



***mecA* positive *Staphylococcus* spp. in bovine mastitis, milkers, milking environment, and the circulation of different MRSA clones at dairy cows farms in the Northeast region of Brazil**

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ABSTRACT: *This study detected the presence and distribution of mecA in Staphylococcus spp. in the dairy production environment at farm level in Brazil. We analyzed 335 samples of mastitis cow milk, 15 samples of nostrils and hand swabs from milkers, 14 teat cup swabs, and 9 milking buckets swabs. Initially, the samples were subjected to microbiological analysis to detect Staphylococcus spp. and then S. aureus and mecA positive isolates were identified by PCR. All S. aureus isolates carrying the mecA genes were subjected to DNA macro-restriction analysis by Pulsed-Field Gel Electrophoresis (PFGE). The mecA gene was detected in 6/335 (1.78%) of mastitis cow milk, 5/15 (33.3%), and 5/15 (33.3%) of nostrils and hand swab, and 4/14 (28.5%) of the teat cup isolates. MRSA genotyping was performed by PFGE, a total of seven pulsotypes were grouped in two clusters. This study identified the occurrence and spread of MRSA at dairy environment of farms, and also the existence of distinct genetic profiles between isolates.*

Key words: *S. aureus, mecA gene, multidrug resistance, animal health, dairy production.*

***Staphylococcus* spp. *mecA* positivo em mastite bovina, ordenhadores, ambiente de ordenha e circulação de diferentes clones de MRSA em fazendas de vacas leiteiras na região Nordeste do Brasil**

RESUMO: *Este estudo teve como objetivo detectar a presença e distribuição de mecA em Staphylococcus spp. no ambiente de produção leiteira em fazendas no Brasil. Foram analisadas 335 amostras de leite de vaca com mastite, 15 amostras de swabs de narinas e mãos de ordenhadores, 14 swabs de teteiras e nove swabs de baldes de ordenha. Inicialmente, as amostras foram submetidas a análises microbiológicas para detecção de Staphylococcus spp. e os isolados positivos foram identificados por PCR para S. aureus e mecA. Todos os isolados de S. aureus portadores do gene mecA foram submetidos à análise de macrorrestricção do DNA por Pulsed-Field Gel Electrophoresis (PFGE). O gene mecA foi detectado em 6/335 (1,78%) de leite de vaca com mastite, 5/15 (33,3%) e 5/15 (33,3%) de swab de narinas e de mãos, e 4/14 (28,5%) de teteiras. A genotipagem de MRSA realizada por PFGE identificou um total de sete pulsotipos, que foram agrupados em dois clusters. Este estudo identificou a ocorrência e disseminação de MRSA no ambiente das fazendas leiteiras, e também a existência de perfis genéticos distintos entre os isolados.*

Palavras-chave: *S. aureus, gene mecA, multirresistência a medicamentos, saúde animal, produção de leite.*

INTRODUCTION

Mastitis is a plurietiological and multifactorial disease that is often caused by bacteria of the genus *Staphylococcus*. These bacteria is opportunistic pathogen associated with a wide variety of infections in humans and animals (WILLE et al., 2014; WONG, 2002).

Another concern related to mastitis caused by *Staphylococcus* spp. is the emergence of

antimicrobial resistant strains, such as Methicillin-Resistant *Staphylococcus* (MRS) (LIM et al., 2013). Emergence of MRS strains results from modifications in the sites of antimicrobial action in the *Staphylococcus* Chromosome Cassettes (SCC*mec*), the acquisition of SCC*mec* occurred in the early 1960s, being considered a determining event in the evolution of this bacterial genus, which result in multiresistant isolates, especially to beta-lactams (CRISOSTOMO et al., 2001).

Twelve types of SCCmec that harbor *mecA* and/or *mecC* are now known; both these genes encode an altered penicillin binding proteins (PBP) called PBP2a/PBP2' and a peptidoglycan transpeptidase that result in a decreased affinity for this class of antimicrobials (KIM et al., 2012; BAIG et al., 2018; LEE et al., 2018). This encoding allows *Staphylococcus* spp. to maintain its biosynthesis even at concentrations considered to be inhibitory to these antimicrobials (LIVERMORE, 2000; PATERSON et al., 2014).

MRS strains have been identified in cases of bovine mastitis in various parts of the world such as Great Britain, Korea, and Germany (FEßLER et al., 2010; LIM et al., 2013; PATERSON et al., 2014). Contact between humans and animals positive for MRS and vice versa, may also favor pathogen transmission between species, and the environment may be a source of infection for both (JUHÁSZ-KASZANYITZKY et al., 2007). In Brazil, few studies have identified cases of mastitis caused by MRS (SOARES et al., 2012; MATOS, 2014; SANTOS et al., 2016; MELO et al., 2018). However, no study conducted in the country has evaluated and detected the presence of MRS isolates in cases of mastitis, workers, and the milking environment, and a possible genetic correlation between MRS isolates from various sources in the agricultural environment. This demonstrated the need to conduct research that contributes to the epidemiology of infection with this pathogen in Brazil.

Considering the aspects mentioned above, this study detected the presence and distribution of *mecA* in *Staphylococcus* spp. in the dairy production environment at farm level in Brazil and determined the genetic relationship between *mecA* positive *S. aureus*.

MATERIALS AND METHODS

Sample collection

Five dairy farms (A, B, C, D and E) located in different regions of Pernambuco State, Northeastern Brazil, were included in the present study. These five family farms had a total of 169 animals in lactation, being the milk from all those sampled. Milk samples collected from 676 udder quarters were subjected to the California Mastitis Tests (CMT) and those that were equal to or greater than one cross (+) (319 samples) or reported positive in the routine strip cup test (16 samples) were selected for microbiological testing. A total of 335 individual quarters were collected after antisepsis with alcohol at 70° GL. A veterinarian using sterile gloves

performed the collection. Then they were stored in sterile microtubes.

Also, samples from dairy environment (mechanical milking equipment and milking buckets) and milkers (hands and nasal cavities) were obtained by using swabs and subjected to microbiological analysis. Of the five farms studied, two used mechanical milking; in these farms, swabs were collected from the teat sets (forming pools of each equipment). In the three farms that performed manual milking, swabs were collected from the buckets used for milking.

A total of 15 samples of nostrils and hand swabs from milkers, 14 samples from teat taps and nine samples of milking buckets were also obtained. The distribution of collected samples by farm is described in table 1. All samples were transported under refrigeration to the laboratory, where microbiological and molecular analyses were performed.

Isolation and preliminary identification of *Staphylococcus* spp.

Isolation of *Staphylococcus* spp. of the milk samples was performed by direct plating of all samples on manitol salt agar (Difco Laboratories Inc., Detroit, USA), followed by incubation at 37 °C for 24-48 h. All swabs (samples of nostrils and hand swabs from milkers, samples from teat taps and samples of milking buckets) were inoculated into 9 mL Mueller Hinton broth (Difco Laboratories Inc., Detroit, USA) containing 6.5% NaCl, incubated overnight at 37 °C and plated on manitol salt agar (Difco Laboratories Inc., Detroit, USA). Then, after incubation at 37 °C for 24-48 h isolated colonies which showed a distinct morphology (size and color) were subjected to Gram staining, catalase and coagulase tests, as well as mannitol fermentation (CARTER, 1998).

Subsequently, the isolates were subjected to molecular techniques for proper identification and characterization and determination of oxacillin minimum inhibitory concentration.

DNA extraction and PCR reactions

The genomic DNA of all isolates identified in preliminary tests as *Staphylococcus* spp. was extracted as previously described by FAN et al. (1995). The DNA obtained was quantified using a spectrophotometer at 260 nm absorbance readings. The identification of *S. aureus* was performed by PCR targeting *nuc*, whereas detection of MRS was performed by targeting *mecA* gene and *mecC* gene (Table 2). Reactions for both genes contained a final volume of 12.5 µL, consisting of 100 ng of isolated

Table 1 - Distribution of collected samples in the five dairy farms (A-E) included in the present study.

Dairy farms	Lactating cows	-----Milk-----		-----Milkers-----		-----Environment-----	
		CMT ¹	SCT ²	Hands	Nasal	Teat cup	Milking buckets
A	32	55	6	3	3	-	2
B	15	15	0	2	2	-	2
C	43	96	1	3	3	4	-
D	61	100	8	4	4	-	5
E	18	53	1	3	3	10	-
Total	169	319	15	15	15	14	9

¹Reagent teat in the California Mastitis Test; ² Reagent teat in the Strip Cup Test.

DNA, 0.5 µL of each primer (10 pmol), 6.25 µL of Go Taq Green Master Mix (Promega Corporation, Madison, USA), and 2.5 µL of ultrapure Milli-q water. The PCR products were subjected to 2% agarose gel electrophoresis for 60 minutes at 100 V. At the end of the run, BlueGreen-stained gels (LGC biotechnology) were visualized under ultraviolet light and imaged. For *mecA* and *nuc* genes detection, the *S. aureus* N315 strain was used as a positive control and for *mecC* detection, *S. aureus* LGA251 was used as positive control in all PCR reactions.

Determination of minimum inhibitory concentration (MIC)

All isolates harboring the MRS genes were subjected to a broth microdilution assay to assess their resistance to methicillin, according the protocol described by the Clinical and Laboratory Standards Institute (CLSI, 2018). Oxacillin was used as a reference antimicrobial at concentrations of 16, 8, 4, 2, 1, 0.5 and 0,25 µg/mL.

For inoculum preparation, isolates were plated in non-selective solid medium (Mueller Hinton Agar, SIGMA-ALDRICH) and incubated at 37 °C

for 24h. Isolated colonies were suspended in 0.9% saline until turbidity equivalent to 0.5 McFarland standard; this suspension contains approximately 1 x 10⁸ CFU/mL. Subsequently, the bacterial suspension was inoculated into Mueller Hinton broth (SIGMA-ALDRICH). Samples were processed in triplicate and a final volume of 100µL was distributed to each well of the 96-well micloplate. Plate microdilutions were incubated at 35 °C for 24h and OD readings at 620 nm were taken on the Elisa microplate reader (Multiskan Go Thermo Scientific).

MRSA PFGE genotyping

All *S. aureus* isolates carrying the MRS genes were subjected to DNA macro-restriction analysis according to the methodology described by ANDRÉ et al. (2008). Digestion with restriction enzymes was performed after 1/5 of the plugs were cut and transferred to tubes containing 150 µL of 1x TE buffer, where 30 U of *SmaI* (Promega Corporation, Madison, WI, USA) restriction enzyme was added. The reaction was performed overnight at 25 °C and plugs were transferred to 1% agarose gel wells. Electrophoresis performed with CHEF-

Table 2 - Target genes and oligonucleotide sequences used to identify *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus*.

Gene	Primer	Sequence	Fragment size (pb)	Reference
<i>nuc</i>	1	GCGATTGATGGTGATACGGT	270	BRAKSTA et al. (1992)
	2	AGCCAAGCCTTGACGAACATAAAGC		
<i>mecA</i>	2W	TGGTATGTGGAAGTTAGATTGGGAT	155	NAKAGAWA et al. (2005)
	2X	CTAATCTCATATGTGTTCCCTGATTGGC		
<i>mecC</i>	F	CATTAATAATCAGAGCGAGGC	188	PATERSON et al. (2012)
	R	TGGCTGAACCCATTTTTGAT		

DR III System (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed following the following parameters: 5-40 s for 21 h, at an angle of 120°, 6V/cm, in 0.5x TBE buffer maintained at 14 °C. The *Salmonella enterica* serotype Braenderup H9812 (CDC) digested with 20 IU of *XbaI* (Promega Corporation®, Madison, WI, USA) was used as a marker (RIBOT et al., 2006). The obtained gels were developed in an immersion bath with intercalating UniSafe Dye (Uniscience, Brazil) and were visualized with a transilluminator under ultraviolet light and imaged for further analysis. Images were analyzed using BioNumerics v.6.6.4 software (Applied Maths, Kortrijk, Belgium). A dendrogram was obtained by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) grouping, using the Dice similarity coefficient and the 2% tolerance and optimization degree (TENOVER et al., 1995).

RESULTS

The results of isolation, identification of *Staphylococcus* spp. and the *mecA* carriage of the samples of mastitis cow milk, milkers, and milking utensils are shown in table 3. All isolates were negative for *mecC* gene. The frequency of MRS mastitis was 1.78% (6/335), and of this total, most cases were caused by MRSA (83%; 5/6). At MIC all MRS isolates were sensitive for oxacillin as determined by CLSI (2018).

The genetic profiles of 10 MRSA isolates identified in the present study were revealed by PFGE after macrorestriction performed with the enzyme *SmaI* (Figure 1). The isolates were grouped into seven pulsotypes (I-VII) within two clusters (I and II). Cluster I grouped four MRSA isolates (12 MNC,

6 MH, 1 TC and 4 MNC) which shared at least 73.1% similarity. Interesting, only MRSA isolates recovered from human and teat cup (from dairy farms B, C, and D) were grouped in this cluster.

Cluster II grouped five MRSA isolates (163 RAT, 164 RRT, 30 RRT, 24 LAT and 32 RAT) recovered from samples of mastitis cow milk at farms A and E. It was possible to detect a greater genetic similarity between them (at least 94.2%). Isolates 163 RAT, 164 RRT and 30 RRT shared the same genetic profile (pulsotype V); although, they originated from farms far away (over 200 km), indicating that genetically similar MRSA strains are widespread in different regions of Pernambuco state, Brazil. The 3MN isolate was not digested by the enzyme *SmaI*; and therefore, it was not possible to determine its genetic correlation with other MRSA isolates.

DISCUSSION

Staphylococcus spp. is one of the main causative agents of mastitis in dairy herds (KREWER et al., 2015; PRIBUL et al., 2011). Our data are in accordance with the literature, that reports infection rates of MRS from 0.05% to 47.6% in bovine mastitis (PU et al., 2014b; SAINI et al., 2012). It is important to highlight that only samples with positive results in CMT or in routine strip cup test were tested for MRS in the present study.

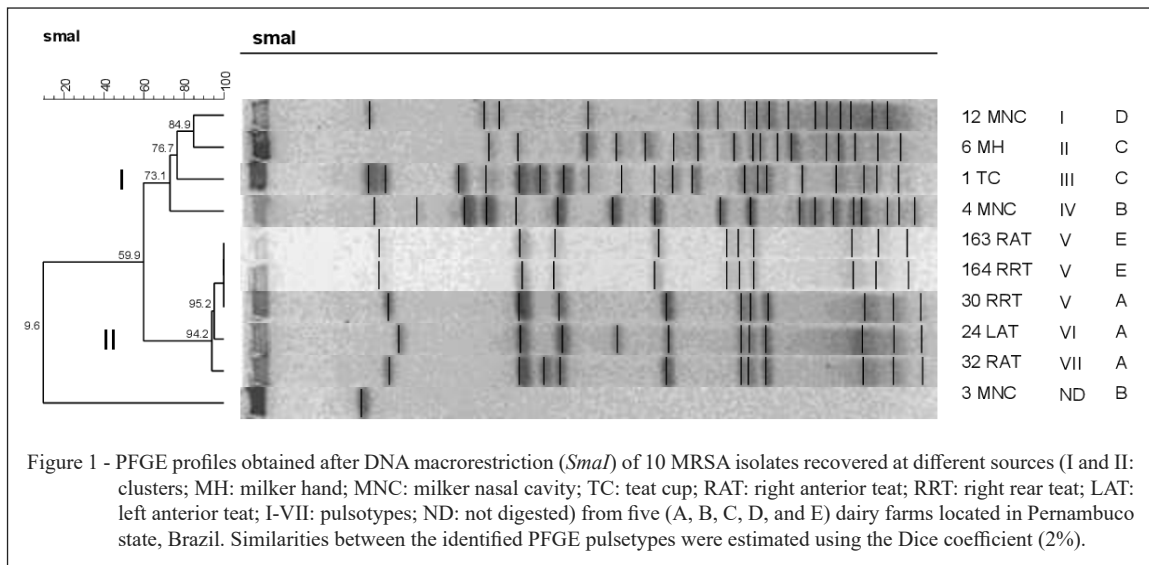
Nowadays, antimicrobial resistant bacteria are considered one of the major public health concern worldwide. Mastitis is the most common cause of antimicrobial use in dairy herds, so bovine milk is considered as potential source of multidrug-resistant bacteria in the agricultural environment (CHANDRASEKARAN et al.,

Table 3 - Isolation, molecular identification of *Staphylococcus* spp. and *mecA* positive samples from mastitis cow milk, milkers, and milking utensils.

Sample	n ¹	Isolation of <i>Staphylococcus</i> spp. ²	Molecular identification of <i>Staphylococcus</i> spp. ³		Molecular identification of methicillin resistance ⁵	
			<i>S. aureus</i>	<i>Sna4</i>	MRSA6	MRSNA7
Mastitis cow milk	335	114/335 (34.3%)	42/114 (36.8%)	72/114 (63.1%)	5/335 (1.49%)	1/335 (0.29%)
Milkers hand swab	15	11/15 (80%)	7/11 (63.6%)	4/11 (36.3%)	3/15 (20%)	2/15 (13.3%)
Milkers nostrils swab	15	13/15 (86.6%)	2/13 (15.3%)	11/13 (84.7%)	1/15 (6.6%)	4/15 (26.6%)
Teat cup swab	14	14/14 (100%)	4/14 (28.5%)	10/14 (71.5%)	1/14 (7.1%)	3/14 (21.4%)
Milking bucket swab	9	7/9 (88.8%)	6/7 (85.7%)	1/7 (14.3%)	0/9 (0%)	0/9 (0%)

¹Samples collected. ²Based on phenotypical characteristics. ³Based on PCR for *mecA*. ⁴*Staphylococcus non-aureus*. ⁵Based on PCR for *mecA*.

⁶MRSA - Methicillin Resistant *Staphylococcus aureus* (MRSA); ⁷MRSNA - Methicillin Resistant *Staphylococcus non-aureus* (MRSNA).



2014a). This minimizes the effectiveness of current treatments and the ability to control infectious diseases in animals and humans, facilitating the spread of bacteria resistant to antimicrobials (LEE, 2006; JAMROZY et al., 2012; LIM et al., 2013; CHANDRASEKARAN et al., 2014b; SILVA et al., 2020), mainly due to the close relationship established in dairy farms between different animal species and humans (KAMAL et al., 2013; SILVA et al., 2014a; RAYMUNDO et al., 2018).

Methicillin is not an antimicrobial used in the treatment of mastitis in cows. However, MRS is a generic term that corresponds to a virtual resistance against practically all β -lactams, except the last generation cephalosporins; when MRS identification occurs, the isolate is considered multiresistant (PEACOCK & PATERSON, 2015).

The results presented in this study are in addition to others performed in the country that have identified cases of bovine mastitis due to MRSA and MRS. It should serve as a warning to animal health authorities regarding the improvement of programs for the control and rational use of antimicrobials in veterinary medicine (SOARES et al., 2012; MATOS, 2014; SILVA et al., 2014b; SANTOS et al., 2016; MELO et al., 2018). High use of antimicrobials or their indiscriminate use contributes to the selection and persistence of MRS strains; and consequently, their spread throughout the agricultural environment. One of the strategies to verify MRS spread in this environment is to collect samples from animals and other sources

such as workers and the environment (JAMROZY et al., 2012; LIM et al., 2013).

In this study, MRS was identified in the nostrils and hands of milkers and in environmental samples (teat cups). With respect to samples from milkers, a high percentage of MRSA was detected on the hands, whereas MRSA was the most identified in the nostrils. Identification of MRS in humans has been reported frequently, especially in cases of nosocomial infection, which has generated considerable concern among public health professionals (RABELO et al., 2014). As in this study, there are also reports of microorganism identification in dairy farm workers in other countries. VAN LOO et al. (2007) they investigated the origin of a new type of MRSA in the Netherlands and concluded that the new strain is of animal origin (pigs and probably cows). The authors suggested the occurrence of MRSA transmission between animals and humans. LIM et al. (2013) determined the presence and persistence of MRSA in milk, farm environment and farmers on 22 dairy farms in Korea, the microorganism was detected in all types of samples. The two studies suggested that people who work or live in close contact with cows are at increased risk of being colonized and infected with MRSA. However, humans can also serve as a source of infection for the environment and animals if they are already infected (JUHÁSZ-KASZANYITZKY et al., 2007). Thus there is a need to monitor microorganisms at the interface of dairy cows and humans (SCHMIDT et al., 2017).

As indicated in the literature, the detection of MRS in teat cups indicate a possible circulation between cows isolates and the environment or humans and the environment, as these utensils are handled by the milkers (FEBLER et al., 2012; LIM et al., 2013). Sample collection from teat cups was performed immediately after milking according to the method proposed by PLETINCKX et al. (2013). Detection of MRS in these utensils soon after milking indicates that these may be a source of transmission and a good indicator of the presence of MRS in dairy farms.

The results at MIC (values of 0.5 µg / mL - 2.0 µg / mL) may indicate the existence of susceptible oxacillin *mecA* Positive (OS-MRS) isolates. Divergence between genotypic and phenotypic resistance has been reported in literature (KAMAL et al., 2013; PU et al., 2014). Phenotypic tests may have limitations due to the occurrence of hetero resistance, where only certain subsets of bacterial cells express the resistant phenotype, and generate false negatives (PENN et al., 2013). Further, regulatory systems such as *mecI-mecR1* and *bla*, present in some strains carrying the *mecA* gene, may control the oxacillin resistance phenotype (MCKINNEY et al., 2001). According to LIU et al. (2016), higher the expression of *bla* gene regulators, the lower is the expression of *mecA* gene, and consequently, the isolates have phenotypic sensitivity to oxacillin. OLIVEIRA & DE LENCASTRE (2011) suggested that other unidentified determinants are involved in the transcriptional control of *mecA* and that elucidating the nature of these determinants is relevant for a complete understanding of the molecular mechanisms controlling the phenotypic expression of resistance, and that this may contribute to the development of new therapeutic strategies.

Our results suggested the existence of different sources of contamination in the studied farms, since different MRSA subtypes were identified by PFGE in animals, environment and humans. However, the identification of isolates with similar genetic profiles in samples of mastitis cow milk from different dairy farms indicates that some strains are disseminated in this region, and may be endemic MRSA clonal profiles (SANTOS et al., 2016). According to our results, genetic related strains adapted to colonize the mammary glands of animals may be disseminated in the state of Pernambuco, Brazil. In addition, at least one MRSA isolate was identified in each on farm included in the study.

CONCLUSION

The present study described the existence of MRS in samples of mastitis cow milk, milkers, and milking utensils and the presence of distinct clonal complex MRSA in dairy cow farms in the Northeast region of Brazil. It also serves as a reference for conducting other studies related to the epidemiology of multiresistant bacteria in the country and implementing control programs and taking measures to improve biosecurity in the dairy farms to avoid the spread of MRS strains.

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The entire experimental procedure is in accordance with the ethical principles adopted by the Ethics Committee for the Use of Animals from Universidade Federal Rural de Pernambuco (UFRPE), Recife, Brazil (license number 106/2017) and the Research Ethics Committee of the Universidade de Pernambuco (UPE), Recife, Brazil (CAAE number: 28833619.7.0000.5207). All persons gave their informed consent prior to their inclusion in the study.

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