Risk factors associated with the antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis[1]

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ABSTRACT.- Beuron D.C., Cortinhas C.S., Botaro B.G., Macedo S.N., Gonçalves J.L., Brito M.A.V.P. & Santos M.V. 2014. **Risk factors associated with the antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis.** *Pesquisa Veterinária Brasileira 34(10):947-952.* Departamento de Nutrição e Produção Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Duque de Caxias Norte 225, Pirassununga, SP 13635-900, Brazil. E-mail: mveiga@usp.br

The objective of this study was to evaluate herd management practices and mastitis treatment procedures as risk factors associated with *Staphylococcus aureus* antimicrobial resistance. For this study, 13 herds were selected to participate in the study to evaluate the association between their management practices and mastitis treatment procedures and *in vitro* antimicrobial susceptibility. A total of 1069 composite milk samples were collected aseptically from the selected cows in four different periods over two years. The samples were used for microbiological culturing of *S. aureus* isolates and evaluation of their antimicrobial susceptibility. A total of 756 samples (70.7%) were culture-positive, and *S. aureus* comprised 27.77% (n=210) of the isolates. The *S. aureus* isolates were tested using the disk-diffusion susceptibility assay with the following antimicrobials: ampicillin 10mg; clindamycin 2μg; penicillin 1mg; ceftiofur 30μg; gentamicin 10mg; sulfa-trimethoprim 25μg; enrofloxacin 5μg; sulfonamide 300μg; tetracycline 30μg; oxacillin 1mg; cephalothin 30μg and erythromycin 5μg. The variables that were significantly associated with *S. aureus* resistance were as follows: the treatment of clinical mastitis for ampicillin (OR=2.18), dry cow treatment for enrofloxacin (OR=2.11) and not sending milk samples for microbiological culture and susceptibility tests, for ampicillin (OR=2.57) and penicillin (OR=4.69). In conclusion, the identification of risk factors for *S. aureus* resistance against various mastitis antimicrobials is an important information that may help in practical recommendations for prudent use of antimicrobial in milk production.

INDEX TERMS: Risk factors, antimicrobial resistance, *Staphylococcus aureus,* mastitis, treatment, management practices, *in vitro* susceptibility.

RESUMO.- [**Fatores de risco associados com a resistência antimicrobiana de *Staphylococcus aureus* isolados da mastite bovina.**] Objetivou-se com este estudo avaliar os fatores de risco associados às práticas de manejo e tratamento de mastite e a resistência aos antimicrobianos de *Staphylococcus aureus* isolados de vacas com mastite. Foram selecionados para o presente estudo 13 rebanhos localizados na região de Pirassununga/SP. Foi aplicado um questionário contendo informações para o levantamento de fatores de risco relacionados à resistência aos antimicrobianos e às práticas de manejo e tratamento de mastite. Após a seleção dos rebanhos e aplicação dos questionários, foram utilizados 210 isolados de *S. aureus* de amostras compostas de leite coletadas durante 24 meses, em quatro períodos, para realização dos testes de resistência. Os antimicrobianos testados foram: ampicilina 10μg, clindamicina 2μg, penicilina 1μg, eftiofour 30μg, gentamicina 10μg, sulfatrimetropin 25μg, enrofloxacina 5μg, sulfonamida 300μg, tetraciclina 30μg, oxacilina 1μg, cefalotina 30μg e eritromicina 5μg. As variáveis que foram significativamente as-

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sociadas à resistência de *S. aureus* foram: o tratamento da mastite clínica para ampicilina (OR = 2,18), o tratamento da vaca seca para enrofloxacina (OR=2,11), e o não envio de amostras de leite para a cultura microbiológica e testes de sensibilidade, para ampicilina (OR=2,57) e penicilina (OR=4,69). Em conclusão, a identificação dos fatores de risco para a resistência *S. aureus* frente aos principais agentes antimicrobianos, utilizados para tratamento da mastite, pode auxiliar o estabelecimento do uso prudente de antimicrobianos na produção de leite.

TERMOS DE INDEXAÇÃO: Fatores de risco, resistência antimicrobiana, *Staphylococcus aureus,* mastite bovina, tratamento, práticas de manejo, *in vitro* susceptibilidade.

INTRODUCTION

Mastitis is the most common disease in dairy herds and is the main cause of antibiotic use in adult dairy cows (Ruegg 2009). On dairy farms, antimicrobials such as penicillin, cephalosporin, and tetracycline, among others, are used to treat and prevent mastitis that is caused by Gram-positive and Gram-negative bacteria (Oliver & Murinda 2012). However, the efficacy of these antimicrobials can be compromised by the emergence of antimicrobial resistance in the relevant mastitis pathogens, such as *Staphylococcus aureus*. The intensive use of antibiotics in human and veterinary medicine may increase bacterial resistance (Hawkey 2003). Thus, dairy farms and the current management practices employed for milk production might be associated with the dissemination of antibiotic-resistant bacterial strains (Acar & Moulin 2006).

Staphylococcus aureus is one of the most important causes of clinical mastitis and is the pathogen that is most frequently isolated in cases of subclinical mastitis worldwide (Sampimon et al. 2009, Waage 1997). *S. aureus* is also the main microorganism investigated in antimicrobial susceptibility studies due to its high prevalence in dairy cow mastitis (Malinowski et al. 2002). Additionally, staphylococcal food poisoning is caused by the ingestion of staphylococcal enterotoxins, which are resistant to heat treatment and are generally produced by *S. aureus* strains (Roberson et al. 1994). Thus, *S. aureus* is responsible for major losses on dairy farms and is resistant to several of the antimicrobials that are routinely used in mastitis treatment (Freitas et al. 2005). Several mechanisms of resistance to the available antimicrobial drugs have been described; however, many aspects of antimicrobial resistance development and dissemination remain uncertain. Resistance among mastitis pathogens has been reported for nearly four decades, but despite this, there is no scientific evidence to determine whether it is an emergent phenomenon or is in progress (Oliver et al. 2011).

Some studies have evaluated the trends of antimicrobial resistance of mastitis-causing bacteria for various periods of time in different regions (Erskine et al. 2002, Myllys et al. 1998, Petrovski et al. 2011). The results of these studies indicated variation among the different regions studied; however, there is no scientific evidence of a significant increase in the antimicrobial resistance of mastitis-causing pathogens over time (Oliver et al. 2011). Some studies have reported that the susceptibility of *S. aureus* varied from 7 to 63% for penicillin (Guler et al. 2005, Watts & Salmon 1997) from 0 to 93% for erythromycin (Wang et al. 2008), from 0 to 28% for tetracycline (Guler et al. 2005, Watts & Salmon 1997), and from 4.5 to 7.5% for sulfadimethoxine (Makovec et al. 2003, Sabour et al. 2004). However, no studies concerning the management- and treatment-associated risk factors for the antimicrobial resistance of *S. aureus* in bovine mastitis are available. The objective of this study was to evaluate the association between the management practices and treatment procedures and the antimicrobial resistance of *S. aureus* that was isolated from bovine mastitis samples.

MATERIALS AND METHODS

Selection of dairy herds and cow sampling

Thirteen out of 60 dairy herds from the members of the dairy industry located in Pirassununga (São Paulo State, Brazil) were selected according the number of lactating cows per herd, the herd's milk production, a maximum distance of 80 km from the laboratory where the evaluation was performed and the farmers' willingness to participate in this study. Selected dairy herds were randomly selected in the municipalities of Leme, Santa Rita do Passa Quatro, Descalvado, São João da Boa Vista and Pirassununga.

To determine the total number of herds to be included in this study, the number of milk samples required to estimate the frequency of *Staphylococcus* spp. antimicrobial resistance according to the recommendations described by the OIE (2008) was considered. A variation in the prevalence of *S. aureus* antimicrobial resistance to penicillin of 35% (90% confidence interval: 33.4-36.7%) was used, based on the results reported by Brito et al. (1999). The number of milk samples collected per herd was based on the methodology of Frankena & Graat (1997) (Table 1).

The on-farm data about the possible risk factors associated with antimicrobial susceptibility were collected during the first farm visit based on a questionnaire that included questions about mastitis treatment, prevention procedures, and herd management. Milk samples were collected on four different occasions over a period of two years (May 2010, August 2010, January 2011 and July 2011).

Bacteriological analyses

Composite milk samples (40mL) from four mammary gland quarters of each selected cow were collected aseptically in a sterile vial before milking. The milk samples were immediately cooled

Table 1. Number of dairy herds and cows selected, according to the prevalence of *Staphylococcus aureus* antimicrobial resistance to penicillin

Number of lactating cows	Herd frequency (%)	Number of selected	
		Dairy herds	Cows†
10-19	29	6	All cows
20-29	13	3	All cows
30-39	8	2	All cows or 30 if > 30
40-49	2	0	-
50-60	4	1	30 (randomization)
>60	4	1	30 (randomization)
Total	60	13	-

† Considering a prevalence of S. *aureus* antimicrobial resistance to penicillin of 35% (90% confidence interval: 33.4-36 (Britto et al. 1999).

to 4°C, transported to the laboratory and stored at -20°C. Microbiological analyses of the milk samples were performed as recommended by the National Mastitis Council (Oliver et al. 2004). A total of 10µl of milk was plated on 5% sheep blood agar and incubated at 37°C for 24 and 48 hours. The bacteriological cultures were classified as positive based on the presence of three or more identical colonies. The colony morphology and Gram-stain reaction of the bacterial isolates were analyzed. In the case of Gram-positive isolates, the catalase test was performed to distinguish *Streptococcus* spp. from *Staphylococcus* spp. In the case of Staphylococci, the coagulase test was performed to classify the bacteria as coagulase positive or negative, and Voges-Proskauer test was performed on the coagulase positive *Staphylococcus* to identify *S. aureus*. All of the bacterial isolates were cryopreserved in brain heart infusion broth (BHI, Becton, Dickinson and Company, Sparks, MD, USA) with 20% glycerol at -20°C for further analyses.

Antimicrobial susceptibility testing

The antimicrobial susceptibility tests of all of the *Staphylpococcus aureus* isolates were performed using agar dilution as described by the Clinical and Laboratory Standards Institute (CLSI 2008). In brief, the isolates were inoculated in BHI broth and incubated for 24 hours at 36°C; subsequently, the turbidity of the cultures were standardized 0.5 on the MacFarland scale. All the standardized bacterial suspensions were plated over the entire surface of a plate of Mueller-Hinton agar using a sterile swab. Twelve antimicrobial discs (Oxoid, Basingstoke, England) were deposited on the medium with the aid of a dispenser (Oxoid, Basingstoke, England). The plates were incubated at 35°C, and the inhibition zones were measured after 18 hours of incubation. The inhibition zones were recorded in millimeters and were interpreted according to the criteria of the CLSI (2008). Quality control was also performed in accordance with Clinical and Laboratory Standards Institute guidelines using *S. aureus* ATCC 25923 and ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Streptococcus pneumoniae* ATCC 49619**.**

The following antimicrobials were tested: ampicillin 10mg, clindamycin 2µg, penicillin 1µg, ceftiofur 30µg, gentamicin 10mg, sulfa-trimethoprim 25mcg, enrofloxacin 5µg, sulfonamide 300µg, tetracycline 30µg, oxacillin 1µg, cephalothin 30µg and erythromycin 5µg.

Questionnaire about mastitis treatment and management practices

Previously formulated questionnaires were submitted to the dairy herd manager during the first farm visit, which included questions about the following: the use of dry cow therapy; the mastitis treatment records; the use of treatment for clinical mastitis cases; the therapeutic treatment record; the use and frequency of microbiological culture tests; and the use and frequency of antimicrobial susceptibility tests of the mastitis pathogens that were identified. The following additional information about the general herd characteristics was recorded: identification and location; number of lactating cows; total milk production; individual milk yield, and milking system.

Statistical analyses

The statistical analyses were performed using the Statistical Analysis System (SAS Institute Inc. 2010). The frequencies *S. aureus* isolates resistance was calculated using the chi-square test (PROC FREQ). Also, the frequencies of responses to the questionnaire for each antimicrobial that had been tested were analyzed using the chi-square test (PROC FREQ), considering a probability of ≤ 0.2 to screen for potential risk factors for the antimicrobial resistance of *S. aureus* that could be inserted into the logistic regression models. The other criteria that were used to select the variables for inclusion in the logistic regression model were those that offered a coherent biological explanation for an increase in *S. aureus* antimicrobial resistance. A multivariate logistic regression model (PROC LOGIT) was used determine risk factors at *S. aureus* positive samples level as described by Frankena & Graat (1997), using a probability of ≤0.05. The odds ratios were estimated using the respective confidence interval (CI) of 95%.

RESULTS

In this study, 77% of the herds that were evaluated were composed of crossbred cows (Holstein x Gir) and were composed of 23% of Holstein cows. The number of lactating cows ranged from 11 to 90/herd and the milk yield ranged from 80 to 1000 liters/day / herd. Regarding the management characteristics, manual milking was performed in four herds and mechanical milking was performed in 56 herds.

A total of 1069 milk samples were collected and subjected to microbiological culture. Of these, 756 samples (70.7%) were culture-positive and 313 (29.3%) were culture-negative. The frequencies with which the mastitis-causing pathogens were isolated are as follows: 28.57% of coagulase-negative *Staphylococcus* (CNS) (n=216); 27.77% of *S. aureus* (n=210); 10.97% of *Corynebacterium* spp. (n=83); 8% of *Streptococcus agalactiae* (n=60); 6.2% of *Streptococcus dysgalactiae* (n=47); 6% of coagulase-positive *Staphylococcus* (n=45); 3.2% of esculin-positive *Streptococcus* (n=24); 4.9% of *Arcanobacterium* spp. (n=37), and 2.5% of *Bacillus* spp. (n=19).

The on-farm potential risk factors for *S. aureus* antimicrobial resistance according to the antimicrobial tested that were inserted into the final logistic regression model are presented in Table 2. The following risk factors for the antimicrobial resistance of *S. aureus* were identified (Table 3): ampicillin- treatment of clinical mastitis cases (OR=2.18) and not sending milk samples for microbiological culture and antimicrobial susceptibility tests (OR=2.57); enrofloxacin- dry cow treatment (OR=2.11); penicillin- not sending milk samples for microbiological culture and antimicrobial susceptibility tests (OR=4.69).

The risk of *S. aureus* resistance on farms that did not send samples for culture and susceptibility tests was also higher for penicillin (OR=4.69) and ampicillin (OR=2.57) compared with *S. aureus* isolated from farms that performed this practice. In this study, the treatment of mastitis was identified as a risk factor for *S. aureus* resistance to ampicillin*. S. aureus* isolates from farms on which all of the clinical mastitis cases were routinely treated were 2.18 times more likely to be resistant to ampicillin than were *S. aureus* isolates from farms on which clinical mastitis was not treated. Additionally, the practice of dry cow treatment was identified as a risk factor for *S. aureus* resistance to enrofloxacin and penicillin. Farms using dry cow treatment had 2.11 times greater chances to have *S. aureus* that was resistant to enrofloxacin than did farms where this practice was not employed.

On the other hand, a protective factor identified in this study for *S. aureus* antimicrobial resistance was not using

Table 2. *Staphylococcus aureus* antimicrobial resistance frequencies according to antimicrobial and evaluated risk factors

Antimicrobial	Total of resistant (%)	Questionnaire questions	Resistant (%)	P†
Ampicillin	71 (33.8)	Use of clinical mastitis treatment	56 (78.9)	0.005
		Not sending milk samples for microbiological culture and susceptibility tests	56 (78.9)	0.003
Ceftiofur	57 (27.1)	Use of clinical mastitis treatment	43 (75.4)	0.048
		Not respecting the withdrawal periods	46 (80.7)	0.011
Enrofloxacin	34 (16.2)	Use of dry cow treatment	17 (50.0)	0.049
Erythromycin	10 (4.8)	Not sending milk samples for microbiological culture and susceptibility tests	7 (70.0)	0.070
		Not using a mastitis treatment protocol	7 (70.0)	0.165
Gentamicin	38 (18.1)	Use of clinical mastitis treatment	29 (76.3)	0.145
		Not keeping records of mastitis treatment	32 (84.2)	0.189
Oxacillin	25 (11.9)	Use of clinical mastitis treatment	13 (52.0)	0.110
		Use of dry cow treatment	6 (24.0)	0.066
		Not using a mastitis treatment protocol	18 (72.0)	0.013
Penicillin	60 (28.6)	Use of clinical mastitis treatment	34 (56.7)	0.065
		Use of dry cow treatment	39 (50.0)	0.016
		Not sending milk samples for microbiological culture and susceptibility tests	50 (83.3)	0.178
		Not using a mastitis treatment protocol	39 (65.0)	0.003
		Not keeping records of mastitis treatment	58 (96.7)	0.042
Sulfa-trimethoprim	35 (16.7)	Use of clinical mastitis treatment	17 (50.0)	0.029
		Not sending milk samples for microbiological culture and susceptibility tests	33 (97.1)	0.078
		Not using a mastitis treatment protocol	20 (61.8)	0.093
Sulfonamide	30 (14.3)	Use of clinical mastitis treatment	17 (56.67)	0.004
		Not using a mastitis treatment protocol	19 (63.3)	0.081
Tetracycline	19 (9.0)	Use of dry cow treatment	11 (57.9)	0.115
		Not sending milk samples for microbiological culture and susceptibility tests	11 (57.9)	<0.001
		Not respecting the withdrawal periods	4 (21.0)	0.115
		Not using a mastitis treatment protocol	12 (63.2)	0.182

† P = probability calculated using the chi-squared test.

a mastitis treatment protocol for clinical mastitis cases. *S. aureus* isolates from farms that did not use this treatment protocol were 0.45 times more likely to be resistant to tetracycline than were *S. aureus* isolates from farms that used a mastitis treatment protocol. The factors that showed a odds ratio < 1 were considered as protective factors.

DISCUSSION

This study evaluated the association between herd management and the treatment practices for mastitis in dairy cows and the resistance of *Staphylococcus aureus* to antimicrobial agents. According to the logistic regression model, the OR of *Staphylococcus aureus* resistance to penicillin and ampicillin was higher on farms that did not sendi milk samples for microbiological culture and susceptibility tests. Hoe & Ruegg (2006) reported that the use of microbiological culture testing for mastitis diagnosis was also associated with the size of the herd. These authors reported a total of 38.9% of the farms with large herds had culture tests performed for all of the clinical mastitis cases, compared with the farms with smaller herds. On farms with medium and large herds, it was 2-3 times more likely that bulk tank cultures to identify mastitis pathogens were performed compared with on farms with smaller herds, and 49% of the farms with smaller herds had never had microbiological bulk tank cultures conducted. According to Hoe & Ruegg (2006), the larger farms had more access to bulk tank diagnostic methods than did the farms with smaller herds.

The practice of dry cow treatment was identified as a risk factor for *S. aureus* resistance to enrofloxacin. Studies of the association between dry cow therapy and the development of antimicrobial resistance in mastitis pathogens are limited. Pol & Ruegg (2007) evaluated the relationship between antimicrobial use and the susceptibility of Gram-positive mastitis pathogens in organic and conventional dairy herds. In that study, the antimicrobials that were most frequently used for dry cow treatment were penicillin, streptomycin and tetracycline (90%), and the daily doses of these antimicrobials was calculated at the mammary quarter level. *S. aureus* isolates from the conventional herds were 7.7 times more likely to be resistant to ampicillin and 6.3 times more likely to be resistant to penicillin compared to the isolates from the organic farms (Pol & Ruegg 2007). The differences in the resistance on the conventional farms were attributed to the intramammary treatment of clinical mastitis (66%) and dry cow treatment (44%). In addition, the isolates from multiparous cows were more likely to be resistant than were the isolates from primiparous cows. These observations suggested that long-term exposure to antimicrobials (for example, continuous use of dry cow treatment) significantly increases the level of antimicrobial resistance (Pol & Ruegg 2007), as observed in the present study.

Table 3. Risk factors associated with *Staphylococcus aureus* antimicrobial resistance as estimated using logistic regression

Antimicrobial	Risk factors	OR†	95% CI‡	P§
Ampicillin	Use of clinical mastitis treatment	2.18	1.10-4.32	0.026
	Not sending milk samples for microbiological culture and susceptibility tests	2.57	1.06-6.24	0.037
Enrofloxacin	Use of dry cow treatment	2.11	1.01-4.44	0.049
Penicillin	Not sending milk samples for microbiological culture and susceptibility tests	4.69	1.10-20.05	0.037

† OR = odds ratio, ‡ CI = confidence interval, § P = probability.

Rajala-Schultz et al. (2009) determined both the prevalence of antimicrobial resistance of coagulase-negative staphylococci (CNS) isolates before and after dry cow treatment and the diversity and persistence of this group of microorganisms during the dry cow period. These authors reported a strong association between antimicrobial resistance to most beta-lactam antibiotics and the age of the animals, dry cow treatment, high somatic cell counts and a history of clinical mastitis. Similarly, in the present study, one of the main risk factors for the antimicrobial resistance of *S. aureus* was dry cow treatment.

Treatment of mastitis is the major reason for antimicrobial use in dairy herds (Sawant et al. 2005). Guidelines on the prudent and judicious use of antimicrobials have recently been developed by international organizations, including the World Health Organization (WHO) and the International Organization of Epizooties (OIE 2008). As part of good veterinary practice, these guidelines aim to maximize therapeutic efficacy and minimize the selection of resistant organisms. Sawant et al. (2005) described some of these practices, including record keeping and the treatment protocols for cases of mastitis.

In this study, the practice of not using a mastitis treatment protocol for clinical mastitis cases was identified as a protective factor identified for *S. aureus* tetracycline resistance. According to Hoe & Ruegg (2006), written treatment protocols are recommended to establish practices and treatment decisions. In a study that Hoe & Ruegg (2006) conducted in Wisconsin (USA), a written protocol for the treatment of mastitis was highly associated with the herd size ($P<0.001$). Most of the farmers in that study had large herds (75%) and reported that they maintained a written protocol for the treatment of mastitis, compared with 29 and 21% of the farmers with medium or small herds, respectively. Regardless of the herd size, 60% of the treatment protocols were written by a veterinarian, approximately 30% of the treatment protocols were written by farm managers and less than 1% of the treatment protocols were from the pharmaceutical companies.

Some of the limitations of the present study were the small number of herds sampled (n=13) and the small number of variables tested (n=6). Therefore, careful consideration should be taken when comparing the data presented in this study with the data from other studies. Additionally, the results of this study are from a specific Brazilian region and may be not representative of the entire country. Thus, future studies may discover the profile of trends in antimicrobial resistance in Brazilian dairy herds.

There was an association between the resistance of *S. aureus* to antimicrobials and the management practices and mastitis treatment on the farms. The results of the present study suggest that the frequency of *Staphylococcus aureus* resistance is greater for ampicillin than other antimicrobial tested. Additionally, *Staphylococcus aureus* resistance for penicillin is higher in dairy farms that do not send milk samples for microbiological culture and susceptibility tests. The identification of risk factors for *S. aureus* resistance against various mastitis antimicrobials is an important information that helps in practical recommendations for a prudently antimicrobial use in milk production.

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