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Prevalence of mastitis and characterization of bacterial agents isolated from dairy herds in the microregion of Garanhuns, state of Pernambuco, Brazil

Prevalência de mastite e caracterização de agentes bacterianos isolados de rebanhos leiteiros da microrregião de Garanhuns, estado de Pernambuco, Brasil

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ABSTRACT: The aims of the present study were to establish the prevalence of bovine clinical and subclinical mastitis in dairy herds located in the microregion of Garanhuns in the state of Pernambuco, Brazil, determine antimicrobial susceptibility as well as identify antimicrobial resistance mechanisms and the production of biofilm in the bacterial groups isolated. The study was conducted in 10 herds of dairy cattle. The number of lactating cows per herd ranged from 22 to 90, totaling 477 animals. The prevalence of clinical and subclinical mastitis ranged from 2.7 to 18.2% and 38.9 to 76%, respectively. The bacterial genera isolated from the cows with mastitis were *Staphylococcus*, *Streptococcus* and *Corynebacterium*, with respective frequencies of 75.2, 17 and 2.6%. Among the species identified, the most frequent was *Staphylococcus aureus* in both clinical and subclinical mastitis. The antimicrobial susceptibility test of the isolates of *Staphylococcus* and *Streptococcus* revealed high rates of resistance to penicillin and tetracycline. Biofilm production was found in 95.4% of *Staphylococcus* genera whereas none of the samples presented efflux pumps. In conclusion, high rates of mastitis were found in the herds analyzed. Moreover, the etiological agents are bacteria that have the potential for biofilm production and are resistant to the main groups of antimicrobials used for the control of the disease, making bovine mastitis a challenge for veterinarians who work in the studied region.

KEYWORDS: Intramammary infection; antimicrobial resistance; biofilm; bacteria.

RESUMO: O objetivo deste estudo foi estabelecer a prevalência de mastite clínica e subclínica em vacas pertencentes a rebanhos localizados na microrregião de Garanhuns, estado de Pernambuco, como também avaliar a susceptibilidade antimicrobiana, identificar a presença de mecanismos de resistência antimicrobiana e a produção de biofilmes nos grupos bacterianos isolados. O estudo foi realizado em 10 rebanhos de bovinos leiteiros, com número de fêmeas em lactação que variou de 22 a 90, totalizando 477 animais. A prevalência de mastite clínica nos rebanhos avaliados variou de 2,7 a 18,2% e de mastite subclínica de 38,9 a 76%. Os gêneros bacterianos isolados do leite de animais com mastite foram *Staphylococcus*, *Streptococcus* e *Corynebacterium*, com frequências de 75,2, 17 e 2,6%, respectivamente. Dentre as espécies identificadas, *Staphylococcus aureus* foi a mais frequente, tanto dos casos de mastite clínica quanto de mastite subclínica. O perfil de susceptibilidade das amostras de *Staphylococcus* e *Streptococcus* evidenciou altas taxas de resistência frente à penicilina e tetraciclina. A produção de biofilme foi observada em 95,4% dos estafilococos, enquanto em nenhuma dessas amostras foi demonstrada a presença de bomba de efluxo. Conclui-se que nos rebanhos avaliados a mastite está presente em taxas elevadas e os agentes etiológicos são bactérias que apresentam resistência aos principais grupos de antimicrobianos utilizados no controle da enfermidade, além de apresentarem potencial para a produção de biofilme, tornando a mastite bovina um desafio para o profissional médico veterinário que atua na região.

PALAVRAS-CHAVE: Infecção intramamária; resistência antimicrobiana; biofilme; bactérias.

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INTRODUCTION

Bovine mastitis is the main illness affecting dairy herds throughout the world. This condition is characterized by inflammation of the mammary gland which has a multifactorial etiology but those of infectious origin stand out, particularly caused by bacteria, such as those of the genera *Staphylococcus, Streptococcus* and *Corynebacterium* as well as Gram-negative bacteria, such as *Escherichia coli*, *Klebisiella* sp. and *Pseudomonas* sp. (Ferreira; Ribeiro, 2022; Lima *et al.*, 2022; Ulsenheimer *et al.*, 2022).

The prevalence of bovine mastitis varies depending on the type of animal management practices as well as predisposing factors, such as trauma to the mammary gland, type of milking and milk production (Vieira *et al.*, 2022; Youssif *et al.*, 2021). In Brazil, the prevalence of the clinical and subclinical forms in dairy herds ranges from 2.38 to 38% and 20.6 to 87.5%, respectively (Niero *et al.*, 2021; Oliveira *et al.*, 2020).

The high prevalence of the disease exerts a negative impact on dairy cattle farming due to the cost of treatment, increase in labor, the discard of contaminated milk, reduction in milk production and the loss of animals (Lopes *et al.*, 2020).

Antibiotic therapy is an important component of mastitis control and prevention programs, with the indication for treatment and prevention of the clinical form during the lactation and dry period, respectively (Ordoñez *et al.*, 2022). However, such treatment favors the selection and dissemination of resistant bacterial strains, especially when antimicrobials are used incorrectly and excessively, which poses public health risks throughout the world (Carvalho *et al.*, 2021).

Bacterial resistance to antimicrobials is an evolutional phenomenon determined by genetic changes that define the expression of different resistance mechanisms either alone or in combination in the same bacterial strain, such as enzyme inactivation, efflux pump, the blocking of the drug entrance and a change in the antimicrobial target site (Nascimento *et al.*, 2023; Silva *et al.*, 2022). Besides antimicrobial resistance, pathogenic bacterial can express other survival strategies, which are known collectively as virulence factors, to invade and multiply in an infection site, causing tissue damage that characterizes and composes the symptoms of a bacterial disease (Åvall-Jääskeläinen *et al.*, 2021). The formation of biofilms and resistance to antimicrobials are examples of virulence factors that interfere with the control of bacterial diseases such as mastitis (Israel *et al.*, 2022; Lucas *et al.*, 2021a).

Considering this context, the aims of the present study were to establish the prevalence of bovine clinical and subclinical mastitis in dairy herds located in the microregion of Garanhuns, state of Pernambuco, Brazil, determine antimicrobial susceptibility as well as identify antimicrobial resistance mechanisms and the production of biofilm in the bacterial groups isolated.

MATERIALS AND METHODS

This study was conducted in 10 herds of dairy cattle. The number of lactating cows ranged from 22 to 90, totaling 477 animals. The herds were on rural properties located in the municipalities of Iati (n = 8), Bom Conselho (n = 1) and Águas Belas (n = 1), which are part of the microregion of Garanhuns in the state of Pernambuco. Visits to the properties, the assessment of the animals and the collection of samples were performed between August 2020 and July 2021. This study received approval from the Ethics Committee on Animal Use of the Federal Rural University of Pernambuco (CEUA-UFRPE protocol nº 4403240520) and the Ethics Committee in Research (CEP, Plataforma Brasil, protocol nº 4.428.064).

To establish the prevalence of clinical mastitis in the herds, information was collected from the property owners on the occurrence of previous cases, clinical examinations of the udder and the black-bottomed mug results. The prevalence of subclinical mastitis was established using the California Mastitis Test (CMT) on all lactating cows. Milk samples were taken from all animals with clinical mastitis as well as 10 to 25% of those with a positive CMT result (1+, 2+ and 3+), except in herds in which the number of cows with a positive CMT was less than or equal to 10 animals; in such cases, samples were collected from all positive animals. The standard method was used for collection, transport, isolation, and identification of the etiological agents (NMC, 2017).

Antimicrobial susceptibility of the bacterial isolates was determined by the disk diffusion method (CLSI, 2018) using the following drugs and respective concentrations: amoxicillin-clavulanic acid (30 μg), cefoxitin (30 μg), cephalothin (30 μg), enrofloxacin (5 μg), penicillin G (10 UI), gentamicin (10 μg) and tetracycline (30 μg). The interpretation of the inhibition zones was carried out according to CLSI (2018).

The investigation of the efflux pump was performed using the method proposed by Ugwuanyi *et al.* (2021) based on the capacity of the bacteria to expel ethidium bromide (EtBr). Plates with Mueller-Hinton agar were prepared containing different concentrations of EtBr (0.5, 1.0, 1.5 and 2.0 mg/L). Plates without EtBr were used as the control. Bacterial samples were inoculated in a radiated star shape and incubated under aerobiosis at 37°C for 24 hours, followed by the transilluminator reading and recording of the results. The interpretation was based on the presence (negative for efflux pump) or absence (positive for efflux pump) of any degree of fluorescence.

Biofilm production was investigated using the microtiter plate (MTP) method (Darwish; Asfour, 2013). The plates were read in a microplate reader (Analítica, Asys UVM 340) at 570 nm. The interpretation of the results was in accordance with the criteria suggested by the same authors. After establishing the cutoff point from the arithmetic mean and standard deviation of the optical density (OD) of the negative control (ODc), the arithmetic mean of the test samples (ODt) was used to classify the bacterial isolate as follows: ODt ≤ 0.070

= non- biofilm producer; 0.070 < ODt ≤ 0.140 = weak biofilm producer; 0.140 < ODt ≤ 0.280 = moderate biofilm producer; and ODt > 0.280 = strong biofilm producer. All tests were performed in triplicate.

The data were organized on Excel® spreadsheets, with the calculation of the mastitis cases, frequencies of the bacterial types isolated, and each characteristic analyzed. The following equation was used for the calculation of the prevalence of clinical and subclinical mastitis (Cortês, 1993):

$$\text{Prevalence} = \frac{\mathit{Number\ of\ affected\ animals}}{\mathit{Total\ number\ assessed}} \text{ x } 100$$

RESULTS AND DISCUSSION

Table 1 displays the prevalence of clinical and subclinical mastitis in the 10 herds analyzed, with high rates in all of them. Surveys conducted by other authors in different Brazilian regions also report high prevalence of subclinical mastitis in bovine dairy herds, i.e., 76.9% in the Santa Catarina State (Niero *et al.*, 2021), 55.9% in the Rio Grande do Norte State (Oliveira *et al.*, 2020) and 40.2% (Lima *et al.*, 2022) in the Paraiba State. For clinical mastitis, prevalence of 0.99% (Santos; Mendonça; Muniz, 2020) to 28.3% (Ferreira; Roquete, 2019) has been reported.

In the evaluated herds the presence of mastitis probably is causing huge economic loss to the milk producer as well as to the dairy industry in the region. It's important to highlight that the subclinical form is responsible for the largest portion of the economic harm caused by mastitis in dairy herd, exerting negative impacts on the production, composition and shelf life of milk and other dairy products (Lopes *et al.*, 2020; Massote *et al.*, 2019; Ribeiro; Caliman; Gasparotto, 2023). On the other hand, high rates of clinical mastitis in dairy herds also increase the production cost of milk, exerting a negative impact on the entire chain of dairy products (Romã; Madureira, 2020) and posing a serious risk to the health of consumers, as milk from animals with this form of the disease could carry traces of antimicrobials, subproducts of microbial metabolism and bacterial strains carrying resistance genes (Peréz *et al.*, 2020).

Table 1. Prevalence of clinical and subclinical mastitis in dairy herds located in the microregion of Garanhuns, Pernambuco, Brazil.

Herd	Animals	Prevalence	
		Clinical mastitis	Subclinical mastitis
1	27	2 (7.4%)	13 (48.1%)
2	39	3 (5.7%)	30 (72.9%)
3	53	4 (18.2%)	31 (58.5%)
4	22	0 (0.0%)	11 (50,0%)
5	22	3 (7.0%)	9 (40.9%)
6	43	5 (7.0%)	21 (48.8%)
7	71	3 (3.3%)	54 (76.0%)
8	90	9 (12.5%)	35 (38.9%)
9	72	1 (2.7%)	48 (66.7%)
10	38	1 (6.7%)	28 (73.7%)
TOTAL	477	31 (6.5%)	280 (58.7%)

In the present study, 242 milk samples from 31 animals with clinical mastitis and 211 with the subclinical form were submitted to the microbiological culture. Microbial growth was seen in 153 samples (63.2%) and no growth was found in 63 (36.8%). Among the positive samples, the genus *Staphylococcus* was the most frequently isolated, followed by *Streptococcus* and *Corynebacterium* (Table 2). The present finding is similar to those reported by other investigators who have observed a high frequency of isolation of the genus *Staphylococcus* in the milk of cows with mastitis in Brazilian herds (Carvalho *et al.*, 2021; Lima *et al.*, 2022; Pinto *et al.*, 2021), demonstrating the importance of this group of bacteria in the etiology of this disease.

The lower frequencies of the genera *Streptococcus* and *Corynebacterium* in the present study agree with the results of previous surveys in the same area (Krewer *et al.*, 2013; Mota *et al.*, 2012), indicating that mastitis in cattle of the microregion of Garanhuns are predominantly caused by *Staphylococcus*.

As shown in Table 3, *Staphylococcus aureus* (*S. aureus*) was the most frequently isolated species in the milk from animals with clinical and subclinical mastitis (48.3 and 49.2%, respectively). This species has been reported in previous studies as the main etiological agent of bovine mastitis, with similar rates, i. e. 65% (Pinto *et al.*, 2021) or higher than to those found in the present study, i. e. 90% (Freitas *et al.*, 2018). This pathogen frequently causes persistent intramammary infections, determining an intense inflammatory response and high somatic cells count (SCC) in the milk of infected animals, with negative impacts on the quality of the raw milk (Ordoñez *et al.*, 2022; Romã; Madureira, 2020) and posing a serious risk to public health since this bacterial species may carry determinants of antimicrobial resistance and other virulence factors (Lucas *et al.*, 2021a).

The Coagulase-negative *Staphylococcus* (CNS) group was isolated in six (20.7%) and 37 (29.8%) milk samples from cows with clinical and subclinical mastitis, respectively, either as a single agent or in combination with other bacteria. In a

Table 2. Frequencies of bacterial genera isolated from cows with clinical and subclinical mastitis.

Genus	AF*(N)	RF*(%)
Staphylococcus	115	75.2
Streptococcus	26	17
Corynebacterium	4	2.6
Staphylococcus + *Corynebacterium*	4	2.6
Staphylococcus + *Streptococcus*	4	2.6
TOTAL	153	100

*AF=absolute frequency; RF=relative frequency study carried out in the Federal District and its surrounding areas, Carvalho *et al*. (2021) reported a 30.7% rate of CNS isolated in cases of bovine mastitis, representing the most prevalent isolated group in the herds analyzed. As found in the present investigation, other researchers have reported the association of the CNS group with bovine mastitis, but at a lower frequency than *S. aureus* and species of *Streptococcus* (Almeida *et al*., 2021). The occurrence of CNS in dairy herds is a cause of concern, as these emerging microorganisms can carry antimicrobial resistance genes, as demonstrated by Lucas et al. (2021b) and Klibi *et al*. (2018), besides have the potential to transfer these genes to other bacterial types (Fluit *et al*., 2013).

The frequency observed for *S. agalactiae* in this study agrees with that published by Lakew; Fayera; Ali (2019), who found a 10.3% frequency for this specie. Differently from the present results, Bettanin *et al*. (2019) observed a 1.7% frequency for *S. agalactiae* as etiological agent of bovine mastitis. About the frequencies observed for *S. uberis*, they were higher than those reported by Bettanin *et al*. (2019), i.e., 1.3%, and Almeida *et al*. (2021), i. e., 3.05%.

It is important to reinforce the importance of these species in the epidemiology of bovine mastitis. *S. uberis* is an environment-associated straw bedding and pasture pathogen that is difficult to control, emerging as an important pathogen of the mammary gland in recent decades (Abd El-Aziz *et al*., 2021) and *S. agalactiae* is considered an obligate contagious agent of the bovine mammary gland that causes mainly subclinical mastitis and elevated milk SCC (El-Razik *et al*., 2021). Thus, and even at a lower frequency than that observed for *Staphylococcus*, the results of the present study suggest that in herds in the studied region *Streptococcus* species are important etiological agents of bovine mastitis.

In the herds analyzed, bacteria of the genus *Corynebacterium* were isolated from 5.7% of the milk samples from cows with subclinical mastitis either as a single agent or associated to *Staphylococcus*, and as a single agent in a case of clinical mastitis. Although divergent opinions are found on the role of these agents in the pathogenesis of bovine mastitis, some authors have reported cases of clinical and subclinical mastitis caused by these bacteria as well as an increase in somatic cells in milk from these animals (Gonçalves *et al*., 2016; Lücken *et al*., 2021). The importance of the genus *Corynebacterium* was recently determined by the identification of a new species isolated repeatedly in cases of bovine mastitis (Kittl *et al*., 2022).

The *in vitro* susceptibility of the isolates of *Staphylococcus* is shown in Tables 4 (*S. aureus*) and 5 (CNS). Sensitivity rates higher than 80% were found for the majority of antimicrobials in all samples analyzed. However, two drugs stood out with regards to resistance: penicillin G and tetracycline. Resistance to penicillin was found in 93.2% of *S. aureus* and 77.8% of CNS isolates. Moreover, 16.4% of *S. aureus* and 59.7% of CNS exhibited resistance to tetracycline.

Regarding *S. aureus*, studies conducted since 2009 in herds from the south Agreste region of the state of Pernambuco, which includes the microregion of Garanhuns, have demonstrated high rates of resistance to penicillin, indicating the perpetuation of this phenotype (Krewer *et al*., 2013; Medeiros *et al*., 2009; Silva *et al*., 2012). The presence of this resistance phenotype over the decades suggests that the selective pressure exerted by the β-lactams antibiotics is a continual event

Table 3. Bacterial agents isolated from cows with clinical and subclinical mastitis in dairy herds located in microregion of Garanhuns, Pernambuco, Brazil.

Bacterial agent	Clinical mastitis		Subclinical mastitis	
	N	%	N	%
S. aureus	14	48.3	61	49.2
Coagulase-negative *Staphylococcus* (CNS)	5	17.3	33	26.6
Streptococcus uberis	2	6.9	9	7.3
Streptococcus agalactiae	2	6.9	4	3.2
Streptococcus spp.	4	13.8	5	4.0
Corynebacterium spp.	1	3.4	3	2.5
Streptococcus spp. + *S. aureus*	-	-	2	1.6
S. aureus + *Corynebacterium* spp.	-	-	2	1.6
Corynebacterium spp. + CNS	-	-	2	1.6
Streptococcus spp. + CNS	-	-	1	0.8
Streptococcus uberis + CNS	-	-	1	0.8
S. aureus + CNS	1	3.4	-	-
Staphylococcus spp.	-	-	1	0.8
TOTAL	29	100	124	100

Table 4. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis.

Antimicrobial	Profile					
	Susceptible		Intermediate		Resistant	
	N	%	N	%	N	%
Amoxicillin-clavulanic acid	72	98.6	-	-	01	1.4
Cephalothin	73	100	-	-	-	-
Cefoxitin	71	97.3	-	-	2	2.7
Enrofloxacin	67	91.7	5	6,8	1	1.5
Gentamicin	68	93.2	-	-	5	6.8
Penicillin G	5	6.8	-	-	68	93.2
Tetracycline	61	83.6	-	-	12	16.4

Table 5. Antimicrobial susceptibility of Coagulase-negative staphylococci isolated from bovine mastitis.

Antimicrobial	Profile					
	Susceptible		Intermediate		Resistant	
	N	%	N	%	N	%
Amoxicillin-clavulanic acid	25	89.3	-	-	02	10.7
Cephalothin	25	89.3	-	-	02	10.7
Cefoxitin	27	100	-	-	-	-
Enrofloxacin	26	96.3	01	3.7	-	-
Gentamicin	24	85.7	-	-	03	14.3
Penicillin G	06	22.2	-	-	21	77.8
Tetracycline	11	40.7	-	-	16	59.7

in herds in that region. In different countries, high rates of resistance to penicillin of *Staphylococcus aureus* isolated from bovine mastitis also continue to be reported (Kaczorek-Łukowska *et al.*, 2022; Neelam *et a*l., 2022; Ren *et al.*, 2020; Rychshanova *et al.*, 2022).

Considering the importance of *S. aureus* as an animal and human pathogen, the results highlighted above are worrisome due to the potential risk of the transference of resistance genes by *S. aureus* to other bacterial types and because resistance to penicillin G implies the impossibility of using some classes of β-lactams as well as non-β-lactams, hampering or even impeding the treatment of animal and human diseases (Barkema; Schukken; Zadoks, 2006).

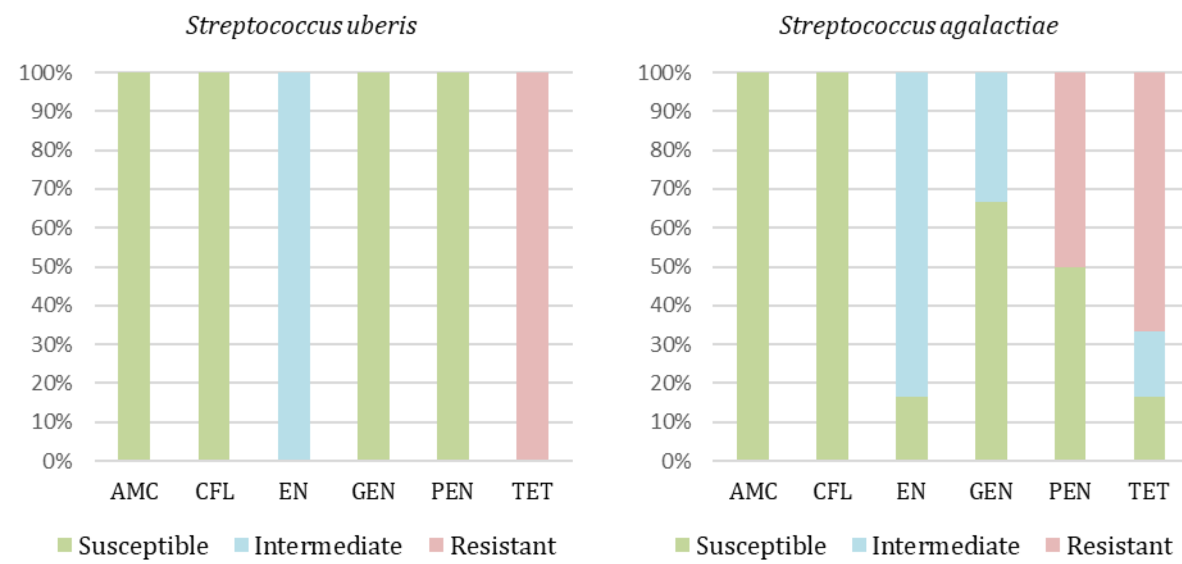
Besides the challenging scenario described above, two (2.7%) methicillin-resistant *S. aureus* (MRSA) isolates were detected in the present study using cefoxitin disk. The detection of MRSA strains causing mastitis in dairy herds is of utmost importance to animal health, as this phenotype impedes the use of all the β-lactams, leading to failures in the treatment of staphylococcal mastitis, favoring the persistence of the agent in the mammary gland and its dissemination in the herd (Lucas *et al.*, 2021b). These MRSA strains will be submitted to genotypic analysis to confirm this phenotype.

Unlike *S. aureus*, resistance to tetracycline was greater than 50% in the CNS group, suggesting that species of this group are submitted to selective pressure due to the intense use of these drugs in the farm environment for the treatment of bacterial infections whether located in the mammary gland or other organs. Indeed, the use of this antimicrobial is frequent among the producers in the region.

Figure 1 demonstrates the antimicrobial susceptibility of eleven isolates of the genus *Streptococcus*. As discussed above for the genus *Staphylococcus*, the species of *Streptococcus* isolated in the present study were likely submitted to selective pressure due to the indiscriminate use of drugs such as tetracycline and penicillin derivatives, which underscores the importance of surveillance and monitoring of the emergence and persistence of resistant phenotypes in these agents (Archer *et al.*, 2017; Mesquita *et al.*, 2019).

The ability to produce biofilm *in vitro* and the presence of the efflux pump were investigated in 66 isolates of the genus *Staphylococcus* (46 *S. aureus* and 20 CNS). Of the total, sixty-three (95.4%) were positive for biofilm production and three (4.6%) were negative. In a separate analysis, all 46 samples of *S. aureus* were positive, with 19 (47.5%) strong producers, eight (5%) moderate producers and 19 (47.5%) weak biofilm producers. Among the CNS samples, 18 (85%) were positive, with six (33%) strong producers, two (11%) moderate producers and 10 (56%) weak biofilm producers.

High rates of positivity for biofilm production in *Staphylococcus* sp. isolated from cases of bovine mastitis



AMC= Amoxicillin-clavulanic acid; CFL= Cephalothin; EN= Enrofloxacin; GEN= Gentamicin; PEN= Penicillin G; TET= Tetracycline.
Source: author's own production

Figure 1. Antimicrobial susceptibility of *Streptococcus uberis* and *Streptococcus agalactiae* isolated from bovine mastitis.

have also been reported by other researchers (Felipe *et al.*, 2017; Israel *et al.*, 2022; Ren *et al.*, 2020; Rychshanova *et al.*, 2022). Biofilm is considered an important virulence factor in species of the genus *Staphylococcus*, contributing to the colonization and persistence of these agents in infection sites (Felipe *et al.*, 2019). The importance of biofilm formation by bacteriological agents of mastitis is associated with the failure of therapeutic protocols. Besides the greater resistance to antimicrobials by physical factors that impede the distribution of the drug in the infection sites, the transference of resistance genes also occurs in biofilm, expanding resistance within the bacterial population (Felipe *et al.*, 2017; Pedersen *et al.*, 2021). In the present study, 42 samples (91.3%) of *S. aureus* and 12 (66.6%) of CNS were simultaneously biofilm producers and resistant to penicillin, which are phenotypes that favor the dissemination and persistence of resistant strains (Rychshanova *et al.*, 2022).

The efflux pump was not detected in any of the *Staphylococcus* isolates in the present study. However, other researchers have reported the presence of this resistance mechanism in *Staphylococcus* species isolated from bovine mastitis, such as Guimarães *et al.* (2012) and Krewer *et al.* (2014), who reported frequencies of 36.7% and 0.9%, respectively. The efflux pump is one of the mechanisms of resistance to tetracycline and in the present study, resistance to this drug was found in 76.1% of the *Staphylococcus* isolates. Considering the absence of the efflux pump, one may raise the hypothesis that other resistant mechanisms may be involved, being important to investigate such mechanisms.

CONCLUSION

The results of the present study indicate high rates of mastitis in the herds located in microregion of Garanhuns, Pernambuco. Moreover, the etiological agents are bacteria that have the potential for biofilm production and are resistant to the main groups of antimicrobials used for the control of this disease, making bovine mastitis a challenge for veterinarians who work in the studied region.

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