphylococcous aureus* - detected during the 2009-2011 period - in respect of the antibiotics most commonly used in the treatment of mammary infections in the cattle.

II - MATERIAL AND METHODS

Through the program aganst mastitis carried out in the period 2009-2011, 574 strains of *Streptococcus agalactiae* and 933 strains of *Staphylococcus aureus* were isolated from mastitis milk samples. Each strain was analyzed to evaluate the antimicrobial susceptibility in agreement with the Kirby Bauer method described in CLSI guidelines. Using a sterile inoculating loop or needle, we touched four or five isolated colonies of organisms to be tested; then the colonies were suspended in 2 ml of sterile saline solution and the tube was vortexed to create a smooth suspension (adjusting the turbidity to 0.5 McFarland standard). A sterile swab was dipped into the inoculum solution and rotated against the side of the tube using firm pressure, to remove the excess fluid. Mueller-Hinton Agar (for strains of *Staphylococcus aureus*) or Blood Mueller-Hinton Agar (for strains of *Streptococcus agalactiae*) was inoculated by streaking the swab three times over the entire agar dried surface. Appropriate antimicrobial-impregnated disks were placed on the surface of the agar, using forceps to dispense each antimicrobial disk one at a time. Then, all plates were incubated at 37°C for 22 to 24 hours. Kirby-Bauer test protocol provides for the measurement of inhibition zone of microbial growth around the disk. Using a caliper each zone was measured with the unaided eye while viewing the back of the petri dish. In particular, we have tested the following molecules pharmacologically actives against different bacteria: amoxicillin, amoxicillin and clavulanic acid, ampicillin, cephalexin, cephalothin, cefoperazone, cefuroxime, cloxacillin, enrofloxacin, neomycin, cloxacillin, penicillin, spiramycin, streptomycin, tetracycline and thiamphenicol. On the recording sheet, depending on the inhibition zone size, the strain was classified as susceptible (S), intermediate (I), or resistant (R) for each molecule, compared to the reference values. The data regarding the prevalence and antibiotic resistance of *Streptococcus agalactiae* and *Staphylococcus aureus* were finally examined with the Cochran-Armitage trend test to evaluate the linearity of the trends in the three years. For this purpose we used the software SAS@9.2

III - RESULTS

For *Streptococcus agalactiae* resistances the most significant (> 10 % of not-sensitive strains) concerned cloxacillin (54.6 %), neomycin (81.4 %) and tetracycline (25.5 %), while for *Staphylococcus aureus* amoxicillin (41.7 %), amoxicillin and clavulanic acid (40.2 %), ampicillin (41.8 %), penicillin (41.4 %), cloxacillin (42.6 %), tetracycline (13.9 %) and cefoperazone (15.2 %). See table 1 and Graphs 1 and 2 for all results.

IV - DISCUSSION

Regarding the susceptibility to antibiotics, the most significant resistances, in other words these with more than 10 % of the strains not sensitive, involved the following molecules: cloxacillin, neomycin, streptomycin and tetracycline for *Streptococcus agalactiae*, amoxicillin, amoxicillin and clavulanic acid, ampicillin, penicillin, cefoperazone, cloxacillin and tetracycline for *Staphylococcus aureus*. About *Streptococcus agalactiae*, the percentage of strains resistant to neomycin records a statistically significant increase from 2009 to 2011 (Test for trend: 5.041 p <.0001), whereas for cloxacillin (Test for trend: 2.25 p <.02) and tetracycline (Test for trend: 3.63 p <.0003) appears statistically significant the reduction.

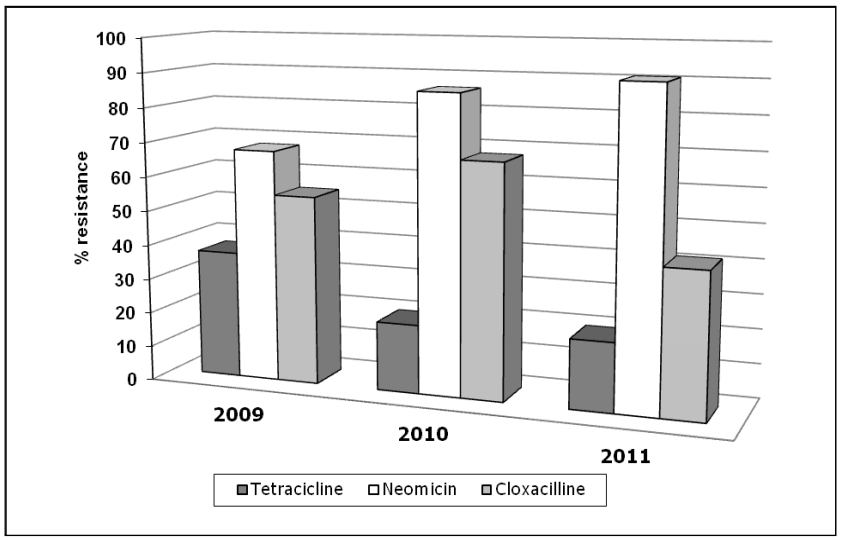
Table 1

Prevalence of resistance strains of *S. aureus* and *S. agalactiae* in the 2009-2011 period

	***S. agalactiae* (%)**			***S. aureus* (%)**		
	2009	**2010**	**2011**	**2009**	**2010**	**2011**
Amoxicillin	2.4	2.0	1.8	39.7	38.1	47.2
Amoxicillin+Clavulanic Ac.	3.3	2.0	1.2	37.9	37.9	44.9
Ampicillin	2.3	2	1.8	39.3	38.9	47.3
Cephalexin	4.8	3.3	1.2	8.6	8.8	10.8
Cefalotine	2.9	4.5	0.6	6.9	8.4	7.4
Cefoperazone	2.7	1.3	0	10.3	11.5	23.7
Cefuroxime	1.9	0.7	0	6.5	4.9	3.5
Cloxacilline	54.8	67.6	41.6	42.5	54.4	31
Enrofloxacin	4.1	0.7	2.4	0.9	0.8	0.8
Neomicin	67.5	86.1	90.6	1.5	1.6	1.7
Oxacillin	5.9	4.6	1.8	7.8	5.6	6.0
Penicillin	7.4	2.7	4.2	40.1	37.1	46.3
Spyramicin	6.2	4.7	93.0	2.5	3.6	3.9
Streptomicin	91.1	88.1	93.0	9.8	5.2	5.9
Tetracycline	36.9	19.9	19.9	13.7	13.2	14.9
Thiamphenicol	3.9	1.3	1.9	6.9	6.4	7.4

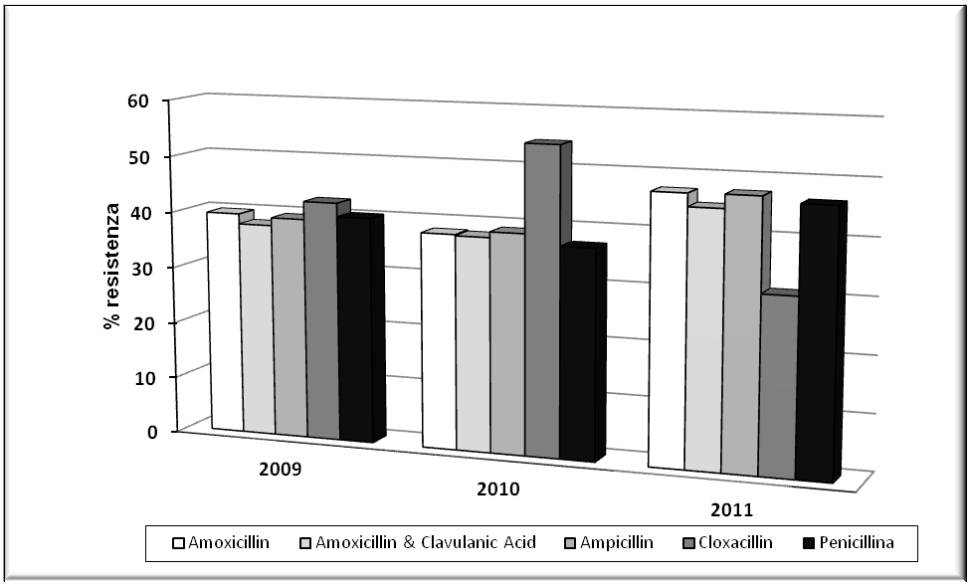
Graph 1

Prevalence of resistant strains of *S. agalactiae* to tetracycline, neomycin cloxacillin during 2009-2011



Graph 2

Prevalence of resistant strains of *S. aureus* to penicillins during 2009-2011



The values of sensitivity, especially as regards the penicillins, appear similar to those found in other European and non- European geographical areas: in USA, among 71 strains of *Streptococcus agalactiae* no one has highlighted resistance to penicillin [Roberts and Brown, 1994]; in Poland, Malinowski *et al.* [1992] showed that 86.7 % of *Streptococcus agalactiae* strains was sensitive to penicillin, while a Canadian study [Messier *et al*., 1994] has reported that no one of 68 strains of *Streptococcus agalactiae* isolated from bovine udder showed resistance to penicillin and erythromycin; in France, among 8 *Streptococcus agalactiae* strains isolated from bovine mastitis all were sensitive to penicillin, while only 62.5 % to tetracycline [Guérin-Faublée *et al*., 2002]. Regarding the high prevalence of strains resistant to streptomycin, it is likely that this is linked to a natural resistance of *Streptococcus agalactiae* to this molecule, depending on some mutation of the ribosomal proteins [Whittem and Hanlon, 1997].

As far as *Staphylococcus aureus*, it is worth noting the increase of strains resistant to cefoperazone (third generation cephalosporin), while stable but very high remains the prevalence of strains resistant to tetracycline and especially to natural or semi-synthetic penicillin; only for cloxacillin the percentage of resistant strains show a linear trend of reduction statistically significant (Test for trend: 3.34 p <.0008). The data are similar to that already mentioned by other authors, starting from Italy: in Veneto, among the strains of *Staphylococcus aureus* isolated from bovine mastitis in the period 1996-1999, 63 % of isolates in 1996 and 53 % in 1999 was penicillin-resistant [Barberio *et al*., 2001]; in Sweden, Bengtsson *et al.* in 2009 found that the penicillin resistance of *Staphylococcus aureus* is largely unchanged for the last 25 years (about 7 % of the strains isolated) in contrast to coagulase-negative staphylococci for which it is registered a clear decrease in non-susceptibility to this principle from 1995 (25.7 %) to 2009 (12.5 %), year of publication of the study; in Finland, through a national surveillance study conducted for defining the prevalence of mastitis, 32 % of the strains of *Staphylococcus aureus* was identified resistant to penicillin [Pitkälä *et al.*, 2004]. As suggested by some Swedish authors [Aarestrup and Jensen, 1998], it is likely that even in the Valle d'Aosta the resistance to penicillins in staphylococci is due to the spread of not-sensitive clones (often producing beta-lactamase) by animal reservoir with unresolved infections, an event favored by selective pressure determined by the wide use of the antibiotics. In the latter regard, some authors attribute the development of resistance to beta-lactams in staphylococci to the practice, particularly widespread, of performing a preventive treatment during the dry period [Botrel *et al.*, 2010]. As for *Streptococcus agalactiae*, also for the prevention of mastitis caused by *Staphylococcus aureus* is important the travel ban

and the separate milking of infected animals as well as the culling of the cows with chronic infections for which the therapeutic intervention is ineffective and it's more likely the presence of a multidrug-resistant strain. The decrease or increase of some antibiotic resistance observed for both contagious bacteria is probably to be ascribed to the different use in the years of the active principles: when there is an increase in sensitivity it may correspond to a greater aware in the use of the molecule and conversely a decrease in the prevalence of strains sensitive reveals a greater use of the molecule with a consequent increase of the selective pressure. Given the high levels of resistance against beta-lactam antibiotics and - through these - especially natural and semi-synthetic penicillins (the molecules most frequently Indicated for the pharmacological treatment of mammary infections), regular monitoring of the anti-microbial susceptibility of the udder pathogens bacteria is essential to ensure the success of the therapy's mastitis in this geographical area and, in the same time, to check the diffusion of multi-drug resistant strains. Nevertheless, it should be noted that the transmission of resistant strains through food or directly from animals can be a threat for human health. There are numerous reports of nosocomial infections especially in immunocompromised patients or neonates caused by strains of *Staphylococcus aureus*, methicillin-resistant for the most part, and *Streptococcus agalactiae* [Claier and Poyart al., 2003] characterized by non-susceptibility to antibiotics genetically based. However, considering that the processes of sterilization, pasteurization and caseification commonly used in the dairy industry compromise the viability of the microorganisms, the risk of transmission can be considered far lower than other food chains that end with the use of final uncooked products (eg. that of meat).

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