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Temporal profile of antimicrobial resistance exhibited by strains of *Staphylococcus* spp. isolated from cases of bovine mastitis for 20 years (1992-2011)

Perfil temporal da resistência antimicrobiana exibido por cepas de *Staphylococcus* spp. isoladas de casos de mastite bovina durante 20 anos (1992-2011)

Ananda Paula Kowalski[I] Grazieli Maboni[I] Julia Pires Espindola[I] Ariane Foletto[I] Guerino Bandeira Junior[I] Luciana Pötter[II] Sônia de Avila Botton[I] Agueda Castagna de Vargas[I*]

ABSTRACT

*Records of in vitro susceptibility tests performed between 1992 and 2011 were retrospectively reviewed in order to evaluate the dynamic profiles of possible changes in antimicrobial resistance of **Staphylococcus** spp. isolated from milk samples of cows with mastitis during two decades. The results of 2,430 isolates tested by disk diffusion technique for susceptibility to oxacillin, penicillin, ampicillin, cephalexin, norfloxacin, tetracycline, sulfazotrim, gentamicin, and neomycin were analysed. Comparisons were performed between the percentages of resistance to antimicrobials and their classes and also between the decades studied. Additionally, the possible tendency or changes in the behaviour of these pathogens against the major drugs used in the last two decades were evaluated using regression analysis. The highest rates of resistance (P<0.0001) were observed for the beta-lactams (34.3%), with exception of cephalexin (6.9%), and for the tetracyclines (28%). Similar resistance rates (7.6% to 15.7%) were observed among the other drugs. Regression analysis showed a reduction in resistance to penicillin and ampicillin throughout the period, whilst for oxacillin and neomycin a decrease in the resistance was observed during the first decade, followed by an increase. A trend towards decreased resistance was found for sulfazotrim, whereas for the other antimicrobials no decrease was observed. The results indicated no trend towards increased resistance for most antimicrobials tested. Nevertheless, it is necessary to monitor the resistance patterns of these pathogens in order to save these drugs as a therapeutic reserve.*

Key words: *cows, milk, **Staphylococcus** spp., susceptibility, surveillance.*

RESUMO

*Os registros de resultados de testes de suscetibilidade in vitro realizados no período de 1992 a 2011 foram revisados, retrospectivamente, a fim de avaliar a dinâmica de possíveis mudanças dos perfis de resistência de isolados de **Staphylococcus** spp., oriundos de amostras de leite de vacas com mastite ao longo do tempo. Os resultados de 2430 isolados testados pela técnica de difusão em disco para oxacilina, penicilina, ampicilina, cefalexina, norfloxacina, tetraciclina, sulfazotrim, gentamicina e neomicina foram analisados. Comparações entre as médias de resistência para os antimicrobianos e suas respectivas classes e em relação ao período analisado foram estabelecidas. O comportamento da resistência para cada antimicrobiano foi avaliado por meio de análise de regressão. As maiores taxas de resistência (P<0,0001) foram observadas para os betalactâmicos (34,3%), com exceção da cefalexina (6,9%), e tetraciclina (28%). Frente aos demais fármacos, os isolados apresentaram médias de resistência semelhantes entre si (de 7,6% a 15,7%). A análise de regressão demonstrou redução da resistência para penicilina e ampicilina ao longo do período analisado. Para a oxacilina e a neomicina, houve um decréscimo da resistência dos micro-organismos testados durante a primeira década; entretanto, houve um aumento no segundo período. Em relação ao sulfazotrim, foi verificada uma tendência à diminuição da resistência dos isolados, ao passo que, para os demais antimicrobianos, nenhum comportamento foi destacado. Os resultados não indicaram tendência de aumento da resistência dos isolados de **Staphylococcus** spp. para a maioria dos antimicrobianos testados. Contudo, é necessário monitorar os padrões de resistência destes patógenos, a fim de que estes fármacos sejam preservados para garantir uma reserva terapêutica.*

Palavras-chave: *vacas, leite, **Staphylococcus** spp., suscetibilidade, vigilância.*

INTRODUCTION

Mastitis is the major infectious disease in dairy herds (CARVALHO et al., 2007; GETAHUM et al., 2008) and it is responsible for major economic

[I]Departamento de Medicina Veterinária Preventiva, Centro de Ciências Rurais (CCR), Universidade Federal de Santa Maria (UFSM), Av. Roraima, 1000, Prédio 44, Sala 5137, Campus, Camobi, 97105-900, Santa Maria, RS, Brasil. E-mail: agueda.vargas@gmail.com. [*]Corresponding author.
[II]Departamento de Zootecnia, CCR, UFSM, Santa Maria, RS, Brasil.

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losses in milk industry. Additionally, it represents the major reason for using antimicrobials in animals worldwide (SARAN & CHAFFER, 2000). Among the etiological agents, ***Staphylococcus*** spp. is cited as the most prevalent micro-organism involved in subclinical mastitis (KREWER et al., 2013). ***Staphylococcus aureus*** is the most pathogenic species (CARVALHO et al., 2007) and is characterized by high invasiveness and formation of fibrous tissue at the site of infection, leading to chronic cases that are characterised by low cure rates (SANTOS & FONSECA, 2007). Moreover, coagulase-negative ***Staphylococcus*** has received attention as an emerging etiological cause of subclinical mastitis, and as ***S. aureus***, it is reported as resistant to multiple antimicrobials commonly used in mastitis treatment (WEESE & DUIJKEREN, 2010).

The emergence of antimicrobial resistance has consequences in both human and animal health. The selective pressure imposed by the constant use of antimicrobials and the presence of resistance genes are the most important features in the occurrence of this phenomenon (PHILLIPS et al., 2004). Several studies with microorganisms isolated from the milk of cows with mastitis reported different patterns of susceptibility to antimicrobial agents (FREITAS et al., 2005; MEDEIROS et al., 2009; KREWER et al., 2013). Nevertheless, there is a lack of information regarding the temporal changes in the susceptibility of these pathogens to antimicrobial agents used in the treatment of this disease (LINDEMAN et al., 2013). The aim of this retrospective study was to determine the antimicrobial resistance profile of ***Staphylococcus*** spp. isolated from bovine milk samples with mastitis over a period of 20 years (1992-2011).

MATERIALS AND METHODS

The results of antimicrobial susceptibility tests of 2,430 isolates of ***Staphylococcus*** spp. were reviewed and analysed. Data were obtained from records of bovine milk samples analysed between 1992 and 2011 in the diagnostic service of the Bacteriology Laboratory of the Federal University of Santa Maria (LABAC-UFSM).

The samples were isolated in blood agar (Himedia Laboratories®) supplemented with 5% of sheep blood, and incubated aerobically at 35±2°C for 4h. Bacterial identification was performed using morphological analysis and biochemical tests (QUINN et al., 1994). Since in most of the cases the identification of ***Staphylococcus*** spp. species was not realized, the results were organized into the genus ***Staphylococcus*** spp.

The antimicrobial susceptibility tests were performed *in vitro* using the method of agar disk diffusion (BAUER et al., 1966) for the following antimicrobials: 1) beta-lactams: oxacillin (1μg), penicillin (10IU), ampicillin (10μg) and cephalexin (30μg); 2) fluoquinolone: norfloxacin (10μg); 3) tetracycline: tetracycline (30μg); 4) sulphonamide: sulphazotrim (25μg); 5) aminoglycosides: gentamicin (25μg) and neomycin (30μg). The results were analysed in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2002).

Isolates were classified as susceptible, intermediate or resistant to antimicrobials. In order to facilitate the calculation, intermediate results were considered resistant. For quality control of the susceptibility tests, a reference strain from the American Type Culture Collection (ATCC) (***S. aureus*** ATCC 25923) was used. During the study period, the number of isolates varied in relation to antimicrobial agents in the same year, possibly due to unavailability of certain antibiotic discs in the laboratory.

The resistance of ***Staphylococcus*** spp. to each antimicrobial was evaluated based on the percentage of resistant isolates compared to the number of samples tested in each year. Comparisons using the Kruskal-Wallis test were performed. The data regarding to the resistance of ***Staphylococcus*** spp. between two decades were analyzed, regardless of antimicrobial tested by means of chi-square. A Bonferroni test was applied to compare the means when differences between classes or antimicrobials were identified. The SAS (2001) statistical program was used to perform the analyses. In order to evaluate the temporal trends in antimicrobial susceptibility during the study period, regression analysis was performed with data transformed to arcsine square root, for which the choice of models was based on the significance of linear, quadratic and cubic coefficients using a "t" test at 5% probability (SAS 2001).

RESULTS

The results of the *in vitro* susceptibility tests showed that 729 (30%) of ***Staphylococcus*** spp. were susceptible to all antimicrobials tested. The resistance profiles of the isolates are summarized in table 1. It was possible to conclude that the resistance to the antimicrobials was higher in the first decade (1992-2001: 29.2%) than the second decade (2002-2011: 17.1%) ($P<0.0004$).

Among the classes of antimicrobials, beta-lactams (34.3%) and tetracycline (28%) showed the highest resistance mean, however there is no

Table 1 - Profile of antimicrobial resistance of ***Staphylococcus*** spp. derived from bovine milk samples (1992-2011)[1].

Year Antimicrobials	OXA	PEN	AMP	CEF	NOR	TET	SUT	GEN	NEO
1992	6/6 100%	6/6 100%	43/47 91.5%	NT	NT	17/45 37.8%	5/45 11.1%	3/47 6.4%	2/5 40%
1993	58/62 93.5%	40/63 63.5%	49/61 80.3%	NT	NT	8/30 26.7%	12/84 14.3%	3/63 4.7%	11/58 19%
1994	51/82 62.2%	61/83 73.5%	58/83 69.9%	NT	NT	NT	23/83 27.7%	6/81 7.4%	23/82 28%
1995	76/117 64.9%	67/127 52.7%	58/135 42.9%	NT	7/47 14.9%	11/28 39.3%	14/131 10.7%	7/135 5.2%	15/129 11.6%
1996	104/176 59.1%	81/171 47.4%	97/180 53.9%	NT	15/161 9.3%	27/73 37%	30/169 17.7%	9/180 5%	29/173 16.7%
1997	91/192 47.4%	75/164 45.7%	84/182 46.1%	5/99 5%	8/194 4.1%	53/192 27.6%	7/193 3.6%	17/197 8.6%	14/188 7.4%
1998	78/389 20%	252/419 60.1%	261/463 56.4%	70/423 16.5%	41/463 8.8%	146/469 31.1%	44/415 10.6%	36/387 9.3%	86/453 19%
1999	42/368 11.4%	180/364 49.4%	179/365 49%	18/360 5%	23/359 6.4%	76/361 21%	12/357 3.4%	14/372 3.7%	23/367 6.3%
2000	36/220 16.4%	117/222 52.7%	104/207 50.2%	11/163 6.7%	26/208 12.5%	60/221 27.1%	21/218 9.6%	21/154 13.6%	33/221 14.9%
2001	13/105 12.4%	41/107 38.3%	39/107 36.4%	5/107 4.7%	7/107 6.5%	25/107 23.4%	4/107 3.7%	3/107 2.8%	7/106 6.6%
2002	22/73 30.1%	22/73 30.1%	22/73 30.1%	2/73 2.7%	1/73 1.4%	13/73 17.9%	8/73 11%	8/73 11%	20/73 27.4%
2003	11/61 18%	19/61 31.1%	15/61 24.6%	1/61 1.6%	5/61 8.2%	13/61 21.3%	13/61 4.9%	4/61 6.6%	11/61 18%
2004	4/43 9.3%	14/43 32.6%	11/43 25.6%	1/43 2.3%	1/43 2.3%	9/43 20.9%	2/43 4.6%	2/43 4.6%	3/43 7%
2005	10/43 23.2%	17/43 39.5%	14/43 32.6%	3/43 7%	6/43 13.9%	14/43 32.6%	1/43 2.3%	2/43 4.6%	6/43 13.9%
2006	5/54 9.3%	12/54 22.2%	10/54 18.5%	1/54 1.8%	0/54 0%	14/54 25.9%	2/54 3.7%	2/54 3.7%	1/54 1.8%
2007	12/49 24.5%	15/49 30.6%	9/49 18.4%	2/49 4.1%	2/49 4.1%	7/49 14.3%	6/49 12.2%	3/49 6.1%	3/49 6.1%
2008	14/50 28%	14/50 28%	18/50 36%	7/50 14%	10/50 20%	22/50 44%	5/50 10%	7/50 14%	6/50 12%
2009	9/32 28.1%	22/69 31.9%	18/67 26.9%	4/69 5.8%	12/68 17.6%	17/68 25%	1/69 1.4%	1/69 1.4%	3/69 4.3%
2010	5/26 19.2%	12/20 60%	12/27 44.4%	3/35 8.6%	4/48 8.3%	12/39 30.8%	4/39 10.3%	5/35 14.3%	7/33 21.2%
2011	5/30 16.7%	11/31 35.5%	14/31 45.2%	0/31 0%	2/31 6.4%	9/31 29%	3/31 9.7%	6/31 19.3%	10/31 32.3%

[1]Number of resistant ***Staphylococcus*** spp. OXA = oxacillin; PEN = penicillin; AMP = ampicillin; CEF = cephalexin; NOR = norfloxacin; TET = tetracycline; SUT = sulphazotrim; GEN = gentamicin; NEO = neomycin; NA = not analysed.

statistical difference between the results of these two classes (P<0.0001). The other classes presented similar resistance means (from 8.5% to 11.6%). Among the antimicrobials from the same classes, different resistance rates were observed (P<0.0001). The highest resistance was observed for beta-lactams, with 46.2% of the isolates resistant to penicillin, 43.9% to ampicillin and 34.7% to oxacillin. Different

results were found for cephalexin, since only 6.9% of the isolates were resistant to this beta-lactam antibiotic. 8.5% of the isolates were resistant to norfloxacin, which was similar to the results found for sulphazotrim (9.1% of resistance). Among aminoglycosides, the highest rate of resistance was demonstrated for neomycin (15.7%), while 7.6% of isolates were resistant to gentamicin.

The dynamic behaviour of the resistance of each isolate is presented in figure 1. During the 20 years studied, there was a reduction in the resistance of ***Staphylococcus*** spp. to penicillin (R^2=50.38; P=0.0005) and ampicillin (R^2=53.65; P=0.0002). However, a decrease in the resistance rates to oxacillin was observed during the first decade, followed by an increase during the second decade (R^2=82.72; P<0.0001) (Figure 1a). It was not observed by regression analysis any evolution pattern of resistance to cephalexin (Figure 1a), norfloxacin (Figure 1b) and tetracycline (Figure 1c). Nevertheless, it was possible to verify a trend towards decreased resistance of the isolates to sulphazotrim (R^2=18.80; P=0.0561) (Figure 1d). The regression analysis for the class of aminoglycosides showed a decline in the resistance

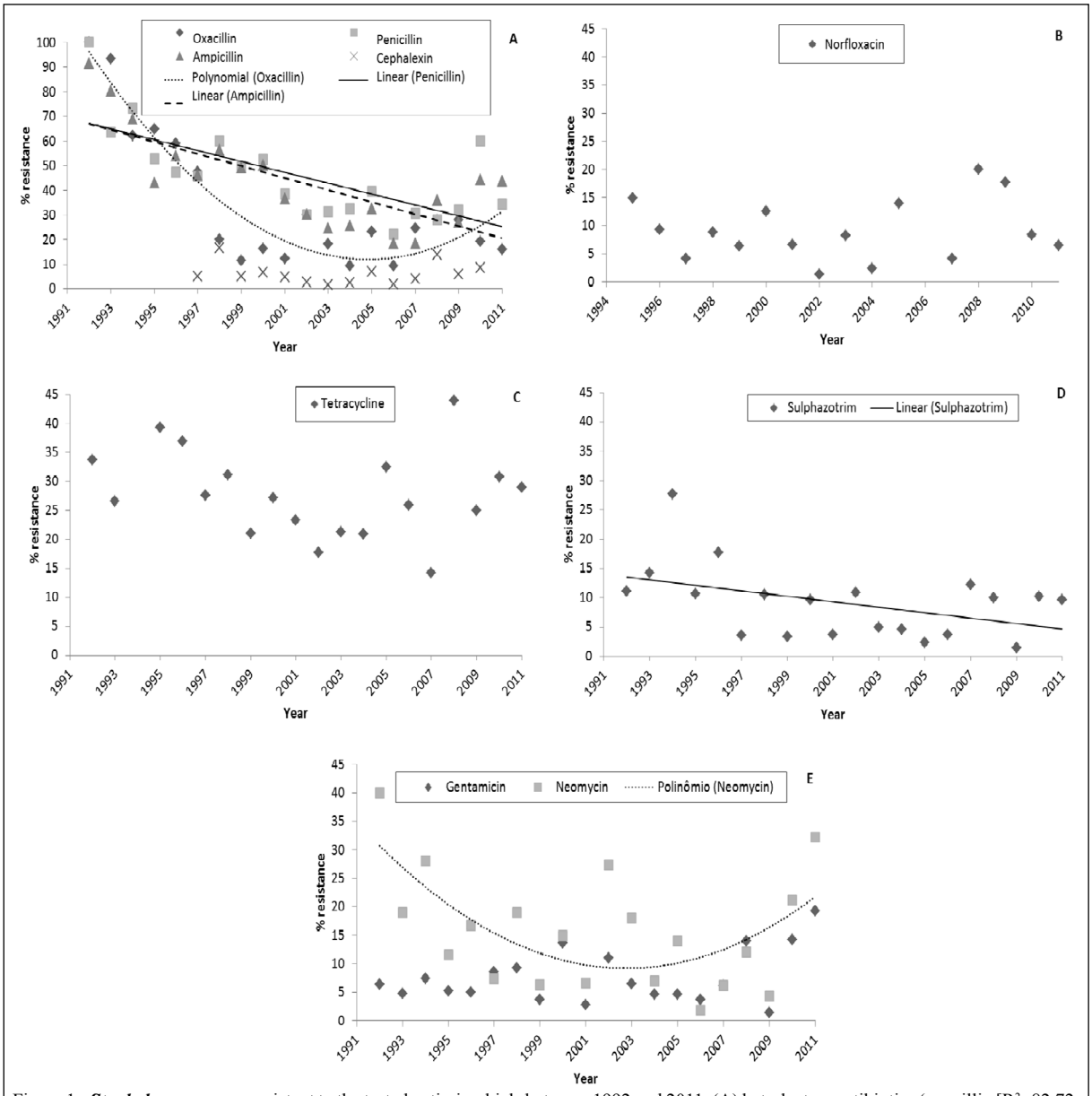

Figure 1 - ***Staphylococcus*** spp. resistant to the tested antimicrobials between 1992 and 2011. (A) beta-lactam antibiotics (oxacillin [R^2=82.72; P<0.0001], penicillin [R^2=50.38; P=0.0005], ampicillin [R^2=53.65; P=0.0002] and cephalexin). (B) Norfloxacin. (C) Tetracyclin. (D) Sulfazotrim [R^2= 18.80; P=0.0561]. (E) aminoglycosides (neomycin [R^2=30.43; P=0.0372] e gentamicin).

to neomycin during the first decade, followed by an increase in resistance in the second decade (R^2=30.43; P=0.0372). However, it was not possible to observe any trend for gentamicin (Figure 1e).

DISCUSSION

Antimicrobial resistance has been the subject of several studies, and currently it is considered the main risk to global health (SPELLBERG et al., 2013). Therefore, it is important to know the dynamics of antimicrobial resistance in order to select effective drugs to eradicate causative agents (GUARDABASSI et al., 2010). Similar to the results found in the present study, other authors also reported high resistance rate to penicillin and ampicillin (MACHADO et al., 2008; MEDEIROS et al., 2009). This result was expected since historically beta-lactams are widely used in mastitis therapy, as well as other bacterial diseases in cattle (MEDEIROS et al., 2009).

At the same time, the regression of resistance to penicillin and ampicillin over the 20 years observed, confirms some evidences observed in previous studies. For instance, Gram-positive bacteria isolated from cows with mastitis showed decreased resistance to beta-lactams (ERSKINE et al., 2002; MAKOVEC & RUEGG, 2003). Additionally, other reports showed an increased availability of several classes of antimicrobials for veterinary use. This range of new available antimicrobials in veterinary can partially explain the decreased resistance of ***S. aureus*** to penicillin in countries with historically high levels of resistance (AARESTRUP & SCHWARZ, 2006).

The lower resistance of ***Staphylococcus*** spp. against cephalexin might be attributed to its relatively recent use in veterinary medicine and due to the fact that cephalosporins are stable to β-lactamases (SPINOSA et al., 2002). Furthermore, other reasons might be the lower availability of veterinary medicine formulations containing this drug (SINDAN, 2013) and the high cost of these products.

The rate of new intramammary infections is approximately four times higher during the dry period compared to the lactation period (SANTOS & FONSECA, 2007). Thus, it is crucial to preserve the susceptibility to beta-lactams and aminoglycosides, since the available formulations for the treatment of mastitis in the dry period consist predominantly of these two antibiotic classes (SINDAN, 2013). Especially in herds where contagious mastitis is well controlled and they are more susceptible to develop environmental mastitis, it is important that these drugs remain effective against the causative microorganisms.

The high level of ***Staphylococcus*** spp. resistant to gentamicin has been attributed to the indiscriminate use of this drug (FREITAS et al., 2005); however, in this study, the average of resistance was relatively low. Similar results were reported in other regions of Brazil where isolates from cases of mastitis showed high sensitivity to gentamicin (MEDEIROS et al., 2009). Among aminoglycosides, the highest resistance rate was found for neomycin suggesting that this antimicrobial was used more often in cattle over the study period.

Although the high level of tetracycline resistant isolates was observed, it was not possible to note any trend of increasing or reducing them over the 20 years. In general, antimicrobial tetracycline class has a broad spectrum of action and is widely used in veterinary medicine mainly for mastitis therapy, respiratory diseases and other bovine diseases (MEDEIROS et al., 2009). Although the *in vitro* sensitivity tests have been requested because of the occurrence of mastitis, it was not possible to determine the form of presentation of the disease or previous antimicrobial treatments for most of the samples. However, the resistance profiles observed in the present study, suggest the occurrence of different degrees of selection pressure from different antimicrobials on strains of ***Staphylococcus*** spp. over the years.

The reduction of resistance is a phenomenon occurring in certain microorganisms, which might happen when selection pressure is removed in certain populations, allowing resistant strains to be replaced by susceptible strains (PHILLIPS et al., 2004). Possible reasons for this phenomenon might be the greater prudence of the use of antimicrobials in dairy herds (ERSKINE et al., 2002) and the improvement of the biochemical constitution of the drugs, which has been correlated with an increase in the antimicrobial susceptibility of ***S. aureus*** isolated from humans (DUARTE & SÁ, 2011). Suggested strategies to reduce the use of antimicrobial treatments in dairy cattle include the treatment of subclinical mastitis cases in the dry period only, and selective dry cow therapy, a strategy that restricts the treatment to infected quarters. The use of this measure can help to reduce the probability of selection of resistant strains among bacterial populations (GUARDABASSI et al., 2010).

Although similar results have been reported to those found in previous studies of antimicrobial agents used in bovine mastitis in other countries (ERSKINE et al., 2002; MAKOVEC & RUEGG, 2003; LINDEMAN et al., 2013), one should be cautious when establishing comparisons

to patterns of resistance of these studies due to (i) sampling criteria, (ii) regional differences of pathogen population, (iii) and differences between laboratory protocols and guidelines for interpretation (AARESTRUP & SCHWARZ, 2006). Furthermore, knowledge and monitoring of local resistance patterns are fundamental to the construction of effective treatment strategies. Prospective studies in this direction, with the comparison of the change in antimicrobial susceptibility after therapy are also highly desirable (ERSKINE et al., 2002).

CONCLUSION

The results indicated no trend towards increased resistance of ***Staphylococcus*** spp. to the most tested antimicrobials. However, it is necessary to monitor the resistance of these antimicrobial agents constantly in order to guarantee a therapeutic reserve.

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