



MONITORING THE RESISTANCE PROFILE OF *Staphylococcus* sp. AND *mecA* GENE DETECTION OF *Staphylococcus aureus* FROM LACTATING COWS IN A DAIRY FARMING PROPERTY

Renata Olivotto Agostinis¹; Isabela Carvalho dos Santos²; Priscila Luiza Mello³; Maria de Lourdes Ribeiro de Souza da Cunha⁴; Lisiane de Almeida Martins^{5*}.

¹Mestre em Ciência Animal. Universidade Paranaense, Umuarama, PR.

²Bolsista do Programa de Pesquisa Básica e Aplicada da UNIPAR em Parceria com a Fundação Araucária.

³Docente da Universidade Guarulhos Ser Educaional. Guarulhos – SP.

⁴Instituto de Biociências – UNESP – Botucatu – SP.

^{5*}Autor correspondente: lisiane.almeida.martins@gmail.com

Universidade Paranaense - UNIPAR. Praça Mascarenhas de Moraes, s/n, Centro, Umuarama, Paraná, CEP 87502-10, Brasil.

Recebido em: 22/09/2018 – Aprovado em: 23/11/2018 – Publicado em: 03/12/2018
DOI: 10.18677/EnciBio_2018B121

ABSTRACT

The main mastitis causing agent is the *Staphylococcus*, and among them, the most pathogenic species is the *Staphylococcus aureus*. The present paper aims to verify the frequency of mastitis causing *Staphylococcus aureus*, trace the susceptibility profile from these to antibiotics and detect the *mecA* gene in *Staphylococcus aureus* from a dairy property in Umuarama-PR. Isolated samples used were taken from lactating cows in a seven months period. After the samples were isolated in blood agar and respectively identified, these were kept at -20 °C to further analysis (antibiograms, polymerase chain reaction to detect the *mecA* gene, beta-lactam detection and minimum inhibitory concentration detection). 189 samples were collected, and, from these, 30,68% showed no growth, 39,15% were negative coagulase *Staphylococcus*, 5,29% *Staphylococcus aureus*, 7,40% *Streptococcus* and 17,46% other micro-organisms (BGP, BGN, *Corynebacterium* and yeast). The antibiotics that showed higher resistance levels were sulfonamide, penicillin, ampicilin and tetracycline, meanwhile, the ones that showed higher sensibility levels were sulpha+trimethopim, gentamicin, ceftiofure and enrofloxacin. In the samples, 86,41% of the *Staphylococcus* sp. and 60% of the *Staphylococcus aureus* were multiresistant. All of the *S. aureus* samples were negative to the *mecA* gene and positive to the beta-lactam test.

KEYWORDS: Antibiogram, Mastitis, *mecA*, Multiresistant, MRSA.

MONITORAMENTO DO PERFIL DE RESISTÊNCIA DE *Staphylococcus* sp. E DETECÇÃO DO GENE *mecA* DE *Staphylococcus aureus* ISOLADOS DE VACAS EM LACTAÇÃO EM UMA PROPRIEDADE LEITEIRA RESUMO

O principal agente causador da mastite é o *Staphylococcus* e dentre estes a espécie mais patogênica o *Staphylococcus aureus*. O presente trabalho teve como objetivo verificar a frequência de *Staphylococcus* sp. causadores de mastite, avaliar o perfil de suscetibilidade de diversos antibióticos frente a esses isolados, além de detectar

o gene *mecA* em *Staphylococcus aureus* de um rebanho produtor de leite de uma propriedade em Umuarama-PR. Foram utilizadas amostras colhidas de vacas em lactação durante sete meses. Após o isolamento das colônias em ágar sangue e respectiva identificação do micro-organismo, estes foram mantidos a -20°C para posteriores análises (antibiogramas, reação em cadeia pela polimerase para detecção do gene *mecA*, detecção da beta-lactamase e determinação da concentração inibitória mínima). Das 189 amostras coletadas, 30,68% não houve crescimento, 39,15% foram *Staphylococcus* coagulase negativa, 5,29% *Staphylococcus aureus*, 7,40% *Streptococcus* sp. e 17,46% outros micro-organismos (BGP, BGN, *Corynebacterium* e levedura). Os antibióticos em que os agentes apresentaram maiores níveis de resistência foram sulfonamida, penicilina, ampicilina e tetraciclina, já os que apresentaram maior sensibilidade foram sulfa+trimetropin, gentamicina, ceftiofur e enrofloxacin. Foram encontrados 86,41% de *Staphylococcus* sp. e 60% de *Staphylococcus aureus* multirresistentes. Todas as amostras de *S. aureus* foram negativas para o gene *mecA* e positivas para beta-lactamase.

PALAVRAS-CHAVE: Antibiograma, Mastite, *mecA*, Multirresistência, SARM.

INTROCUCTION

Mastitis is the most important disease in milk production, due to the great impact that it generates as much by the loss of quantity as by the quality of the milk produced. Data from Embrapa, 2012, show that in 2008 Brazil produced 27 million liters of milk and the loss was calculated at R \$ 2.3 billion (EMBRAPA, 2012).

Among the microorganisms that cause intramammary infections of dairy cattle, *Staphylococcus* sp. are the most isolated microorganisms in clinical and subclinical mastitis (DE VLIEGHER et al., 2012). *Staphylococcus aureus* stands out because of its pathogenicity and because it causes contagious mastitis, that is, it can be transmitted during milking (SÁ et al., 2004). For this reason, it is important to have a strict control of this micro-organisms in dairy farms.

In order to guarantee control and low rate of infection of the herd, the immediate treatment of animals with clinical mastitis with antibiotics is indicated, and the treatment in the dry period for cases of subclinical mastitis (BRADLEY; GREEN, 2004).

The success of this therapy can be hampered by the high number of microorganisms resistant to some antibiotics, mainly due to the indiscriminate use of antibiotics in dairy cattle. For this reason, it is important to evaluate the resistance profile of the isolates, since this bad use has contributed to the appearance of multiresistant bacteria. It is worrisome worldwide that the prevalence of mastitis-causing multi-resistant *Staphylococcus aureus* (ZAFALON et al., 2008). The family of beta-lactams that was frequently used effectively in the treatment of intramammary infections in some herds is no longer as effective (AARESTRUP et al., 2001).

This phenomenon is partly due to strains called MRSA (methicillin-resistant *Staphylococcus aureus*), which are not only resistant to methicillin but also to other beta-lactams (ANVISA, 2005). Resistance to methicillin can be attributed to the presence of the *mecA* gene, which modifies penicillin-binding proteins (PBP's) (AARESTRUP et al., 2001).

Considering the possibility of transmission of *Staphylococcus* sp. multiresistants during milking and the great economic importance of milk production, this study aims to verify the frequency of *Staphylococcus* sp. of mastitis in a dairy

herd of a property in Umuarama-PR, to evaluate the susceptibility profile of the isolates against several antibiotics, to detect the presence of betalactamase and minimal inhibitory concentration of oxacillin in strains of *Staphylococcus aureus* and to detect the *mecA* gene in *Staphylococcus aureus*.

MATERIALS AND METHODS

A total of 189 samples of lactating dairy cows were collected from the Experimental Farm of the Paranaense University, located in the municipality of Umuarama, in the Northwest region of the state of Paraná, with an average of 27 animals (18 to 35 animals / month) dutch breed, with varying ages. The milking is mechanized in the shape of a fishbone, with no calf at the foot and performed twice a day.

During the period from September 2012 to March 2013, monthly collections of all lactating cows were carried out, totalizing seven collections, where from each one of them was collected milk of the four ceilings, obtaining a sample for each animal.

For the collection, the ceilings were washed with running water, dried with disposable paper towels and then 70% alcohol antiseptics was performed, the first jets were discarded, and then the milk was collected in sterile tubes, which were properly identified, stored in isothermal boxes and sent to the Laboratory of Preventive Veterinary Medicine and Public Health of the Master in Animal Science of the University of Parana for analysis.

In the laboratory, the samples were analyzed according to the National Mastitis Council (NMC, 2004). They were seeded in petri dishes containing base agar plus 5% defibrinated sheep blood, incubated in an incubator at 37° C for up to 72 hours and after growth, the colonies were identified (NMC, 2004).

On the identification, the colonies that presented themselves as Gram-positive cocci in the Gram staining method were subjected to the catalase and coagulase assay using rabbit plasma. The positive samples in both tests were subjected to the Voges-Proskauer (VP) test, and the positives were considered *Staphylococcus aureus*. All the *Staphylococcus sp.* were stored at -20 ° C for further analysis. For the freezing the samples were inoculated in 4 mL of Brain Heart Infusion (BHI) broth to incubation at 37° C for 24 hours; after the growth the samples were centrifuged at 2.500 rpm for 15 minutes, the supernatant were discarded and the sediment were suspended in 2mL of BHI. After, 0,5 mL of sterile glycerol were added and 1,2 mL of the samples were distributed in microvials for freezing.

All the strains of *Staphylococcus sp.* were subject to antibiogram using 12 antibiotics, which were selected as the most commonly used in herds and/or of public significance. They were, gentamicin (10µg), enrofloxacin (5µg), sulphonamide (300µg), penicillin (1µg), ampicillin (10µg), oxacillin (1µg), cephalothin (30µg), ceftiofur (30µg), clindamycin (2µg), tetracycline (30µg), erythromycin (5µg) and sulfamethoxazole-trimethoprim (25µg). For running the test, the colonies were diluted saline prior according to the Mac Farland scale (0,5), later they were sown with the aid of a swab in petri plates with Muller Hinton agar, and after were add the antibiotics disks above mentioned. These plates were incubate at 37° C for 24 hours and after this period was realize the reading of inhibition halos in millimeters (CLSI, 2008).

The *Staphylococcus aureus* were also tested for beta-lactamase production and determination of minimum inhibitory concentration (MIC) to the drugs by the e-test method. The detection of beta-lactamase production was made through the use

of disks impregnated with Nitrocefin (chromogenic cephalosporin-cefinase BBL. The disks were moistened with one to two drops of sterile distilled water and deposited on *Staphylococcus aureus* colonies previously incubated at 35 °C/24 h on the Mueller-Hinton agar plate with oxacillin E-test tape. The positive reaction was evidenced by the development of a red color and the negative by not changing color. For the correct analysis of the results, the discs in tests were always compared with the negative control. International reference lines were used as positive control (*Staphylococcus aureus* ATCC 33591 e *Staphylococcus aureus* ATCC 29213) and negative control (*Staphylococcus xylosus* ATCC 29979).

The determination of minimum inhibitory concentration (MIC) to drugs was realized by the E-test method. Were tested in vitro susceptibility to oxacillin from samples of *Staphylococcus aureus*. Therefore, was determinate the minimum inhibitory concentration (MIC) of this drugs through the E-test. This procedure is a quantitative method which uses inert plastic strip, transparent, measuring 60 mm in length by 5.5 mm in width, in which a stabilized concentration gradient of the antimicrobial to be investigated is incorporated. The concentration of the various drugs on E-test varies from 0,002 to 256µg /mL. The technical parameters involved in carrying out the E-test are: Mueller-Hinton culture medium, inoculum with final concentration similar to turbidity corresponding to McFarland scale 0.5; incubation of the plates in aerobiose at 35-37°C; reading by scaling the anterior portion of the tape of the value corresponding to the intersection of the ellipse zone of inhibition of bacterial growth. The results of the study of the MICs of the various drugs were expressed by means of: MIC 50% (drug concentration required to inhibit 50% of bacterial population tested); MIC 90% (drug concentration required to inhibit 90% of bacterial population tested); range of MICs and proportion of drug-sensitive samples, as defined by CLSI (2011).

The *Staphylococcus aureus* were subjected to PCR for *mecA* gene detection in the applied microbiology laboratory of the Department of Microbiology and Immunology in the Institute of Bioscience of UNESP – Botucatu Campus. For the DNA extraction, the total nucleic acid was extracted from samples of *Staphylococcus aureus*, cultivated in blood agar, individually inoculated in BHI and incubated at 37°C for 24h. The extraction was performed with the Kit Illustra ® (GE Healthcare) that consists on an initial digestion of staphylococcal cells with lysozyme (10 mg/mL) and proteinase K (20 mg/mL). Then, 500 µL from the lysis solution were added to the mixture, this was centrifuged at 5.000 x g for 1 min. After that the supernatant was transferred to a column and centrifuged at 11.000 x g for 1 min. The liquid was discarded and 500 µL of the lysis solution were add to the column again. After centrifugation at 11.000 x g for 1 minute and the discard of the liquid part, 500 µL of the washing solution were add to the column, and this was subjected to centrifugation at 11.000 x g for 3 min. After this, the column was transferred for a tube of 1, 5 mL and 200 µL of Milli Q water preheated to 70°C were employed to the elution. The samples were centrifuged at 5.000 x g for 1 min. the column was discarded. The extracted DNA were stocked at -20°C.

Then, the amplification of the DNA (PCR) was performed for detection of lines of Methicillin-resistant *Staphylococcus* (MRS) (*mecA*). The PCR reactions were performed into a 0, 5 ml microfuge tube in total volume of 25 µL containing 20 pmol of each primer, 2,5 U Taq DNA polymerases, 200 µM dNTPs, 20 mM of Tris-HCl, pH 8,4, 1,5 mM MgCl₂, and 3 µL of DNA. The incubation was performed in appropriate thermal cycler, using the primers *mecA*1 (AAA ATC GAT GGT AAA GGT TGG) and

mecA2 (AGT TCT GCA GTA CCG GAT TTG) – 533 (pb) employing the parameter described by Murakami et al. (1991): 40 denaturing cycles at 94°C for 30 seconds, primers ringing at 55,5°C for 30 seconds and extension at 72 °C for 1 min. After complete 40 cycles, the tubes were incubated at 72 °C for 5 min. before cooler at 4°C. In all the reactions performed were used lines of international reference: positive control (*Staphylococcus aureus* ATCC 33591) and negative (*Staphylococcus aureus* ATCC 25923). The equipment used were: Thermal cyler PTC-100 MJ Research (used in PCR). Made in vat from Loccus Biotecnologia LCH – 12X14, time for solidification before applying the samples to the gel: 15 minutes, Photodocumentation: transillumination: Ingenius Singene Bio Imaging (to take picture), Software: GeneSnap from Syngene (used to analyses the gel picture).

RESULTS AND DISCUSSION

During seven months 189 samples (100%) were collected, in 30,68% (58/189) there wasn't growing of microorganisms, 39,15% (74/189) were coagulase-negative *Staphylococcus*, 5,29% (10/189) *Staphylococcus aureus*, 7,40% (14/189) *Streptococcus* e 17,46% (33/189) other microorganisms (BGP, BGN, *Corynebacterium* and yeast).

The table 01 identifies the number of microorganisms isolated during the seven months of collection. There was significant difference between the microorganisms on the monthly collections demonstrated by the G test, because $p < 0,05$ ($p=0,0003$).

TABLE 01- Frequency of microorganisms isolated during the seven monthly collection performed in the period of September 2012 to March 2013. Umuarama, 2013

	M ₁	M ₂	M ₃	M ₄	M ₅	M ₆	M ₇	TOTAL
Month								
Bacteria								
Without growing	16	11	2	5	10	8	6	58
<i>Staphylococcus</i> coagulase-negative	12	10	17	7	10	11	7	74
<i>Staphylococcus aureus</i>	1	2	3	1	1	0	2	10
<i>Streptococcus</i>	0	6	1	0	2	3	2	14
Others (BGP, BGN, <i>Corynebacterium</i>, yeast)	3	4	7	5	0	2	12	33
TOTAL	32	33	30	18	23	24	29	189

The figure 01 allows accompanying the variability of each microorganism during the monthly collection allowing verify the influence of temperature and rain, among other factors that could interfere in the incidence of pathogens responsible for mastitis.

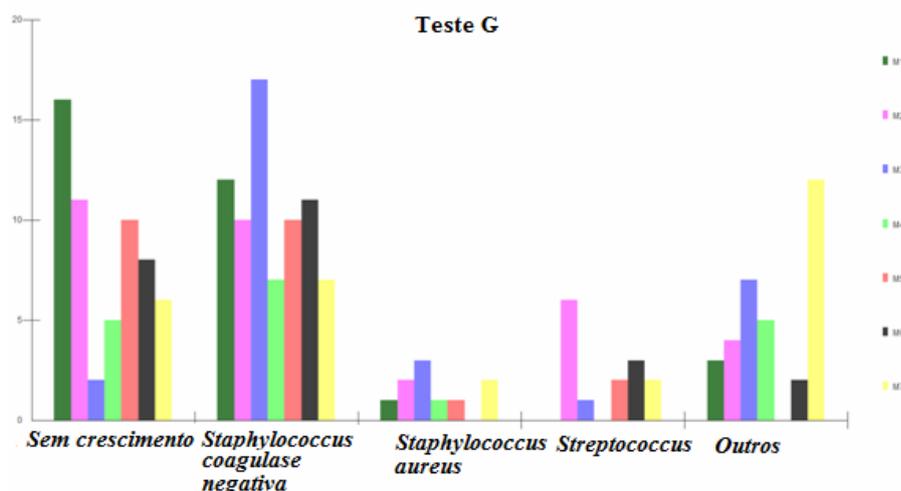


FIGURE 01. Variability of microorganisms isolated during the seven monthly collection performed in the period of September 2012 to March 2013 of a dairy farm from Umuarama, PR, 2013

Laranja; Machado (1994) observed that the most causers of mastitis are the *Staphylococcus* sp. (44,6%), followed by *Corynebacterium* sp. (15,0%), *Streptococcus* sp. (8,2%) and yeast/fungus (5,4%), what is alike to the present work with index of *Staphylococcus* sp. of 44,44%. Medeiros et al. (2009) studying 291 isolates from milk of herds from three regions of Northeast found 170 (58,4%) *Staphylococcus* coagulase-negative, 37 (12,7%) *Staphylococcus* coagulase-positive and 84 (28,9%) *Staphylococcus aureus*. In the present work the results found were 39,15% of *Staphylococcus* coagulase-negative and only 5,29% *Staphylococcus aureus*.

Nowadays the *Staphylococcus* coagulase-negative has been received more emphasis as causative agents of intramammary infections in dairy cattle in all world (GENTILINI et al., 2002; FREITAS et al., 2005), and in this the biggest number of microorganisms isolated were the *Staphylococcus* coagulase-negative.

For analyses the data concerning about the antibiograms we used only the samples of *Staphylococcus* coagulase-negative and *Staphylococcus aureus* (named by *Staphylococcus* sp. when analysed together), and *S. aureus* in isolation. The table 02 shows discriminately the frequency of *Staphylococcus* sp. and the table 03 shows the *Staphylococcus aureus* resistant, with intermediary sensibility and sensitive to each antibiotic.

TABLE 02. Antimicrobial sensitivity profile of *Staphylococcus* sp. samples isolated during the monthly collected performed in the period of September 2012 and March 2013. Umuarama, 2013.

ATB Action	Gent	Enro	Sulf	Peni	Amp	Oxa	Cefa	Ceft	Clin	Tetra	Eritro	Sulf+tri	total
R	23	3	78	61	60	47	28	4	45	52	47	23	468
I	6	3	0	0	0	1	6	10	8	8	11	7	60
S	52	75	3	20	21	33	47	67	28	21	23	51	441

Statistics: Degrees of freedom = 22; G test= 384,4; p < 0,0001

Gent: gentamicin; Enro: enrofloxacin; Sulf: sulphonamide; Peni: penicillin; Amp: ampicillin; Oxa: oxacillin; Cefa: cephalothin; Ceft: ceftiofur; Clin: clindamycin; Tetra: tetracycline; Eritro: erythromycin; Sulf+tri: sulfa+trimethoprim.

R: Resistant; I: Intermediary sensibility; S: Sensitive

TABLE 03. Antimicrobial sensitivity profile of *Staphylococcus aureus* samples isolated during the monthly collected performed in the period of September 2012 and March 2013. Umuarama, 2013.

ATB Action	Gent	Enro	Sulf	Peni	Amp	Oxa	Cefa	Ceft	Clin	Tetra	Eritro	Sulf+tri	TOTAL
R	2	0	8	6	6	3	0	2	5	3	5	1	41
I	0	0	0	0	0	0	1	1	0	2	0	0	4
S	8	10	2	4	4	7	6	7	5	5	5	9	72

Statistics: Degrees of freedom = 22; G test= 47,2; p = 0,0014

Gent: gentamicin; Enro: enrofloxacin; Sulf: sulphonamide; Peni: penicillin; Amp: ampicillin; Oxa: oxacillin; Cefa: cephalothin; Ceft: ceftiofur; Clin: clindamycin; Tetra: tetracycline; Eritro: erythromycin; Sulf+tri: sulfa+trimethoprim.

R: Resistant; I: Intermediary sensibility; S: Sensitive

The antibiotics were separated in 3 groups (A, B and C), to better implementation of the static test (table 04), being possible to verify the *Staphylococcus* sp. sensitive profile. It is possible to realize that there was a big number of resistant samples against the antibiotics in the group A (represented by Sulphonamide (96,29%), penicillin (75,30%), ampicillin (74,07%) and tetracycline (64,19%)). The group B presents the antibiotics with intermediary levels of sensibility against the etiological agents (Oxacillin (58,02%), Cephalothin (34,56%), Clindamycin (55,55%), Erythromycin (58,02%)). And the group C was the group of antibiotics that present most sensibility against the etiological agents (represented by sulfamethoxazole + trimethoprim (62,96%), gentamicin (64,19%), ceftiofur (82,71%), and enrofloxacin (82,59%)).

The *Staphylococcus aureus* singly analyzed, presented the same results of *Staphylococcus* sp., where the group A was the one who presented bigger resistance against the isolated and the C bigger sensibility (table 05).

TABLE 04. Groups of selected antibiotics according to the sensibility level (A – antibiotics with bigger number of resistant samples, B – antibiotics with intermediary number of sensitive samples and C – antibiotics with the bigger number of sensitive samples) of *Staphylococcus* sp. isolated in a dairy farm from Umuarama-PR, 2013.

	R	I	S	TOTAL
A (Sulf, Peni, Amp e Tetra)	251	8	65	324
B (Eritro, Clin, Oxa, e Cefa)	167	26	131	324
C (Sulf+Tri, Gent, Ceft e Enro)	53	26	245	324

Statistics: Degrees of freedom = 4; G test= 267,7; p < 0,0001

TABLE 05. Groups of selected antibiotics according to the sensibility level (A – antibiotics with bigger number of resistant samples, B – antibiotics with intermediary number of sensitive samples and C – antibiotics with the bigger number of sensitive samples) of *Staphylococcus aureus* isolated in a dairy farm from Umuarama-PR, 2013.

	R	I	S
A (Sulf, Peni, Amp e Tetra)	23	2	15
B (Eritro, Clin, Oxa, e Cefa)	16	1	23
C (Sulf+Tri, Gent, Ceft e Enro)	5	1	34

Statistics: Degrees of freedom = 4; G test= 20,8; p = 0,0003

Fontana et al (2010) studying *Staphylococcus* spp. noticed that the most efficient drug was gentamicin (85,9%), and the less efficient were the penicillin and ampicillin, both with 100% of resistance. What are alike to the results on this paper, where, although the gentamicin presented a lower value of sensibility (64,19%), it is in the group of antibiotics with bigger sensibility against the isolated. As to the penicillin and ampicillin are in the group of most resistant antibiotics, with 75, 30% and 74,07% respectively.

In a study performed by Zanette et al (2010) in the far west of Santa Catarina, where they analyzed the sensibility of *Staphylococcus aureus* against various antibiotics, the less efficient were penicillin, with 46,15% of resistance, tetracycline with 30,77% and clindamycin with 20,51%. The current study presents higher values, because the penicillin and tetracycline are in the group that presented bigger resistance (75,30% and 64,19%, respectively) as to the clindamycin is in the intermediary group (B), but in the same way still had a higher frequency of resistance (55,55%) against the isolated.

These results can be allocated to the heavy inadequate use of these antibiotics in the routine of dairy farm to treatment of intramammary infections. The penicillin belongs to the beta- lactam group and was the first antibiotic to be discovered, being largely used all over the world (OGAWARA, 1981). It acts against most of gram-positive bacterias, like streptococcus and staphylococci (WILLEY et al., 2008), and is very used in the field, as to mastitis treatment, as to other diseases. The same thing happens with ampicillin, tetracyclin and sulphonamides for been broad-spectrum antibiotics acting against gram-positives and gram-negatives (SPINOSA, 1996, WILLEY et al., 2008).

Medeiros et al. (2009) worked with samples of *Staphylococcus* spp. in three regions: Metropolitan of Recife (A), Agreste (B) and Wood zone of Pernambuco state (C), and through the sensibility test in vitro with some antibiotics it was possible to verify that gentamicin showed high values of sensibility in the three regions, (A- 96, 8%), (B- 76,2%) and (C- 67,9%).

These results are alike the results of the present study, where gentamicin fits in the group of antibiotics that presents highest levels of sensibility with rate of 64,19%. Langoni et al. (2000) have said that gentamicin still being an effective antibiotic to the treatment of bovine mastitis from bacterial origin.

Costa et al. (2013) studying *Staphylococcus aureus* in Minas Gerais, obtained low rate of resistance to enrofloxacin (0,26%) and ceftiofur (0,40%). These results corroborate with this study, where despite the frequency of resistance be bigger (enrofloxacin (3,70%) and ceftiofur (4,93%)), still are in the group of bigger sensibility.

Santos et al. (2006) using *Staphylococcus* spp. originating from mastitis of herd from Uberlândia-MG found resistance index of 26, 6% for erythromycin; 16,6% the rifampicin 6,6% the tetracycline, chloramphenicol and oxacillin and 3,3% for ciprofloxacin, clindamycin and cephalothin. The results differ from the present study, because the tetracycline was one of the antibiotics that presents increased resistance against the isolated (64,19%), the erythromycin and oxacillin are in the intermediary group, but still presenting a high resistance (58,02%), although, as for the penicillin and the ampicillin, the results are similar, because in both works the antibiotics were the antibiotics that presented resistance against the isolated. These results corroborate with the idea that the penicillin usually present high index of resistance to *Staphylococcus* isolated of mastitis in bovine (ANDRADE et al., 2000; BYARUGABA, 2004).

The table 06 shows the quantity of *Staphylococcus* sp. and *Staphylococcus aureus* that were or not multi-resistant (resistance to three or more antibiotics). It is possible to observe that there was a big number of *Staphylococcus* sp. multi-resistant (70/ 86,41%). About the *Staphylococcus aureus* (10 samples), six were multi-resistant (60%). Through the Fisher test was possible to verify that though the big number of multi-resistant samples there wasn't a significant difference, because $p= 0,0561$. The figure 02 illustrates the difference between the multi-resistant samples and not multi-resistant.

TABLE 06 – Number of samples of *Staphylococcus* sp. and *Staphylococcus aureus* who were multi-resistant or not. Umuarama, 2013.

	Yes	No	Total
<i>Staphylococcus</i> sp.	70	11	81
<i>Staphylococcus aureus</i>	6	4	10
Total	76	15	91

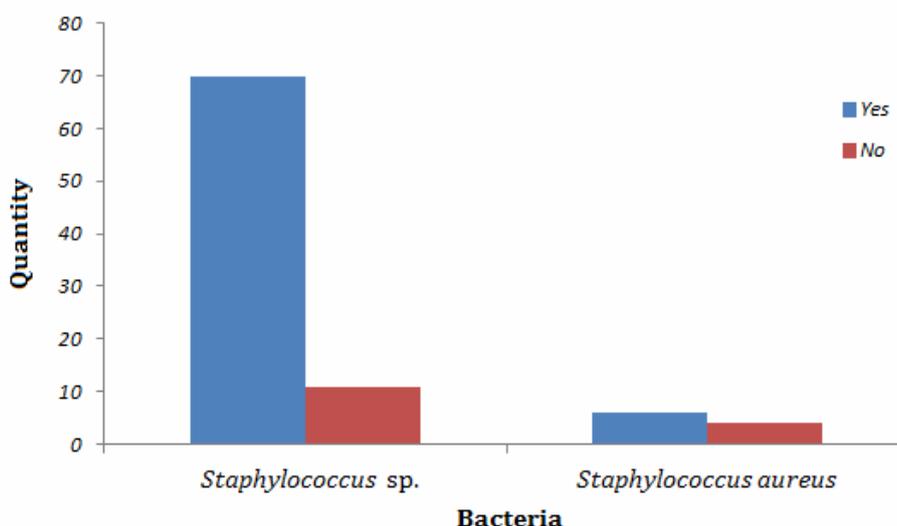


FIGURE 02. Change in volume of *Staphylococcus* sp. and *Staphylococcus aureus* multi-resistant, or not, samples. Umuarama, 2013.

Zanette et al. (2010) have reported that from the 39 positive samples to *S. aureus* searched, nine (23,07%) showed multi-resistance, ranging from three to eight antimicrobial, these results are lower than those founds in the present study, because 60% of *S. aureus* were multi-resistant.

It was possible to observe that the coagulase-negative *Staphylococcus* presented a bigger rate of multi-resistance (79,01%) when compared to *Staphylococcus aureus* (60%), this result can be allocated to the fact of the coagulase-negative *Staphylococcus* have genetic factors that promote the acquisition of resistance (KONEMAN et al., 1997; TORTORA et al., 2002), besides inadequate using of antibiotics and selective pressure. That way stands out the importance of coagulase-negative *Staphylococcus* in intramammary infections, because the resistance to the treatment make appear chronic cases who act as fountain of infections for the herd (ARCHER; CLIMO, 1994).

We found 44 samples of coagulase-negative *Staphylococcus* (35,64%) and 3 (30%) of *S. aureus* resistant to oxacillin. Fontana et al. (2010) found 100% of *Staphylococcus aureus* resistant to oxacillin.

The oxacillin is a semi-synthetic beta-lactam antibacterial and recommended to detect resistance in vitro for methicillin in *Staphylococcus* (CLSI, 2008), for being more stable and present, more trusted results (NCCLS, 1997).

The *Staphylococcus* that presents resistance to oxacillin also presented intrinsic resistance to others antimicrobials (PIRIZ et al., 1995). This is very disturbing, because these samples will be resistant to most of treatment schemes, impeding the bacteriological healing of this herd, resulting in loss to the producer, beyond the problem related to the public health by possible broadcasting of resistant bacterias, resistance gene and antibiotic waste.

All the *Staphylococcus aureus* (10) were submitted to Polymerase Chain Reaction (PCR) for detection of *mecA* gene. Before, these samples were analyzed with catalase and coagulase tests to confirmation of the microorganism species, where was possible to detect that all of them were *Staphylococcus aureus*. Then the antibiogram (test with oxacillin discs) and e-test for determinate the minimum inhibitory concentration were realized, where the *Staphylococcus aureus* presented a variation between 0,19 to 32 µg/mL. All the samples were negative to the *mecA* gene, even those who showed phenotypic resistance to oxacillin. These results can be attributed to the fact of all samples present themselves positives to the beta-lactamase test, so that means, presented other mechanism of resistance (table 07).

The *Staphylococcus aureus* have enzymes that catalyse the cell wall synthesis, as these enzyme are the place of action of penicillin is called PBP's (penicillin binding proteins). The *mecA* gen when present in bacterias is able to synthesize the PBP2a or PBP2' that replace other proteins penicillin binding proteins in the membrane and mean that the antibiotic, not just the oxacillin as others beta lactams cannot connect to effect your action, that way the bacteria keep to synthesize your wall and is not eliminated (LYON; SKURRAY, 1987; KATAYAMA et al., 2000; HIRAMATSU et al., 2002; SCHITO, 2006).

In recent years there has been a worrying increase in the number of microorganisms resistant to antibiotics and among them methicillin resistant *Staphylococcus aureus* (MRSA) may be of great importance, these strains have not been found in animals, but have been reported in some cases of infections in domestic animals (RICH et al., 2005). It is important to monitor dairy herds regarding the appearance of these strains of MRSA, mainly because *Staphylococcus aureus* is one of the major causes of mastitis in cattle.

According Zafalon et al. (2008) the increase of bacterial resistance may not be only caused by the intense and incorrect use of antibiotics, but can occur also through the direct contact or by feeding with antibiotic residues. After that, the gens of resistance can be located in plasmid and be easily transported between bacterias, even from different lines through bacterial conjugation (KONEMAN et al., 1997), when you take this into consideration worth remembering the zoonotic possibility of strains of *Staphylococcus* spp. resistant to oxacillin (SOARES et al., 2008). In this way is very important to monitor the antibiotic resistance profiles of isolated microorganisms not just from dairy herds, but in all farm animals.

The table 06 presents the results related to inhibition halo of oxacillin and beta-lactamase presence, E-test and *mecA* gen detection of *Staphylococcus aureus* samples from the seven milk collections. These results can demonstrate that even

the isolated samples present important pathogenicity factors that inhibit the action of oxacillin, was not detected the *mecA* gen in none of ten samples of *Staphylococcus aureus*. With this, is confirmed the possibility of *Staphylococcus aureus* resistant to oxacillin in the diffusion test in disc did not show the *mecA* gen as a resistance mechanism, but involved to others mechanisms. According Chambers (1997) and CLSI (2008) is important emphasize that the isolates that were positive to *mecA* should be described as oxacillin resistant, and the negatives for *mecA* should be describe as sensible just if the CIM to oxacillin be smaller or same as 2 µg/mL due to others resistance mechanisms like the overproduction of beta-lactamase. And the negatives for *mecA*, however with CIM bigger or the same to 4 µg/mL should be describe as resistant. Taking this into consideration, the present paper where all the *Staphylococcus aureus* were negative for *mecA*, only the samples Re 82, Re 62 and Re 40 can be considered resistant to oxacillin.

TABLE 07. Resistance-related pathogenicity factors of isolates of subclinical mastitis *Staphylococcus aureus* from a dairy farm of Umuarama - PR, 2013.

SAMPLES	OXA DISC	OXA E-TEST	BETA LACTAMASE	SA442	MEC-A
RE 82	0	<256	+	+	-
RE 21	26	0,25	+	+	-
RE 62	16	8	+	+	-
RE 10	20	0,25	+	+	-
RE 75	17	1,5	+	+	-
RE 30	26	0,19	+	+	-
RE 16	28	0,19	+	+	-
RE 33	26	0,25	+	+	-
RE 44	28	0,25	+	+	-
RE 40	0	32	+	+	-

Results of the present study can be confronted with others authors that identify this gen in 11% of the milk samples from refrigeration tank in Minas Gerais (DIAS et al., 2011) and 6,7% in milk samples of cows with subclinical and clinical mastitis from the Distrito Federal (GUIMARÃES et al., 2012). A study performed with 272 milk samples from cows positive to the *California Mastitis Test* from properties of the south Fluminense region didn't detected the *mecA* gen, in other words, the results are similar to the ones found in this work (MENDONÇA et al., 2012).

Although it was not detected the *mecA* gen in this property in the municipality of Umuarama in the present study, it is known that the state of Paraná is an important carrier of this gen. In this way it is necessary to monitor the resistance profile of the properties, in order to detect possible gens of resistance and avoid inappropriate use of antibiotics.

These results shows that in this property is worrisome the big number of multiresistants *Staphylococcus* sp. and *Staphylococcus aureus* this shows how it is important to make adequate use of the antibiotics, as well as to know the sensitivity profile of the herd

CONCLUSION

Among the isolated microorganisms, *Staphylococcus* sp. was the most prevalent etiological agent in the studied property. The antibiotics that presented higher resistance indices to the studied etiological agents were: Sulphonamide, Penicillin, Ampicillin and Tetracycline, followed by those with intermediate levels of sensitivity: Erythromycin, Clindamycin, Oxacillin, and Cephalotin. On the other hand, those that presented greater sensitivity to the etiological agents were Sulph + Trimethoprim, Gentamicin, Ceftiofur and Enrofloxacin. A total of 86.41% of *Staphylococcus* sp. and 60% of multiresistant *Staphylococcus aureus*. All *S. aureus* samples were positive for beta-lactamase and negative for the *mecA* gene.

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