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Research Article

High Frequency of Beta-Lactam Resistance Among *Staphylococcus aureus* Isolated from Bovine Mastitis in Northeast of Brazil

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Abstract

Beta-lactams are antimicrobials commonly used in the treatment of bovine mastitis caused by *Staphylococcus aureus.*

Resistance to beta-lactams may occur through mechanisms such as beta-lactamases production, alteration of the antimicrobial target or reduction in the amount of the antimicrobial that reaches the target caused by decrease permeability or by an exit increase. The objective of this study was to evaluate the resistance profile of *Staphylococcus aureus* isolated from mastitis in Northeastern Brazil against beta-lactam and other antimicrobials. A total of 161 strains of *Staphylococcus aureus* isolated from milk samples with mastitis were analyzed. 68.9% (111/161) of *S. aureus* were positive for the blaZ gene, while mecA and mecC genes were not detected. The highest rates of antimicrobial resistance occurred for amoxicillin, ampicillin and penicillin with 77.6% (125/161), 67.7% (109/161) and 64.6% (104/161), respectively, in the disk-diffusion technique. Amoxicillin had 91.3% (147/161) of resistance in minimal inhibitory concentration detection and 9.3% (15/161) *aureus* were multidrug resistant. It is concluded that there is a high prevalence of beta-lactam resistant *S. aureus* in the Northeast region of Brazil, and the blaZ gene is the main inducer of resistance to this antimicrobial class. These levels of antimicrobial resistance should be considered as an alert to animal and human health.

Keywords: Betalactamase;Blaz; Milk; Multidrug Resistance

Introduction

Mastitis is a plurietiological and multifactorial disease of the mammary gland, responsible for considerable damages to the properties with creations directed to milk production and to the dairy industry, as much for the expenses with control and prophylaxis, as for the decrease in quantity, quality and industrial yield of the milk produced [1].

Main microorganisms involved in the infectious etiology of bovine mastitis are those of contagious origin, especially bacteria of the genus *Staphylococcus* spp., because they have high frequency in the world herds and are difficult to treat due to the high antimicrobial resistance and the presence of several mechanisms of virulence [2]. *Staphylococcus aureus* is the most important bacteria in the etiology of this disease, found in both clinical and subclinical case,[3] and has an impact on public health due to its capacity to cause food poisoning and to transfer antimicrobial resistance [4].

Prophylactic or therapeutic use of antibiotic in mastitis is one of the main reasons for the use of antimicrobials in dairy herds [5] and beta-lactams are the most frequently used antimicrobials in dairy cattle. As a result, worrying rates of resistance to this antimicrobial class are observed in microorganisms involved in mastitis [6].

Considering the importance of *Staphylococcus aureus* as mastitis cause in cows and its impact on the milk production chain in Northeast region of Brazil, aimed to study the resistance profile of this bacterium to beta-lactams.

Material and Methods

Ethical Approval

This study was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal Rural University of Pernambuco (UFRPE), Recife, Brazil (License No. 079/2014).

Bacterial Isolates

A total of 936 cow milk samples**,** from 25 farms**,** located in the states of Alagoas, Bahia, and Pernambuco, Northeast of Brazil, were analyzed. Milk samples with California Mastitis Test (CMT) ≥1+ or positive samples in the screened cup test, 538 in total, were plated in 5% sheep blood agar and incubated at 37°C for 24 to 48 hours. Afterwards, a presumptive classification of *S. aureus* based on colony morphology, dyeing characteristics in the Gram technique and biochemical tests such as DNase production, catalase, coagulase and mannitol fermentation was performed [7].

Genomic DNA Extraction and Polymerase Chain Reaction (PCR)

Isolates classified as *S. aureus*in the biochemical tests had the genomic DNA extracted from 1mL of culture grown in BHI broth (Brain Heart Infusion) using the Wizard Kit SV Genomic DNA Purification System (Promega® - Madison, Wisconsin, USA), accordingtomanufacturer'sinstructions.Formolecularconfirmation of isolates such as *Staphylococcus aureus*, PCR was performed for amplification of the nuc gene, specific for *S. aureus*, and then for the blaZ gene encoding betalactamases, in addition to mecA and mecC genes, inducers of alteration of the antimicrobial target.

Reactions were assembled separately for each gene in a final volume of 15µL per well, containing 100ng DNA template, 10pmol of each oligonucleotide (Table 1), Taq buffer (10mM Tris, 50mM KCl, 2.5mM $MgCl_2$), 200mM dNTPs and 1U Taq DNA polymerase (Cenbiot, Ludwig Biotec, Porto Alegre, RS, Brazil). Thermal profiles of the amplifications were 4 min. at 94°C, followed by 32 cycles of denaturation at 94°C for 30 sec., annealing at 65°C for 30 sec. (nuc gene), 50.5°C for 30 sec. (blaZ gene) or 55 °C (mecA and mecC genes) and extended at 72°C for 30 secs, with final extension at 72°C for 5 min. Then 10µL of each reaction was electrophoresed for 40 minutes at 100V in 1.5% agarose gel stained with BlueGreen, visualized and photo documented under ultraviolet light.

Oligonucleotide	Sequence (5'-3')	Amplicon (bp)	Reference
nucA	F-GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	R-279	19
blaZ	F-AAGAGATTTGCCTATGCTTC GCTTGACCACTTTTATCAGC	R-517	42
mecA	F-TGGTATGTGGAAGTTAGATTGGGAT CTAATCTCATATGTGTTCCTGTATTGGC	R-155	31
mecC	F-CATTAAAATCAGAGCGAGGC TGGCTGAACCCATTTTTGAT	R-188	34
bp=base pairs			

Table 1: Oligonucleotides sequences and sizes of the amplified fragments used in this study

Antimicrobial Susceptibility Test

In vitro antimicrobial resistance was determined by disc-diffusion method for the following drugs: amoxicillin (30µg), ampicillin (10µg), cefotaxime (30µg), cefoxitin (30µg), ceftriaxone (30µg), gentamicin (10µg), norfloxacin (10µg), oxacill in (1µg), penicillin G (10U), sulfazotrim (23.75/1.25µg), tetracycline (30µg) and vancomycin (30µg). Multiple Antimicrobial Resistance (MAR) index was calculated as described previously [8]. Minimum inhibitory concentration (MIC) for antimicrobials (amoxicillin, cephalexin, cefotaxime, ceftriaxone and oxacillin) was also detected according to standards techniques [9].

Statistical Analysis

Statistical differences in antimicrobial resistance frequencies were calculated using the Fisher Exact test ($p \leq 0.05$) (Epi Info™, version 7.2). For the other analyzes, descriptive statistics were used, calculating the absolute and relative frequencies of the results [10].

Results

A total of 298 *Staphylococcus* spp. were recovered from the 538 cultivated samples, of which 161 (54.0%) had a presumptive classification of *Staphylococcus aureus* in the biochemical tests and 100% of them (161/161) were confirmed in the molecular test (nuc gene) (Figure.1).

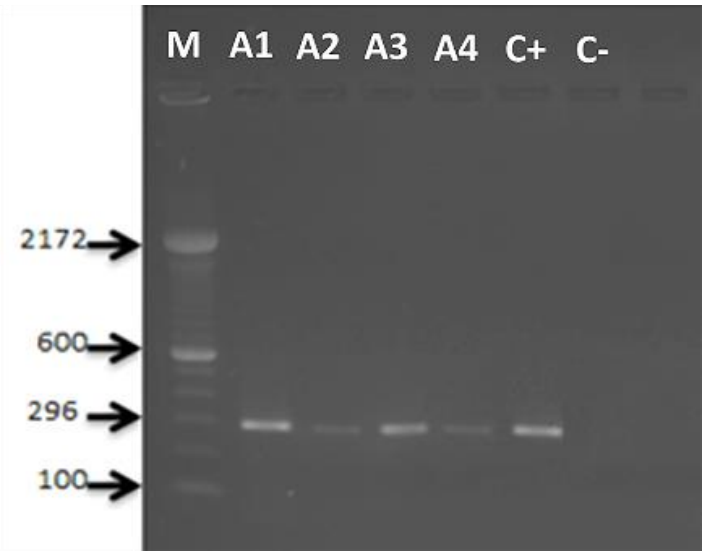

Figure 1: Amplification of nuc gene fragment in *S. aureus*isolated from cow's milk with mastitis. Column M: 100pb molecular weight marker (Invitrogen); Columns A1 to A4: Samples tested; C + column: positive control; Column C-: negative control.

BlaZ gene (Figure 2) was detected in 68.9% (111/161) of the *S. aureus*isolates and the mecA and mecC genes were not detected in any of the isolates tested.

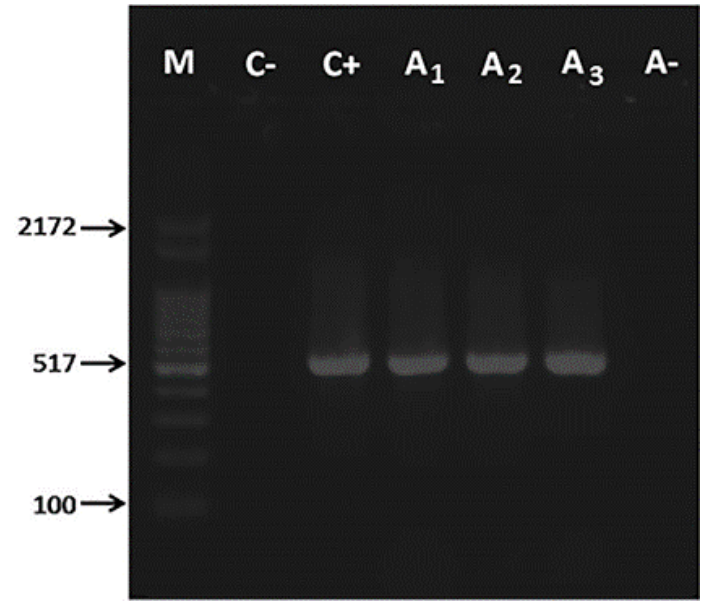

Figure 2: Amplification of blaZ gene fragment in *S. aureus* isolated from cow's milk with mastitis. Column M: 100pb molecular weight marker (Invitrogen); Column C-: negative control; Column C+: positive control; Columns A1 to A3: Samples tested with positive result; Column A- Sample tested with negative result.

Percentage of resistance in the *S. aureus* disc-diffusion technique for the tested antimicrobials is shown in the (Table 2).

	S. aureus(n=161)
Amoxicillin (30µg)	125 (77,6%)
Ampicillin (10µg)	109 (67,7%)
Penicillin G (10U)	104 (64,6%)
Tetracycline (30µg)	24 (14,9%)
Ceftriaxone (30µg)	12 (7,5%)
Oxacillin (1µg)	11 (6,8%)
Cefotaxime (30µg)	7 (4,3%)
Cefoxitin (30µg)	2 (1,2%)
Gentamicin (10µg)	2 (1,2%)
Nnorfloxacin (10µg)	1 (0,6%)
Sulfazotrim (23,75/1,25µg)	0 (0,0%)
Vancomycin (30µg)	0 (0,0%)

Table 2: Percentage of antimicrobial resistance of *Staphylococcus aureus*isolated from mastitis in cows of Alagoas, Bahia and Pernambuco, Brazil, by disc-diffusion technique.

There was a significant difference between the antimicrobial resistance indexes per group, especially amoxicillin, ampicillin and penicillin, belonging to the beta-lactam group, compared with norfloxacin (fluoroquinolones), gentamicin (aminoglycosides), vancomycin (glycopeptides) and sulfazotrim ($p < 0.05$). There was no statistical difference between the representatives of beta-lactams and tetracycline (tetracyclines) ($p \geq 0.05$).

Multiple Antimicrobial Resistance (MAR) index ranged from 0 to 0.6 among the isolates, 9.3% (15/161) of which were considered multidrug resistant, while 26.7% (43/161) of the strains were sensitive to all the drugs tested.

Minimum Inhibitory Concentration (MIC) showed that amoxicillin had a higher resistance index, 91.3% (147/161) were resistant to this drug, requiring at least 128 µg/mL of this drug to have antimicrobial action, while the cutoff point is 8µg/mL. Cephalexin was ineffective for 39.1% (63/161) of *S. aureus*, followed by ceftriaxone with 20.5% (33/161), oxacillin with 18.6% (30/161), and cefotaxime with 7.5% (12/161).

Discussion

These data include the most extensive study on the antimicrobial resistance of *Staphylococcus aureus* to beta-lactams, fluoroquinolones, sulfonamides, tetracyclines, aminoglycosides and glycopeptides in Alagoas, Bahia, and Pernambuco states, and they are widely used in the treatment of bacterial diseases in ruminants, including mastitis.

Staphylococcus aureus were identified by biochemical and molecular tests in 29.9% (161/538) of the cultivated samples. This finding is similar to that described in the worldwide literature on

the etiology of mastitis [11-13]. Studies in Brazil have shown that the frequency of *S. aureus*in bovine mastitis can vary between 3.2% and 70.9% [14,15]. In Northeast of Brazil, where this study was conducted, the dry climate prevails most of the year, mainly in the municipalities where the milk basins are located. Previous study [3] has shown that the dry climate may favor the prevalence of contagious agents in mastitis. In addition, *S. aureus* as well as other contagious microorganisms are generally found in the udder and on the surface of the infected cows' teat, which is the primary source of infection for healthy animals, which usually occurs during milking [16].High prevalence of *S. aureus* has been associated with the absence of pre- and post-dipping, washing and disinfection of the hands of milkers and equipment between milking and non-discarding of cows with chronic mastitis [17].

High *in vitro* resistance rates of *S. aureus* to amoxicillin, penicillin and ampicillin were observed in this study, limiting the indication of these antimicrobials in the treatment of mastitis in this region. These data are in agreement with those described in the Brazilian literature [18-21], and worldwide [22,23,5]. The constant and unaddicted use of these antimicrobials in dairy herds from the studied region has also been reported in other countries in world [5, 22]. In this region, beta-lactams (amoxicillin, ampicillin and penicillin) are widely used in the intramammary or systemic treatment of mastitis and other infectious diseases without sensitivity tests [2]. This strongly contributes to the high rates of resistance to these antimicrobials through various mechanisms such as efflux pump, alteration of target drug in the microorganism, and degradation or modification of drug molecules by enzymes [24]. These mechanisms are conferred by genes that can be acquired by horizontal transfer or by spontaneous mutation, [25,26] being the populations, carriers and resistant, selected through selection pressure, mainly by antimicrobial sub-dosages [27]. Often, the choice by the cattle rancher of the antimicrobials used in the herd is made without laboratory support such as lactoculture and antibiogram, considering only the cost of the drug and the period of discarding the milk after treatment [28]. To reduce the negative impact of antimicrobial use in dairy herds, it is important to obtain guidance from the veterinary service with a technical and judicious basis to reduce the multiplication of resistant microorganisms.

On the other hand, considering the high efficacy observed *in vitro* for antimicrobials: sulfazotrim, gentamicin, cefoxitin and norfloxacin compared to *S. aureus* strains analyzed, these may be indicated as an alternative for the treatment of intramammary infections in the herds of this region.

Based on MAR, which in our study ranged from 0 to 0.6, multidrug resistance observed among *S. aureus* isolates was 9.3% (15/161), being smaller than the results obtained in previous studies [2,19,29] which reported 65.6%, 48.6%, and 39.6% of multidrug resistance, respectively. Although the multidrug resistance rate observed is relatively low, researchers [30] believe that determining antimicrobial sensitivity profiles favours the rational control of mastitis, allowing the selection of drugs more appropriate for the treatment of mastitis and reducing the selection pressure of the microorganisms involved.

High amoxicillin resistance (91.3%) detected in the minimal inhibitory concentration technique reinforced the results obtained in the disk-diffusion technique, in addition to dimensioning the resistance intensity, where a high tolerance of *S. aureus* strains was observed for this antimicrobial base. Although all drugs tested in the MIC are beta-lactams, the mechanisms of resistance involved may vary according to the genetic characteristics of the microorganism, so susceptibility to different beta-lactams may also vary [31]. In this case, resistance to amoxicillin is associated with beta-lactamases production, which is the same mechanism observed in penicillin-resistant strains, while resistance to oxacillin, ceftriaxone and cefotaxime would be related to the modification of the penicillin binding protein (PBP), a mechanism present in Methicillin-resistant Strains (MRSA) [32, 33].

Although the disk-diffusion method is the routine test used to determine susceptibility to antimicrobials, cutoff points for this methodology are based on serum drug concentration [9], and there may be variations between the concentration of circulating antimicrobial and in the sites of action, where these drugs may be more, or less concentrated [34]. In the case of intramammary application, the mammary gland can concentrate the antimicrobial and, with this, the minimum inhibitory concentration (MIC) may be reached at this site. Therefore, many microorganisms considered resistant in the antibiogram may respond to the intramammary treatment of mastitis [35]. To confirm this hypothesis, studies are needed to assess MIC in association with the pharmacokinetics and pharmacodynamics of antimicrobials in the mammary gland.

Presence of the blaZ gene in 68.9% (111/161) analyzed *S. aureus* is compatible with the proportion of isolates resistant to ampicillin and penicillin, which were 67.7 and 64.6% respectively. According to previous study [36] in Gram positive bacteria, the production of beta-lactamase enzyme occurs only in the presence of inducers such as penicillin, which is generally observed in bovine herds studied because they are exposed to antimicrobials more frequently due to the management intensive, exerting selection pressure for resistant microorganisms.

Use of penicillin in the treatment of infections caused by *Staphylococcus aureus* is in disuse in human and veterinary medicine due to the high frequency of resistant isolates for many decades, especially in animal species of production [2,18,23,36]. However, the empirical and constant use of this drug is still a reality in many dairy herds [37,38], and constant monitoring of the circulating strains should be maintained in addition to dose and treatment period attention, avoiding sub -dosages.

Absence of *S. aureus* strains carrying the mecA and mecC genes in this study differs from studies in other countries that have demonstrated the presence of these genes in *S. aureus* isolates from bovine mastitis [39-42]. This absence of mecA and mecC genes reinforces the participation of the blaZ gene in the beta-lactam resistance mechanism verified in this study, once that according to previous study [43] a high production of betalactamases may lead to oxacillin resistance in strains of *Staphylococcus*spp.

Studies of this nature have a great impact on national livestock, as they contribute to the planning of control of Methicillin-resistant *Staphylococcus aureus* (MRSA) in herds, which would make them reservoirs of antimicrobial resistance genes with risk of transmission to humans, in addition to making it increasingly difficult to treat intramammary infections.

Conclusion

Results obtained in this study demonstrate that bacteria of the genus *Staphylococcus* spp. have great importance in the epidemiology of bovine mastitis in this region, with emphasis on *S. aureus*. Levels of beta-lactam resistance observed alert to the risk of transmission of these microorganisms to humans. It is recommended the constant monitoring of circulating microorganisms, as well as the choice and judicious use of antimicrobial drugs to control mastitis, avoiding the selection of resistant bacteria.

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