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RESEARCH ARTICLE

ANTIMICROBIAL RESISTANCE PHENOTYPE AND ANALYSES OF *MEC*A GENE IN *STAPHYLOCOCCUS AUREUS* FROM CHEESE IN BRAZIL

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ABSTRACT

The widespread use of methicillin against bacterial infections, mainly in hospitals, led to the emergence of a resistance mechanism related to the presence and expression of the *mec*A gene, which encodes the protein PBP2a, resistant to penicillin/methicillin. Forty-two *Staphylococcus aureus*, previously isolated from Ricotta cheese sold in Brazil, were tested to antibiotic susceptibility and analyzed for the presence of *mec*A gene. Four isolates were resistant to oxacillin/methicillin, and to other antibiotics, including penicillin. Molecular characterization of the isolates revealed seven isolates carrying *mec*A gene. These results suggest that the resistance mechanism in these isolates is probably given by the expression of this gene, this may negatively regulated in isolates showed no resistance phenotype, since analysis showed that these genes do not have mutations that can explain the sensitive phenotype. Therefore, the food, particularly Ricotta cheese, are a potential way to spread strains of *S. aureus* carrying genes for resistance to antibiotics. Our results show that the presence of resistant strains in non-hospital environments merit special attention because the indiscriminate use of antibiotics by the population can lead to a positive selection of these strains, causing serious public health problems.

INTRODUCTION

Methicillin is a β-lactam semisynthetic antibiotic used to treat bacterial infections, including those caused by *Staphylococcus aureus*, besides its use in phenotypic characterization of bacterial strains. Methicillin is very similar to penicillin (benzylpenicillin or penicillin G), but differing because the presence of methoxy groups in penicillin benzene ring (Stapleton and Taylor, 2002). The antibiotic methicillin, synthesized from 1961, temporarily solved the problem of penicillin-resistance found in some isolates of *S. aureus*, because the enzyme involved in the penicillin-resistance possesses lower affinity to methicillin. However, the widespread use of methicillin in chemotherapy, especially in nosocomial environments, led to the emergence of methicillin-resistant *S. aureus* (MRSA), which turned to be a public health concern worldwide (Lowy, 2003). At least two mechanisms are involved in the establishment of the resistance of *S. aureus* to β-lactam antibiotics: i) the synthesis of β-lactamase enzyme (encoded by the chromosomal gene *bla*Z), which hydrolyze the β-lactam molecule; and ii) the presence and expression of the *mec*A gene (located in the staphylococcal cassette chromossome *mec* – SCC*mec*), which encode the protein PBP2a resistant to penicillin/methicillin. The structure of SCC*mec* and the role of genes contained in it give an ecological advantage to *S. aureus* carrying this genetic element in different media, including the presence of antibiotics. (Ito *et al.*, 2001)

SCC*mec* is similar to other genetic elements, such as transposon and bacteriophage genes, but differs from the first because the absence of *tra* gene complex; and from the second because the absence of head and tail protein-coding genes (Katayama *et al.*, 2000). Detailed study of SCC*mec* revealed the presence of two principal regions: the *mec* gene complex, containing the *mec*A gene and other genes controlling its expression (activators and inhibitors); and the *ccr* gene complex containing genes encoding recombinases enzymes related to the excision and insertion of these elements in chromosomal regions. The combination of these two complexes allow the classification of SCC*mec* into eight subtypes named I – VIII, and subtypes I – III, VI and VIII have been related to resistance to many antibiotics (Malachowa and DeLeo, 2010).

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The sensible PBP2 protein is normally present in bacteria, participating in the synthesis of cell wall peptidoglycan. In the presence of penicillin, PBP2 protein is inhibited by the binding of the antibiotic to its transpeptidase domain, leading the cell to enter in the lytic cycle (Giesbrecht *et al.*, 1998). The PBP2a, on the other hand, has a low affinity for penicillin, and remains active in the synthesis of cell wall. It is known, however, that the presence of sensitive protein is required for the functioning of PBP2a, because despite the inhibition of its transpeptidase site, its transglycosilation domain remains functional and is required for activation of that protein (Pinho *et al.*, 2001a; Pinho *et al.*, 2001b; Scheffers and Pinho, 2005). Currently, although the majority of MRSA infections still occurs in nosocomial environments by the so-called health care associated - methicillin resistant *S. aureus* (HCA-MRSA), involving people considered belonging to the risk group (with frequent contact, direct or indirect, with health service), in recent years MRSA emerged in populations without the predisposing risk factors. This infection is caused by community associated – methicillin-resistant *S. aureus* (CA-MRSA) (Mimica and Mendes, 2007). CA-MRSA clones have been isolated since 1990 in several countries, including Brazil (Ribeiro *et al.*, 2005). Authors report that the HCA-MRSA clones are more related to SCC*mec* I - III subtypes, while CA-MRSA to IV - V subtypes, suggesting these relation with the broader spectrum of multidrug resistance in SCC*mec* I – III subtypes in comparison to SCC*mec* IV – VI (Mimica and Mendes, 2007). Commonly the contamination of people by CA-MRSA occurs by direct contact with fomites (Wallin *et al.*, 2008) or skin (Montgomery *et al.*, 2010; Diep and Miller, 2008), especially in environments shared by many people (Wallin *et al.*, 2008), or among people belonging to specific groups with direct contact and/or sharing equipment and personal belongings (Aiello *et al.*, 2006; Kirkland and Adams, 2008). Another possible contamination pathway by CA-MRSA is the ingestion of contaminated foods, such as cheese, sushi/sashimi, "bento" (Rizek *et al.*, 2011), milk (Kwon *et al.*, 2005) and meat (Corrente *et al.*, 2007). Regarding that the bacterial antibiotic resistance, particularly the presence of MRSA, in foods and food production environments is already recognized as a public health threat, the present study aimed at investigating the resistance profile to some classical antibiotics and the occurrence of *mec*A gene in samples of *S. aureus* isolated from ricotta cheese in Brazil.

MATERIAL AND METHODS

Strains isolation and identification

Forty two strains of *S. aureus*, were isolated from different samples of ricotta cheese marketed in João Pessoa (Brazil) by standard procedures (Downes and Ito, 2001) in the period of October to November of 2009, and were investigated for the presence of *mec*A gene. Stock cultures of the strains were kept on Blood Agar Base (Laboratórios Difco Ltda., Brazil) slants under refrigeration, and prior to use the they were grown overnight at 37°C in Brain Heart Infusion (Laboratórios Difco Ltda., Brazil).

Antibiotic susceptibility tests

The strains were categorized as MRSA by the disk diffusion test (1 µg of oxacillin; zone diameter, < 16 mm) (Kampf et al, 1998). Antibiogram data other than oxacillin were available for the following classical antibiotics: penicillin, streptomycin, tetracycline and erythromycin. The minimum inhibitory concentrations (MICs) were determined by agar dilution methods using an inoculum of *ca* 10^4 CFU/spot on Blood Agar Base (Laboratórios Difco Ltda., Brazil) (Pereira and Siqueira-Junior 1995). The type of erythromycin resistance-constitutive or inducible-was determined by the agar disc diffusion test according to the method of Weisblum and Demohn (1969).

PCR and sequencing

The extraction of genomic DNA from bacterial strains was performed according to Ausubel *et al.* (1987) with minor modifications. The *mec*A gene amplification was performed using the primers *mec*A1 (5'- GTA GAA ATG ACT GAA CGT CCG ATA A-3')and *mec*A2 (5'- CCA ATT CCA CAT TGT TTC GGT CTA A –3') in a thermocycler (Primus) with thermal cycle parameters as follows: initial denaturing step at 94 °C for 10 min; 30 cycles at 94 °C for 1 min; 55 °C for 1 min; 72 °C for 1 min; and a final elongation step of 72 °C for 10 min. As positive control was used a sample of purified PCR of HCA-MRSA isolated from hospital environment and previously characterized. The PCR products were purified prior to DNA sequencing using the NucleoSpin® Extract II PCR purification kit (Macherey-Nagel), and then sequenced on ABI3130 sequencer (Applied Biosystems). The sequences were compared with those of GenBank by mean of BLAST (Altschul *et al.*, 1990).

RESULTS AND DISCUSSION

Four out from the forty two *S. aureus* test isolates were resistant to methicillin/oxacillin and streptomycin (4, 21, 30 and 38). Moreover, isolates 21, 30 and 38 were resistant to penicillin, and 21 and 30 exhibited inducible resistant to erythromycin. Nine isolates (1, 5, 6, 21, 24, 26, 30, 33, and 34) amplified the *mec*A gene by PCR. Samples 21 and 30, which showed resistance phenotype to methicillin, were positive to *mec*A gene. Although the other seven isolates (1, 5, 6, 24, 26, 33 and 34) were positive to *mec*A gene, no resistance phenotype was noted for them in antimicrobial susceptibility test. Comparison of the gene sequence of each *S. aureus* isolate with those deposited in GenBank (GB) revealed closest relativity to SCC*mec* (Acces code dbj|AB505630.1|). The similarity indexes were greater than or equal to 97% for all *mecA* gene positive isolates in comparison with the sample from GenBank, while the assayed control isolate showed a 100% similarity (Table 1). The alignment of these sequences with SCC*mec* (Access code gb|GU370073.2|) (Fig. 1) evidenced that *mec*A genes were well conserved in all *mecA* gene positive isolates.

The resistant phenotype to methicillin/oxacillin found in isolates 4, 21, 30 and 38, besides the presence of the *mec*A gene in the samples 21 and 30, suggests that the mechanism of resistance could be given by the expression of this gene. The absence of resistant phenotype in some positive *mec*A isolates (1, 5, 6, 24, 26, 33 and 34) probably was not a result of mutations in *mec*A gene because the isolates 1 (methicillin-sensitive) and 21 (methicillin-resistant) showed the same

Table 1. Result of BLAST with the nine isolates of *S. strains* from Ricotta cheeses sold in Brazil, that amplified the *mec*A gene by PCR and positive control (C+). * Access code of SCC*mec* sequence of *S. aureus* carrying the *mec*A gene.

Strain	Organism	Gene	Max score	Query coverage	E value	Max identification
C+	*Staphylococcus aureus*	*mecA* (dbj|AB505630.1|)*	366	100%	2e-98	100%
1	*S. aureus*	*mecA* (dbj|AB505630.1|)*	329	100%	3e-87	98%
5	*S. aureus*	*mecA* (dbj|AB505630.1|)*	353	100%	2e-94	99%
6	*S. aureus*	*mecA* (dbj|AB505630.1|)*	344	100%	1e-91	98%
21	*S. aureus*	*mecA* (dbj|AB505630.1|)*	379	100%	3e-102	99%
24	*S. aureus*	*mecA* (dbj|AB505630.1|)*	351	100%	7e-94	98%
26	*S. aureus*	*mecA* (dbj|AB505630.1|)*	327	99%	1e-86	98%
30	*S. aureus*	*mecA* (dbj|AB505630.1|)*	363	100%	3e-97	99%
33	*S. aureus*	*mecA* (dbj|AB505630.1|)*	350	100%	2e-93	98%
34	*S. aureus*	*mecA* (dbj|AB505630.1|)*	339	100%	5e-90	97%

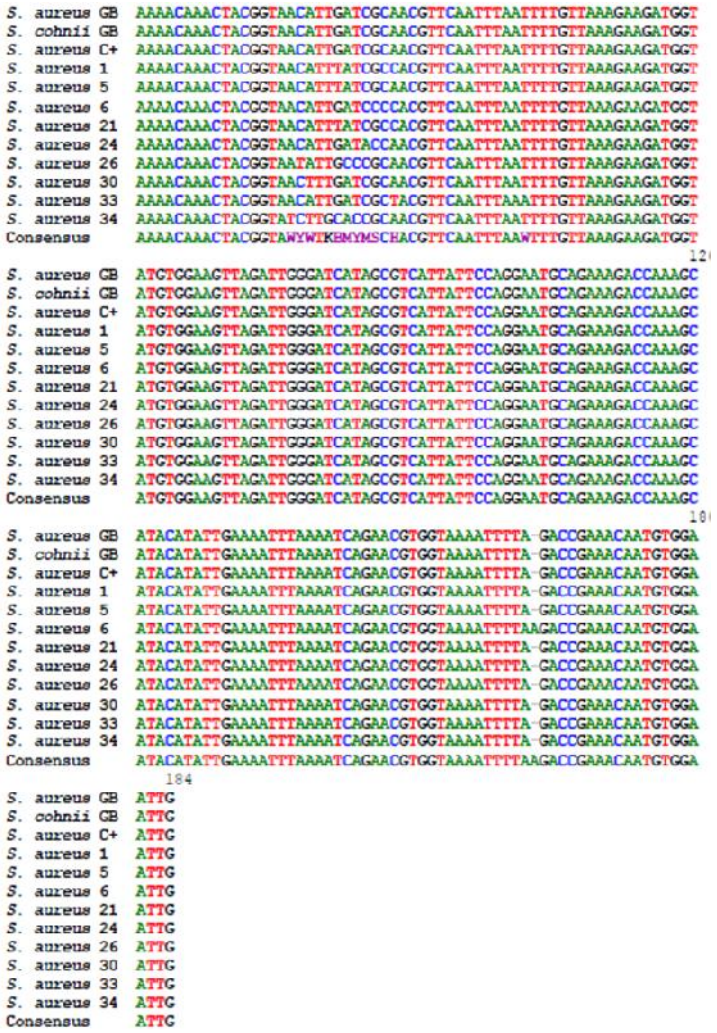

Figure 1. Align ment of sequences of samples studied with the *mec*A gene sequences of *Staphylococcus aureus* SCC*mec[1] and *Staphylococcus cohnii* SCC*mec**[2]. *Sequences obtained in GenBank (GB). [1]Access code dbj|AB505630.1|; [2]Access code gb|GU370073.2|.**

sequence (Fig. 1). The *mec*A gene in isolates phenotypically characterized as methicillin-sensitive should be negatively regulated (Rizek *et al.*, 2011). These findings could propose that the occurrence of selective pressure on these isolates could trigger the expression of the *mec*A gene becoming them potentially resistant to methicillin. Some samples also presented resistance to penicillin, suggesting that even in CA-MRSA the mechanisms to overcome the effects of antibiotics have been accumulating over time, a feature that previously was attributed mainly to HCA-MRSA samples (Mimica and Mendes, 2007). According to McMahon *et al.* (2006) and Rizek *et al.* (2011) Environmental elements can select bacterial strains adapted to the effects of stress, and the methods traditionally used to control the growth of bacteria in food could provide the type of stress required for a selection of MRSA, thus increasing these individuals in the population. These authors also stated that the presence of *S. aureus* carriers of genes for resistance, even if it is inactive, could be considered as a potential threat to public health, and reveal the need for continued monitoring in food and food processing plants.

Conclusions

The results of this study showed the presence of some isolates of *S. aureus* possessing resistance to methicillin/oxacillin, streptomycin, penicillin and erythromycin in Ricotta cheeses sold in Brazil. Moreover, some of these isolates demonstrated the presence of *mec*A gene, revealing the necessity to consider the foods as a potential pathway for spreading strains of *S. aureus* carrying resistance gene, and able to activate the mechanisms involved in the resistance establishment, in outside nosocomial environments.

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