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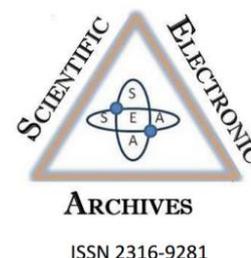
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Microbiological and molecular aspects of *Staphylococcus aureus* isolated from bovine mastitis in West-Central Brazil

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Abstract. Bovine mastitis is a major disease affecting dairy cattle, and *Staphylococcus aureus* is one of the most important agent involved in this condition due to its capacity to produce enterotoxins and develop resistance to antimicrobial agents. This study aimed to detect *S. aureus* strains in milk samples from cows with subclinical mastitis employing microbiological and molecular analysis. Eleven farms were visited and from 187 lactating cows sampled, 33 *S. aureus* strains were isolated. Only one of the 33 strains was positive for *mecA* resistance gene, 23 were positive for *sea* enterotoxin gene, and none was positive for *seb* or *sec* enterotoxin gene. *S. aureus* strains were submitted to *in vitro* antimicrobial susceptibility test and 63.6% (21/33) were susceptible to all antimicrobials tested, while 36.3% (12/33) were resistant to one or more antimicrobial agents. Identification of *mecA* and the *sea* genes highlighted the need to elaborate strategies to reduce problems related to animal. Furthermore, the identification of bovine mastitis caused by *S. aureus* is very important to manage herd and to public health, since milk contaminated by this pathogen can lead to serious health problems.

Keywords: Susceptibility, Testing, PCR, Methicillin

Introduction

Staphylococcus aureus is an important pathogen that causes a wide range of diseases in humans and animals. This specie is the most important in the development of bovine mastitis (Fontana et al., 2010; Mello et al., 2012), which is one of the main diseases affecting dairy cattle, causing economic losses and reduction in milk yield and quality (Bramley et al., 1996). Bovine mastitis is characterized by inflammation of the mammary gland, leading to changes in the glandular tissue and milk characteristics (Radostits et al., 2002). Clinical mastitis displays readily observable symptoms in the udder, including pain in the mammary gland, edema, hyperthermia, yellow-white flakes, purulent discharge and changes in milk characteristics (Fonseca & Santos, 2000). In subclinical mastitis, although macroscopic signs are not evident, there is a reduction in milk quality (Reis et al., 2003).

The occurrence of *S. aureus* as the etiological agent of mastitis is related to the ability of this microorganism to invade and adhere deeply in the mammary gland tissues (Zafalon et al., 2008). In

addition, this pathogen can cause persistent infections that are difficult to eliminate due to the presence of multiresistant strains, which may occur as a result of antimicrobial overuse and *mecA* gene expression that decreases effectiveness of drugs used to treat this condition (Marin, 2002). Moreover, *S. aureus* isolated from bovine mastitis can produce enterotoxins that aggravates the pathogenesis and chronicity of the disease (Fontana et al., 2010). These toxins have been often identified in raw milk (Dias et al., 2011; Havaei et al., 2015; Mathenge et al. 2015) and milk derivatives (Fooladi et al., 2010; Mathenge et al., 2015), thus representing a risk to public health, since consumption of contaminated food with enterotoxins can cause gastroenteritis (Garcia et al., 1980; Fonseca & Santos, 2000).

Due to the lack of information about microbiological and epidemiological characteristics of *S. aureus* in dairy herd in West-Central Brazil, investigation of these aspects is needed. The objective of this study was to evaluate the *S. aureus* occurrence in milk of cows with mastitis from dairy farms located in the city of Sinop, Mato Grosso

(Brazil) and characterize the strains through antimicrobial resistance profile, presence of *mecA* gene, and enterotoxins genes.

Methods

In 2013, eleven farms were visited in the city of Sinop- Mato Grosso (Brazil) and 187 lactating cows were sampled. The California Mastitis Test (CMT) was performed on each animal to detect subclinical mastitis. To perform the test, the teats were washed with water and the first milk squirt was discarded, then CMT was carried out according to procedures described by Fonseca and Santos (Fonseca & Santos, 2000). After CMT, the teats were disinfected with 70% alcohol, milk samples from positive quarters were collected in sterile containers, creating a sampling pool, and microbiological and molecular analysis were realized.

Bacterial isolation was performed by adapting the methodology described by Koneman et al. (2008). The milk samples from CMT-positive tests were centrifuged to be homogenized. Subsequently, 1mL of milk from each sample was inoculated into test tubes containing 5 mL of Brain Heart Infusion Broth (BHI) and incubated at 37°C for 24 hours. Then, an aliquot was seeded in Blood Agar with 5% defibrinated sheep's blood. These plates were incubated at 37 °C for 24 hours.

Identification of *S. aureus* was performed according to the specifications of Edwards and Ewing (1962), Costa & Hofer (1972) and Quinn et al. (1994). The isolated colonies were stained by Gram staining technique, then catalase and oxidase tests were carried out and strains with characteristic reactions of the genus *Staphylococcus* (catalase-positive and oxidase-negative) were submitted to tube coagulase

test. Bacteria that were coagulase-positive were transferred to Mannitol Agar, Baird-Parker Agar, and DNAase with Toluidine Blue Test Agar and incubated for 24 to 96 hours, varying according to the growth medium and following the indications of each manufacturer. Bacteria that showed biochemical characteristic of *S. aureus* were sent to molecular analysis.

The DNA from *S. aureus* strains was extracted using the commercial Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's recommendation. To identify the species through the *coa* gene and detection of the *mecA* gene, two primer pairs were used according to Kearns et al. (1999). For the detection of enterotoxin genes *sea*, *seb*, and *sec*, PCR reactions were performed with one pair of primers for each gene (Table 1) and reactions were realized under the following parameters: 5 minutes at 94 °C, 40 cycles of 30 seconds at 94 °C, 30 seconds at 57 °C, and 30 seconds at 72 °C, with a final extension of 10 minutes at 72°C. The PCR mixture used was as follows: 4µl of DNA template in 21µl of a mixture containing 10 mM Tris / HCl (pH 8.8), 4 mM MgCl₂, 200 µM of each dNTP, 200 uM each primer and 1 U of *Taq* DNA polymerase. In all reactions, a positive and a negative control were used. Positive controls for the *coa* / *mecA*, *sea*, *seb* and *sec* genes were *S. aureus* ATCC 33591, ATCC 13565, ATCC 14458 and ATCC 19095, respectively.

Table 1. Primers used for species identification, *coa*, *mecA* and enterotoxin detection

Gene	Primer Sequence (5'-3')	Amplicon size (pb)
<i>coa</i>	GTAGATTGGGCAATTACATTTTGGAGG	117
	CGCATCAGCTTTGTTATCCCATGT	
<i>mecA</i>	CGGTAACATTGATCGCAACGTTCA	214
	CTTTGGAACGATGCCTAATCTCAT	
<i>sea</i>	GGTTATCAATGTGCGGGTGG	102
	CGGCACTTTTTTCTCTTCGG	
<i>seb</i>	GTATGGTGGTGAAGTACTGAGC	164
	CCAAATAGTGACGAGTTAAGG	
<i>sec</i>	AGATGAAGTTAGTTGATGTGTATGG	451
	CACACTTTTAGAATCAACCG	

For the *in vitro* antimicrobial susceptibility test of *S. aureus* strains against antimicrobials agents, agar disk-diffusion method with the observation of inhibition zone diameter was used (CLSI, 2005). The antimicrobials used to evaluate the sensitivity and

resistance of *S. aureus* strains were: cephalothin (CFL 30); enrofloxacin (ENO 5); ciprofloxacin (CIP 5); vancomycin (VAN 30); rifampicin (RIF 30); cephalixin (CFE 30); neomycin (NEO 30); penicillin G (PEN 10); erythromycin (ERI 15); amoxicillin-clavulanic acid (AMC 30); streptomycin (EST 10);

bacitracin (BAC 10); amikacin (AMI 30); ampicillin (AMP 10); tetracycline (TET 30); chloramphenicol (CLO 30); norfloxacin (NOR 10) and gentamicin (GEN 10).

Results and discussion

No animals were diagnosed with clinical mastitis, and subclinical mastitis was identified by CMT in 46.52% (87/187) of the animals sampled, confirming the bacterial involvement in 45.5% (85/187) of these cases. These results were lower than those obtained by Bandeira et al. (2013) and Martins et al. (2010), which found that 53.6% and 65% of the dairy herd were positive for this disease, respectively. However, our results were higher than those obtained by Silva et al. (2016) and Oliveira et al. (2011) that detected, respectively, 33.8% and 15.6% of subclinical mastitis in dairy herd. The high prevalence of subclinical mastitis observed in the current research can be related to the management and care of the milking process, since the use of inadequate techniques and poor hygiene contribute to the increase of disease occurrence in the herd (Silva et al., 2016; Aqib et al., 2017). Thus, the improvement of milking procedures such as the use of pre-dipping and post-dipping, hygienic milking techniques, appropriate facilities, suitable equipment, and proper training of workers could reduce the occurrence of mastitis in the dairy farms studied (Silva et al., 2016).

Bacterial isolation was obtained in 97.70% (85/87) of milk samples positive for subclinical mastitis. Out of the 85 animals with subclinical mastitis in which one or more microorganisms were identified. From these animals, 599 species of bacteria were isolated, 300 were gram-negative and 299 gram-positive, and 46 strains were biochemically identified as *S. aureus*. *S. aureus* was identified in 24.7% (21/85) of the animals with subclinical mastitis comprising 81.8% of the farms. These results are higher than to those obtained by Bandeira et al. (2013) and Oliveira et al. (2011) that have found 17.6% (40/227) and 17.7% (11/62) cases of subclinical mastitis with *S. aureus* involvement, respectively. The high occurrence of subclinical mastitis due to the presence of *S. aureus* in this study may be related to workers lack the knowledge on how to prevent mastitis (Bandeira et al., 2013), or improper hygiene and erroneous management practices (Hamid et al., 2017), that contributes to the dissemination of the pathogen in the herd since the main route of this microorganism transmission occurs during milking (Bandeira et al., 2013). Furthermore, the prevalence of *Staphylococcus sp.* in cases of subclinical mastitis has been related to the ability of these bacteria to form biofilms (Chagas et al., 2012), which reduces antimicrobial susceptibility of these microorganisms.

Out of 46 strains identified as *S. aureus* using biochemistry tests, 33 (71.7%) were confirmed as *S. aureus* by PCR, based on the identification of the *coa* gene (Kearns et al. 1999, Bes et al. 2018), and only

one strain (3%) was positive for the *mecA* gene. Twenty-three strains (69.69%) were positive for the *sea* gene, and none were positive for the *seb* and *sec* genes.

Another problem related with bovine mastitis caused by *S. aureus* is the methicillin resistance of some strains, which may complicate treatment and increase transmission risk of resistant strains to human populations. The first report of bovine mastitis caused by a strain of Methicillin-Resistant *Staphylococcus aureus* (MRSA) occurred in Belgium in the early 1970s. Later, several researchers isolated this microorganism in cattle (Havaei et al., 2015; Conceição et al., 2017), suggesting that both animals and products destined for consumption can serve as a source of pathogenic strains to humans. The *mecA* gene, a molecular marker of methicillin resistance, encodes a membrane protein called PBP2a that differs from the expressed protein PBP2. The PBP2 protein has a high affinity for beta-lactam antibiotics; nevertheless, the PBP2a protein has low affinity leading to resistance to these drug (Marin, 2002). Marin (2002) further points out that in addition to the resistance against beta-lactams, the *mecA* gene may also confer multiple resistance to quinolones and aminoglycosides.

Guimarães et al. (2017), investigating an outbreak of mastitis in a farm in the state of São Paulo, Brazil, identified that 52.17% (60/115) of the milk samples were contaminated with *S. aureus*, 48.3% of these strains had the *mecA* gene, in which 23.3% displayed phenotypic resistance. Hamid et al. (2017) when investigating the presence of MRSA in 160 samples of milk in Jammu province, India, obtained 36 isolates of *S. aureus*, in which 6 (16.6%) of the isolates were identified as MRSA. Havaei et al. (2015) showed similar results in Isfahan, Iran, when analyzed 450 milk samples from cows with mastitis, in which 54 had *S. aureus*, and 10 out of 54 (18.51%) were resistant and positive for the *mecA* gene. Aqib et al. (2017) found a higher rate of 34% (306/900) of MRSA prevalence in dairy cattle in Faisalabad district of Pakistan.

Compared with other studies, the incidence observed in this study is low. Nevertheless, the presence of *mecA* gene represents a transmission risk of multiresistant bacteria to others cows and human populations. In Brazil, Rizek et al. (2011) already identified the *mecA* gene present in food ready to consumption (cheese), showing that the transmission was no longer restrict to farms. A major concern when dealing with this specific resistance mechanism is that *mecA* gene is part of a mobile chromosome cassette (SCCmec), which facilitates transmission between strains of *S. aureus* (Okuma et al., 2002; RIZEK et al., 2011). The presence of pathogens with antimicrobial resistance is a growing concern in veterinary medicine since it leads to ineffectiveness of antimicrobial agents (Guimarães et al., 2017; Hamid et al., 2017). In addition, the presence of MRSA has a zoonotic implication, and it is considered an occupational risk for veterinarians

and other professionals working with animal production (Köck et al., 2014). Thus, direct contact with animals and products of animal origin MRSA positive, such as raw milk, could serve as a source of antimicrobial resistant strains, which is of great importance to public health.

All 33 strains of *S. aureus* were submitted to *in vitro* susceptibility test against antimicrobial agents. Of these strains, 63.63% (21/33) were susceptible to all antimicrobial agents tested and 36.36% (12/33) were resistant to one or more antimicrobial agents. The 21 strains susceptible to antimicrobial agents

were isolated from 12 cows with mastitis obtained from 6 dairy farms.

The 12 strains resistant to antimicrobial agent were isolated from nine cows raised in five farms and the resistance profile found was: 6% (2/33) of isolates strains resistant to erythromycin; 3% (1/33) to penicillin G; 18.18% (6/33) erythromycin and tetracycline concomitantly; 3% (1/33) to tetracycline and chloramphenicol; 3% (1/33) to erythromycin, ampicillin and tetracycline, and 3% (1/33) to enrofloxacin and penicillin G (Table 2).

Table 2: Relationship of *Staphylococcus aureus* involved in the pathogenesis of subclinical mastitis and number of dairy cows in which this microorganism was resistant to one or more antimicrobial agents tested.

Number of farms	Animals with bacterial mastitis	Animals with mastitis involving <i>S. aureus</i> positive for <i>coa</i> gene	Number of animals with resistant strains of <i>S. aureus</i> *1	(a/ b)*2: antimicrobial agents for which the strains were resistant	Number of animals with susceptible <i>S. aureus</i> strains *1	Number of positive strains for <i>sea</i> gene
1	8	0	-	-	-	-
2	18	0	-	-	-	-
3	3	1	1	(2/1): Erythromycin	0	2 (2/2)
4	1	1	0	-	1	1 (1/2)
5	8	3	0	-	3	4 (4/5)
6	7	1	1	(1/1): Erythromycin and tetracycline	0	1(1/1)
7	8	3	3	(3/2): Erythromycin and tetracycline (1/1): Tetracycline and Chloramphenicol	0	2 (2/3)
8	6	1	0	-	1	1 (1/3)
9	8	4	1	(1/1): Penicillin G	3	6*5 (6/6)
10	9	5	3	(2/2*3): Erythromycin and tetracycline (1/1*3): Erythromycin, ampicillin and tetracycline (1/1): Enrofloxacin and penicillin G	2	3 (3/8)
11	9	2	0	-	2	3 (3/3)
Total	85	21	9	-	12	23 (23/33)*4

*1 Number of cows identified with mastitis involving *S. aureus* resistant and susceptible to antimicrobial agents.

*2 a: Number of antimicrobial resistant strains; b: Number of cows carrying antimicrobial resistant strains.

*3 Two strains of *S. aureus* with different pattern of susceptibility isolated from the same cow.

*4 23 strains of *S. aureus* positive for *sea* gene, out of a total of 33 strains positive for *coa* gene.

*5 One strain positive for *mecA* gene.

Profiles of multiresistant *S. aureus* have been traced in Brazil and other countries. Hamid et al. (2017) found that out of 36 *S. aureus* isolates from cows, 34 (94.4%) were resistant to penicillin, 30 (83.3%) to ampicillin, 28 (77.7%) to amoxicillin-sulbactam, 24 (66.6%) to enrofloxacin, 18 (50%) to ceftriaxone, 10 (27.7%) to methicillin and only two were resistant to all the antimicrobial agent tested. Costa et al. (2013) identified 4.56% (16/352) isolates of bovine mastitis susceptible to all tested antimicrobials. They also found low resistance to gentamicin (1.69%) and high resistance to ampicillin

(80.92%) and penicillin (80.45%). In Iran, Jamali et al. (2014) identified 20.1% (43/207) of *S. aureus* strains in milk of cows affected by clinical mastitis. These strains showed resistance values of 86% to penicillin, 76.7% to tetracycline, 39.5% to erythromycin, 11.6% to chloramphenicol, 7% to gentamicin, and 2.3% to streptomycin. Meanwhile in Chile, San Martín et al. (2003) analyzed 2914 milk samples from cows with clinical mastitis, and isolated 635 *S. aureus* strains which showed resistance to amoxicillin (38.1%), penicillin (28.8%), ampicillin (26%), enrofloxacin (2.5%), and gentamicin (3.3%).

All of the studies mentioned above, exhibited a higher antimicrobial resistance profile when compared with the present one. According to Li et al. (2009), the emergence of resistant strains may be related to the indiscriminate antimicrobial use in cattle treatment. It seems that, in the farms we analyzed, the use of antimicrobials agent still did not showed interferences on the strains susceptibility profile, since the strains analyzed in this study showed little or no resistance to the tested antimicrobials. However, some *S. aureus* strains showed resistance, even in a low proportion. For this reason and to prevent the rise of others resistance mechanisms, it is needed to do *in vitro* susceptibility test before treatment of bovine mastitis, and according to the result choose the appropriate antimicrobial agent to treat each particular case.

When it comes to enterotoxins production, cows with mastitis caused by *S. aureus* are probably the main source of raw milk contamination. Milk is a good substrate for growth of these bacteria that can produce thermostable enterotoxins leading to staphylococcal food poisoning (Havaei et al., 2015). In the present study, the *sea* gene was the only one identified for enterotoxin production, obtained in 69.69% (23/33) of *S. aureus* strains isolated. In a similar study in Iran, Havaei et al. (2015) identified the *sea* gene and *seb* gene in 19% (19/54) and 2% (2/54) of *S. aureus* strains, respectively. In Brazil, Dias et al. (2011) performing a study in the region of Sete Lagos –MG identified *sea* gene in 60% (87/145) of the cases, *seb* gene in 37.93% (55/145) and *sec* gene in 6.89% (10/145). Masud et al. (1993) examined 85 *S. aureus* strains from dairy products, and identified 45 enterotoxigenic strains: 37.7% type A, 17.7% type B and 10.6% type C. All studies mentioned and in our own, the *sea* gene was the most prevalent which represents a serious problem for public health since staphylococcal enterotoxin A has the potential to cause severe food poisoning (Evenson et al., 1998), being the main cause of this disease in the world (Havaei et al., 2015).

Conclusion

S. aureus was found in 24.70% (21/85) of the animals with subclinical mastitis and was widespread among dairy farms in the study. The *sea* gene responsible for the production of the enterotoxin A was the most prevalent among strains (23/33). However, no strain carrying the *seb* or *sec* gene were identified, and *mecA* gene was identified in only one strain. Regarding the susceptibility profile, 63.63% (21/33) of the strains analyzed were susceptible to all drugs tested, and 36.36% (12/33) were resistant to one or more antimicrobial agents. The identification of the *mecA* gene highlights the need to develop strategies to reduce the problems related to public and animal health. In addition, the identification of bovine mastitis caused by *S. aureus* is of great value both for the prevention of infections in the dairy herd and for public health, since the milk contaminated with this pathogen can serve as a source of infection, leading to serious health problems.

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