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Author for correspondence:
Amanda Thaís Ferreira Silva,
Email: amanda.tfs@gmail.com

Antimicrobial resistance profile of non-aureus *Staphylococci* isolates from buffalo, goat and sheep mastitis in the Northeast region of Brazil

André de Souza Santos[1], Débora Costa Viegas de Lima[1], Atzel Candido Acosta Abad[1], Pollyanne Raysa Fernandes de Oliveira[1], José Givanildo da Silva[1], Guilherme Santana de Moura[1], Amanda Thaís Ferreira Silva[1], Vinícius da Silva Amorim[2], Mateus Matiuzzi da Costa[2] and Rinaldo Aparecido Mota[1]

[1]Department of Veterinary Medicine, Federal Rural University of Pernambuco, Recife, Brazil and [2]Department of Veterinary Medicine, Federal University of Vale do São Francisco, Petrolina, Brazil

Abstract

The study described in this Research Communication investigated the genotypic and phenotypic profiles of resistance to beta-lactams and other antimicrobials in non-aureus *Staphylococci* (NAS) isolated from buffalo, goat and sheep mastitis in the Northeast region of Brazil. A total of 190 isolates were analyzed and 42.3, 43.9 and 23.6% of them were positive for *blaZ* gene in buffalo, goat and sheep, respectively. Regarding the animal groups, in goats, amoxicillin was the antimicrobial with highest resistance index (72.7%), followed by penicillin G in buffaloes (51.9%) and ampicillin in sheep (43.1%). With regard to multiple antimicrobial resistance, 30.8% of NAS isolates from buffalo milk samples, 25.8% from goats and 25.0% from sheep presented multidrug-resistance. In the minimum inhibitory concentration (MIC) technique, amoxicillin MIC_{50} and MIC_{90} were 64 and 128 μg/ml, respectively, among isolates of the three animal species. In conclusion, high rates of resistance to beta-lactams are presented among NAS isolated from mastitis cases in buffaloes, goats and sheep in Northeast region of Brazil. These results provide an alert to animal and human health researchers, suggesting that the frequency of NAS needs to be reduced because they carry resistance genes which might increase the existing levels of antimicrobial resistance.

Staphylococcus spp. are the main pathogens causing intramammary infection in small ruminants and buffaloes, with emphasis on non-aureus *Staphylococci* (NAS) (Acosta *et al*., 2016). Mastitis caused by NAS usually occurs without noticeable clinical signs (Taponen and Pyörälä, 2009). However, NAS often cause persistent intramammary infections, resulting in higher somatic cell counts (SCC), besides the emergence of antimicrobial resistance, and are considered to be reservoirs of resistance genes with potential transmission for other species (Taponen and Pyörälä, 2009).

Beta-lactams are the most frequently used drugs worldwide to treat both mastitis and other infectious diseases, either in cattle, goats, sheep, buffaloes or other species (Fejzić *et al*., 2014). It is important to highlight that antimicrobial resistance is rising to dangerously high levels due to the regular use of these drugs (Fernandes *et al*., 2013).

Considering the important role of NAS on mastitis etiology in small ruminants and buffaloes, the objective was to study NAS genotypic and phenotypic resistance profiles to beta-lactams and other antimicrobials in strains isolated from goat, sheep and buffalo mastitis in the Northeast region of Brazil.

Materials and methods

Full details of the methodology are provided in Supplementary File Materials and Methods.

The experimental procedures were approved by the Ethics Committee on the Use of Animals (CEUA) of Federal Rural University of Pernambuco (UFRPE), Recife, Brazil (License No. 079/2014).

Bacterial isolates

A total of 190 NAS strains, isolated from milk samples collected from buffalo ($n = 52$), goats ($n = 66$), and sheep's ($n = 72$) mastitis cases between March 2014 and July 2017 in different

Table 1. Percentage of antimicrobial resistance of *non-aureus Staphylococci* isolated from mastitis in buffaloes, goats and sheep in Northeast of Brazil by disk-diffusion technique

	Buffaloes (*N* = 52)		Goats (*N* = 66)		Sheep (*N* = 72)		
Antimicrobial	*R*	%	*R*	%	*R*	%	*P*-value
Amoxicillin	37	71.2	48	72.7	49	68.1	0.829
Ampicillin	18	34.6	23	34.9	31	43.1	0.5187
Cefotaxime	4	7.7	4	6.1	0	0	0.0711
Cefoxitin	1	1.9	1	1.5	2	2.8	0.8703
Ceftriaxone	7	13.5	8	12.1	8	11.1	0.9246
Gentamicin	0	0	1	1.5	1	1.4	0.6815
Norfloxacin	0	0	2	3.0	2	2.8	0.4606
Oxacillin	8	15.4	11	16.7	7	9.7	0.4536
Penicillin G	27	51.9	27	40.9	29	40.3	0.3714
Sulfazotrim	0	0	2	3.0	2	2.8	0.4606
Tetracycline	2	3.9	8	12.1	12	16.7	0.0873
Vancomycin	1	1.9	1	1.5	2	2.8	0.8703

N, total of samples; R, resistant; %, relative frequency.

states in Brazilian Northeast Region as pointed localizeds in Alagoas: São Luiz do Quitunde (43 buffalo); Viçosa (10 goats)/ Bahia: Valente (14 goats)/Paraíba: Bananeiras (20 sheep)/ Pernambuco: Custódia (3 goats; 11 sheep); Floresta (9 sheep); Limoeiro (8 sheep); Petrolina (9 sheep); Ribeirão (9 buffalo); Santa Maria (18 goats); Serra Talhada (7 sheep); Sertânia (21 goats; 8 sheep). Location of these states is shown in online Supplementary Fig. S1. The isolates were stored under freezing in glycerinated BHI (Brain Heart Infusion) broth at −20°C at the Laboratory of Infectious Diseases (LDIC) – UFRPE. Biochemical identification of the isolates was performed by testing the coagulase and acetoin as recommended by the National Mastitis Council (NMC, 2017).

Antimicrobial susceptibility test

In vitro antimicrobial resistance was determined by disk-diffusion method for the following drugs: amoxicillin (30 µg), ampicillin (10 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), norfloxacin (10 µg), oxacillin (1 µg), penicillin G (10 U), sulfazotrim (23.75/1.25 µg), tetracycline (30 µg) and vancomycin (30 µg). Multiple antimicrobial resistance (MAR) index was calculated according to Krumperman (1983). The minimum inhibitory concentration (MIC) for antimicrobials (amoxicillin, cephalexin, cefotaxime, ceftriaxone and oxacillin) was also detected according to CLSI (2015).

Genomic DNA extraction and polymerase chain reaction (PCR)

Bacterial genomic DNA was extracted from 1 mL of culture grown in BHI (Brain Heart Infusion) broth using the Wizard Kit SV Genomic DNA Purification System (Promega®-Madison, Wisconsin, USA) according to manufacturer's instructions. Polymerase chain reaction (PCR) was performed for amplification of *blaZ* gene (see online Supplementary Fig. S2), which encodes beta-lactamases, in addition to *mecA* and *mecC* genes detection, which are inducers of the beta-lactam site of action modification.

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 7.04 for Windows (GraphPad Software, La Jolla, California, USA) and Epi Info™ version 7.2 for Windows (Epi Info™ software, Atlanta, Georgia, USA). χ^2 test was used to verify the statistical significance in the antimicrobial resistance frequencies and MIC values for different species studied. Kaplan–Meier curves were used to assess the concentration-dependent surveillance probability from the first contact with the drug in vitro until the complete suppression of NAS growth in each group of animals (buffaloes, goats and sheep). The analysis was performed using increasing drug concentration in place of the usual time variable. Comparison of survival curves was evaluated using Wilcoxon test. Statistically significant differences were considered for $P < 0.05$.

Results and discussion

In this study, according to MAR index calculated, 30.8% (16/52), 25.8% (17/66) and 25.0% (18/72) of isolates presented multidrug resistance for buffalo, goat and sheep, respectively. Only 7.7% (4/52) of buffalo isolates, 6.1% (4/66) of goats and 11.1% (8/72) of sheep were susceptible to all antimicrobials simultaneously. None of the isolates were resistant to all antimicrobials at the same time. Despite this, it should be noted that NAS are present in animal and human skin, besides being considered reservoirs of resistance genes for strains of *S. aureus* that are generally more virulent and present greater clinical importance for human and other animal species (Tulinski *et al*., 2012).

Percentages of antimicrobial resistance detected in disk-diffusion technique for NAS analyzed are described in Table 1. Amoxicillin, ampicillin and penicillin had high resistance indexes by phenotypical analyses, corroborating with genotypic findings, since resistance to non-stable penicillins, (e.g. penicillin and ampicillin) is determined by the action of beta-lactamases encoded by blaZ gene (Dias *et al*., 2015). Therefore, gentamicin, norfloxacin, sulfazotrim and vancomycin are alternatives for the treatment of mastitis caused by NAS mastitis. Considering MIC

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Table 2. Relative frequency of isolates inhibited for each tested concentration and MIC_{50} and MIC_{90} values for different beta-lactams in non-aureus *Staphylococci* isolates of buffalo, goat and sheep mastitis in Northeast Brazil

	MIC^2 (µg/ml)													
Antimicrobials	<0.25	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	>128.0	MIC$^3_{50}$	MIC$^4_{90}$	*P*-value
NAS isolated from buffalo (*n* = 52)															
AMO	0.0	1.9	3.8	3.8	9.6	3.8	9.6	5.8	9.6	13.5	30.8	7.7	64.0	128.0	<0.0001
CFX	0.0	3.8	5.8	5.8	13.5	7.7	23.1	3.8	3.8	9.6	1.9	21.2	8.0	>128.0	
CTX	0.0	0.0	0.0	1.9	32.7	19.2	13.5	7.7	7.7	5.8	9.6	1.9	4.0	128.0	
CRO	0.0	0.0	11.5	15.4	5.8	5.8	25.0	9.6	5.8	5.8	3.8	11.5	8.0	>128.0	
OXA	0.0	13.5	11.5	15.4	26.9	9.6	19.2	0.0	0.0	0.0	0.0	3.8	2.0	8.0	
NAS isolated from goat (*n* = 66)															
AMO	0.0	0.0	0.0	4.5	12.1	3.0	9.1	7.6	6.1	39.4	10.6	7.6	64.0	128.0	<0.0001
CFX	12.1	4.5	12.1	7.6	3.0	6.1	24.2	3.0	12.1	1.5	1.5	12.1	8.0	>128.0	
CTX	4.5	6.1	3.0	7.6	6.1	37.9	7.6	7.6	6.1	10.6	3.0	0.0	4.0	64.0	
CRO	7.6	0.0	12.1	7.6	10.6	13.6	14	6.1	4.5	6.1	9.1	1.5	4.0	128.0	
OXA	0.0	53.0	4.5	9.1	12.1	10.6	4.5	1.5	1.5	1.5	1.5	0.0	0.25	8.0	
NAS isolated from sheep (*n* = 72)															
AMO	0.0	0.0	1.4	0.0	6.9	15.3	11.1	2.8	8.3	37.5	11.1	5.6	64.0	128.0	<0.0001
CFX	0.0	1.4	4.2	11.1	6.9	9.7	20.8	19.4	6.9	4.2	4.2	11.1	8.0	>128.0	
CTX	1.4	5.6	8.3	11.1	5.6	18.1	4.2	19.4	4.2	11.1	9.7	1.4	4.0	128.0	
CRO	0.0	0.0	11.1	9.7	4.2	9.7	20.8	4.2	9.7	12.5	2.8	15.3	16.0	>128.0	
OXA	19.4	36.1	4.2	5.6	11.1	6.9	5.6	5.6	2.8	2.8	0.0	0.0	0.25	16.0	

[a]*In vitro* essays for non-aureus *Staphylococci* (*n* = 190).
[b]MIC, minimum inhibitory concentration (µg/ml).
[c]MIC_{50}, minimum inhibitory concentration to inhibit 50% of tested non-aureus *Staphylococci* isolates.
[d]MIC_{90}, minimum inhibitory concentration to inhibit 90% of tested non-aureus *Staphylococci* isolates.

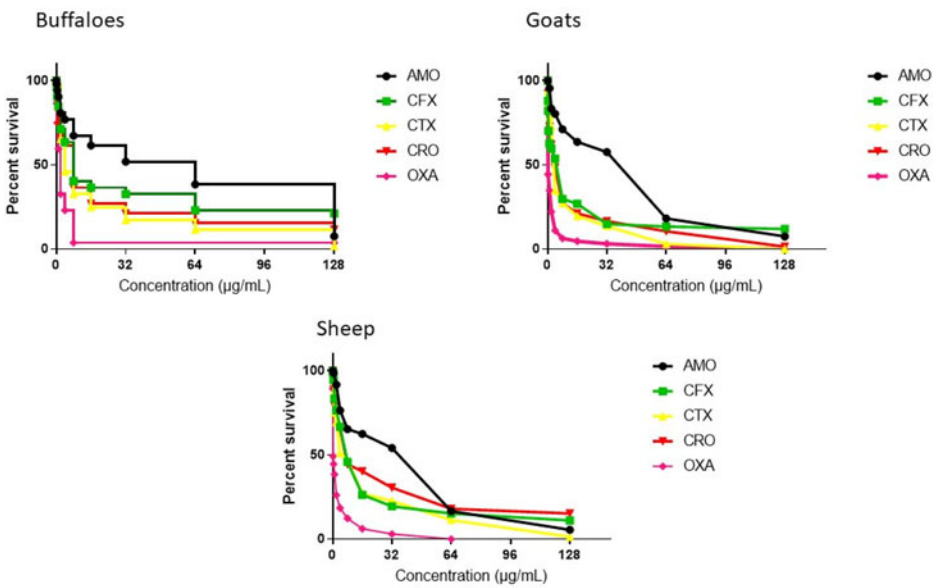

Fig. 1. The comparison of Kaplan–Meier survival curves for MICs of drugs used against NAS isolates from buffaloes, goats and sheep.

results, amoxicillin showed the highest resistance rates with 76.9% (40/52) among NAS from buffalo, 80.3% (53/66) from goat and 76.4% (55/72) from sheep, once again evidence of the ability of beta-lactamases to act as resistance inducing mechanisms in NAS. Despite the variation of only one dilution between the MIC_{50} and MIC_{90} observed, in agreement with results obtained by Oliveira *et al.* (2012), MIC_{50} (64 µg/ml) compared to cutoff point for this antimicrobial (8 µg/ml) demonstrates a worrying tolerance to this antimicrobial. Observed MICs, by species, for each of the beta-lactams tested are described in Table 2.

In this study, 42.3% (22/52) of buffalo origin isolates were positive for blaZ gene. Among isolates from goat origin, this percentage was 43.9% (29/66), while for those of sheep origin 23.6% (17/72) were positive for this gene. All the isolates analyzed were negative for mecA and mecC genes.

The analysis of Kaplan–Meier survival curves constructed for NAS isolates from buffaloes, goats and sheep, is shown in Fig. 1. NAS isolates from buffaloes, goats and sheep had similar sensitivity/resistance profile to the set of studied drugs. In general, higher concentrations of amoxicillin were required ($P < 0.0001$) for complete suppression of NAS growth. Comparison of the survival curves of NAS among animals groups revealed the same pattern for oxacillin (low concentrations of these drugs inhibited the growth of a higher number of NAS strains, $P < 0.0001$).

In the Northeast region of Brazil, sheep are mainly raised for meat and antimicrobial use is less frequent compared to buffalo and goat species. Thus, there is a lower selective pressure of microorganisms carrying resistance genes, such as *blaZ*, causing sheep mastitis. Despite the importance of NAS in mastitis etiology, data on *blaZ* gene detection in this group of microorganisms are still scarce, especially in Brazil. In this study, the detection of *blaZ* gene in all NAS isolates from these three animal species was compatible to phenotypic resistance profiles of penicillin, amoxicillin and ampicillin. According to Dias *et al.* (2015), this usually occurs due to beta-lactamases action, whose production is induced by *blaZ* gene.

All the isolates analyzed were negative for *mecA* and *mecC* genes and the absence of these genes is in agreement with a study carried out by França *et al.* (2012) in this same Brazilian region, suggesting a greater epidemiological importance of *blaZ* gene as an inducer of beta-lactam resistance. The phenotypic results showed a different picture to genotypic analysis. Strains resistant to oxacillin, ceftriaxone and cefotaxime were detected, but they were negative for *mecA* and *mecC* genes. According to Mendonça *et al.* (2012), the absence of *mecA* and *mecC* genes in oxacillin-resistant isolates can be explained by the existence of other resistance mechanisms independent of gene expression, such as the occurrence of homologous genes and the production of other classes of penicillin binding proteins, and also due to a beta-lactamase hyperproduction.

In conclusion, high rates of resistance to beta-lactams are presented among NAS isolated from mastitis cases in buffaloes, goats and sheep in Northeast region of Brazil. Among these isolates, the most active mechanism of resistance is beta-lactamase production that may be causing resistance even to more stable classes of beta-lactams. These results provide an alert to animal and human health researchers, suggesting that the frequency of NAS needs to be reduced because they carry resistance genes which might increase the existing levels of antimicrobial resistance.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029920000771.

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