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MICROBIOLOGY

Methicillin-resistant *Staphylococcus* spp. isolated from curd cheese "requeijão" and "especialidade láctea type requeijão" sold in Brazil

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ABSTRACT: *This study focused on counting Staphylococcus spp. in curd cheeses "requeijão" and "especialidade láctea type requeijão" sold in Brazil, assessing the presence of mecA gene in obtained isolates and establishing antimicrobial resistance profile of the mecA gene positive isolates. To this, a set of 200 samples of these dairy products were evaluated. Low counts of Staphylococcus spp. were observed in these dairy products. All the isolates were determined as coagulase-negative strains using coagulase test and PCR. However, two isolates (3.70%) were carriers of mecA gene and they can be considered as risk for public health. These isolates presented resistance to penicillin, oxacillin and erythromycin. In conclusion, low counts of Staphylococcus were detected in curd cheese "requeijão" and "especialidade láctea type requeijão" sold in Brazil. However, coagulase-negative methicillin-resistant Staphylococcus spp. was detected in these dairy products. This is the first report of the detection of methicillin-resistant Staphylococcus spp. in heat-treated dairy products in Brazil. Results served as a warning to public sanitary authorities to control multidrug-resistant strains in veterinary and human medicine.*
Key words: *antimicrobial resistance, cheese, food safety, MRS, Staphylococcus spp.*

Isolamento de *Staphylococcus* spp. resistente à meticilina de queijos fundidos requeijão e especialidade láctea tipo requeijão comercializados no Brasil

RESUMO: *Este trabalho objetivou realizar a contagem de Staphylococcus spp. em queijos fundidos "requeijão" e "especialidade láctea tipo requeijão" comercializados no Brasil, verificar a presença do gene mecA nos isolados obtidos e estabelecer o perfil de resistência antimicrobiana dos isolados positivos para tal gene. Para isso, 200 amostras desses produtos lácteos foram avaliadas. Baixas contagens de Staphylococcus spp. foram observadas nas amostras. Todos os isolados foram considerados como coagulase-negativos através do teste da coagulase e através da PCR. Entretanto, em dois isolados (3,70%) foi possível detectar o gene mecA e representam potencial risco à saúde pública. Esses isolados apresentaram resistência a penicilina, oxacilina e eritromicina. Conclui-se que baixas contagens de Staphylococcus foram detectadas em queijos fundidos "requeijão" e "especialidade láctea type requeijão". Entretanto, cepas de Staphylococcus spp. coagulase-negativas e resistentes à meticilina foram detectadas nesses derivados lácteos. Esse é o primeiro relato da ocorrência de cepas de Staphylococcus spp. resistentes à meticilina em produtos lácteos termicamente tratados comercializados no Brasil. Os resultados servem como um alerta para as autoridades sanitárias públicas nacionais para o controle de cepas multirresistentes em medicina veterinária e humana.*
Palavras-chave: *resistência antimicrobiana, segurança alimentar, MRS, queijos, Staphylococcus spp.*

INTRODUCTION

"Requeijão" is the third most sold cheese in Brazil (ZACARCHENCO et al., 2012). "Requeijão" is defined as "the product obtained using acid or enzymatic milk coagulation followed by the curd mass fusion (80ºC for 5 seconds), whey removal and washing according to Brazilian legislation (BRAZIL, 1997). Also, addition of milk cream, butter or butter oil in its composition is allowed. Another curd cheese routinely consumed in Brazil is "especialidade láctea type requeijão", a similar cheese produced using the same process but the addition of vegetable ingredients such as corn starch and vegetable oil is allowed in composition.

The genus *Staphylococcus* is among the most important pathogenic bacteria reported in dairy products. This genus is divided into two groups: coagulase-positive and coagulase-negative strains. Among the first one, *S. aureus* is known for its clinical importance and as a foodborne pathogen. The coagulase-negative group is comprised by several species involved in opportunistic infections in immunocompromised patients (MARTINS & CUNHA, 2007).

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Staphylococcus spp. are also frequently associated to mastitis (PITKALA et al., 2004), biofilm formation (OTTO, 2009) and are closely related to improper food handling of food handlers (ARGUDÍN et al., 2012), leading contamination of milk and other dairy products, posing public health into risk. Consequently, *Staphylococcus* spp. antimicrobial resistance is a concern for public health (KOKSAL et al., 2009).

Methicillin resistance is an increasing global problem (MARTINS & CUNHA, 2007), even for domestic animals (CUNY et al, 2010). The presence of these isolates in food and animals highlight the potential risk for human transmission (WEESE & DUJIKEREN, 2010). Some researchers have reported strains of methicillin resistant *Staphylococcus* spp. (MRS) causing mastitis (MOON et al., 2007), in bulk tank milk (VIRGIN et al., 2009), in bovine milk, pecorino and mozzarella cheeses (NORMANO et al., 2009) and in raw meats (BOER et al., 2009). Based on all these information, this study focused on counting *Staphylococcus* spp. in curd cheeses "requeijão" and "especialidade láctea type requeijão" sold in Brazil, assessing the presence of *mecA* gene in obtained isolates and establishing antimicrobial resistance profile of the *mecA* gene positive isolates.

MATERIALS AND METHODS

This research was performed using samples of "requeijão", "requeijão light", "especialidade láctea type requeijão" and "especialidade láctea type requeijão light", obtained from supermarkets located in the municipality of Jaboticabal, state of São Paulo, Brazil, during the year of 2015. Samples were kept refrigerated in cooler boxes with ice, during the transport to laboratory. Each kind of dairy product ("requeijão", "requeijão light", "especialidade láctea type requeijão") had 50 samples bought, from 5 different commercials brands, each one with 2 different production lots (each lot composed by 5 samples). For the "especialidade láctea type requeijão light", the samples were from three different commercial brands and 10 different production lots (each lot composed by 5 samples). In total, 200 samples were used for this study.

For each sample, 25g was placed into a Scott Bottle containing 225mL of 0.1% peptone water (Difco, Detroit, USA). Out of this first dilution (10^{-1}), other serial dilutions were performed, until 10^{-3}. Selective plating was done using 0.1mL of these dilutions in Baird-Parker agar (Difco, Detroit, USA). Plates were incubated for 45-48 hours under 35ºC. Whenever black colonies grew in the agar, it was transferred to a tube with Tryptic Soy Agar (TSA) (Difco, Detroit, USA) medium, and incubated for 24 hours under 35ºC (APHA, 2011). Moreover, the colonies were transferred to tubes containing Brain Heart Infusion broth (BHI) (Difco, Detroit, USA) and incubated for 24 hours under 37ºC. The coagulase test was performed using the isolates from BHI broth and rabbit plasma, in order to assess the presence of coagulase-positive strains (APHA, 2011).

The DNA from the 57 isolates was extracted according to the method described by KESKIMAKI et al. (2001). Presence of *Staphylococcus* spp. and *S. aureus* DNA molecules was detected using primers 16SrRNA (Pereira *et al.,* 2009) and Sa442-1/2 (MOROT-BIZOT et al., 2004). A PCR also was performed to identify coagulase-positive *Staphylococcus* spp. (*S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. lutrae*, *S. delphini*, and *S. pseudintermedius* according to SASAKI et al. (2010) (Table 1). In order to detect methicillin-resistant isolates (mecA), a PCR was performed using the primers as described by GORTEL et al. (1999): 5' AAA ATC GAT GGT AAA GGT TGG C 3' e 5' AGT TCT GCA GTA CCG GAT TTG C 3', with a 533bp product and 55°C annealing temperature.

PCR amplifications were carried out with mixtures containing PCR buffer 1x [100mM Tris-HCl pH 8.8; 500mMKCl; 0.8% (v/v) Nonidet P40]; $MgCl_2$ 2mM; dNTP's 0.2mM, 1.5U de Taq DNA polymerase, 5pmol of each primer, 60ng genomic DNA and sterile water until reaching the volume of 20µL. Cycling conditions consisted of an initial step at 95°C for 5min, followed by 35 cycles at 94ºC for 1min, annealing at the specific temperature of each primer for 1min and 72ºC for 1.5min. The final extension was at 72°C for 10min. The amplified products were visualized in a 1% agarose gel electrophoresis stained with 0.5µg mL^{-1} ethidium bromide (Vetec, São Paulo, Brazil). A previously known molecular weight marker was used as a reference (GeneRuler 1kb DNA Ladder – Fermentas, Thermo Fish Scientific, São Paulo, Brazil).

MRS positive isolates were examined for resistance to 11 antimicrobials by disk-diffusion (Kirby-Bauer) method. The following disks (Laborclin™) were used: oxacillin (1µg), cefepime (30µg), rifampicin (5µg), chloramphenicol (30µg), clindamyicin (2µg), erythromycin (15µg), penicillin G (10un), sulfatrim (23,75/1,25µg), tetracycline (30µg), gentamicin (10µg) and ciprofloxacin (5µg).

Table 1 - Primer, nucleotides sequence, amplicon size (bp), annealing temperature, positive control and reference of each primer used for the detection of *Staphylococcus* spp.

	Primer	Nucleotides Sequence	Size (bp)	Annealing temperature (°C)	Positive control	References
Staphylococcus spp.	16srRNA	GTA GGT GGC AAG CGTTAT CC CGC ACA TCA GCG TCA G	228	55	ATCC 25983	Pereira et al., 2009
S. aureus	Sa442	AATCTTTGTCGGTACACGATATT CTTCACG CGTAATGAGATTTCAGTAGATAA TACAACA	102	55	ATCC 25983	Morot-Bizot et al., 2004
S. aureus	au-F3 au-nucR	TCGCTTGCTATGATTGTGG GCCAATGTTCTACCATAGC	359		ATCC 25983	Sasaki et al., 2010
S. intermedius	in-F in-R3	CATGTCATATTATTG CGAATGA AGGACCATCACCATT GACATATTGA AACC	430			
S. schleiferi subspecie coagulans	sch-F sch-R	AATGGCTACAATGAT AATCACTAA CATATCTGTCTTTCG GCGCG	526			
S. hyicus	hy-F1 hy-R1	CATTATATGATTTGA ACGTG GAATCAATATCGTAA AGTTGC	793			
S. deplhini grupo A	dea-F dea-R	TGAAGGCATATTGTA GAACAA CGRTACTTTTCGTTA GGTCG	661			
S. deplhini grupo B	debF debR4	GGAAGRTTCGTTTTTCCTAGAC TATGCGATTCAAGAA CTGA	1135			
S. pseudintermedius	pseF2 pseR5	TRGGCAGTAGGATT CGTTAA CTTTTGTGCTYCMTT TTGG	926	*56*		

The significance between the *Staphylococcus* spp. counting of the groups was assessed through a variance analysis and the Kruskall-Wallis test with 5% of significance level. Data regarding the "presence or absence" of *Staphylococcus* spp. in the samples group (comparison among light and normal products; and among "requeijão" and "especialidade láctea" products) was performed using the chi-square test with 5% of significance level. The analyses were performed using Software R®.

RESULTS AND DISCUSSION

Table 2 presents *Staphylococcus* spp. counts of the dairy product samples analyzed in this study. Results of *Staphylococcus* spp. count did not meet the necessary requirements to perform the variance analysis. Moreover, when using the nonparametric Kruskal-Wallis test with 5% of significance level, no statistical significance (P>0.05) was observed among these products. The chi-square test performed to evaluate the difference among the presence or absence of these microorganisms in the products also presented no significance (P>0.05)

Staphylococcus spp. count in the samples was low and all the strains were considered as coagulase-negative strains on coagulase-test. *S. aureus* is considered as the most important foodborne pathogen of this group and it is considered as coagulase-positive specie. Based on such results,

Table 2 - Average *Staphylococus* spp. count in "requeijão", "requeijão light", "especialidade láctea type requeijão" and "especialidade láctea type "requeijão light" sold in Jaboticabal municipality, state of São Paulo, Brazil during 2015.

Product	Brand	Lot	*Staphylococcus* spp. count (CFU g^{-1})	Product	Brand	Lot	*Staphylococcus* spp. count (CFU g^{-1})
"Requeijão"	A	1	$<0.1 \times 10^0$	"Especialidade láctea"	A	1	8.6×10^0
		2	$<0.1 \times 10^0$			2	$<0.1 \times 10^0$
	B	1	$<0.1 \times 10^0$		B	1	$<0.1 \times 10^0$
		2	0.3×10^0			2	0.1×10^0
	C	1	$<0.5 \times 10^0$		C	1	$<0.1 \times 10^0$
		2	$<0.5 \times 10^0$			2	$<0.1 \times 10^0$
	D	1	0.8×10^0		D	1	$<0.1 \times 10^0$
		2	$<0.1 \times 10^0$			2	$<0.1 \times 10^0$
	E	1	$<0.1 \times 10^0$		E	1	$<0.1 \times 10^0$
		2	0.4×10^0			2	19.7×10^0
"Requeijão" Light	A	1	$<0.1 \times 10^0$	"Especialidade láctea" light	A	1	$<0.1 \times 10^0$
		2	$<0.1 \times 10^0$			2	$<0.1 \times 10^0$
	B	1	$<0.1 \times 10^0$			3	$<0.1 \times 10^0$
		2	$<0.1 \times 10^0$			4	0.1×10^0
	C	1	$<0.1 \times 10^0$		B	1	0.2×10^0
		2	0.1×10^0			2	$<0.1 \times 10^0$
	D	1	$<0.1 \times 10^0$			3	$<0.1 \times 10^0$
		2	1.9×10^0			4	$<0.1 \times 10^0$
	E	1	$<0.1 \times 10^0$		c	1	$<0.1 \times 10^0$
		2	$<0.1 \times 10^0$			2	0.1×10^0

it is possible to state that the consumption of these dairy products does not directly pose risk to the Brazilian population, with regard to foodborne illness occurrence. Furthermore, the low number of the pathogen observed in the samples could be explained by the high temperature processes used in the production flowchart (BRAZIL, 1997) and good manufacturing practices implementation in these industries (SANTANA et al., 2009).

A set of 57 isolates were subjected to a PCR in order to confirm if they were *Staphylococcus* spp. (PEREIRA et al., 2009), *S. aureus* (MOROT-BIZOT et al., 2004) or other coagulase-positive *Staphylococcus* spp. (*S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. lutrae*, *S. delphini*, and *S. pseudintermedius* (SASAKI et al., 2010). In total 54 isolates were confirmed as *Staphylococcus* spp. with amplification of the corresponding product. There were no amplification products using primers for *Staphylococcus* spp. coagulase-positive, agreeing with the coagulase-test results and allowing us to confirm that all isolates were coagulase-negative strains.

Using PCR to detect the presence of *mecA* gene, one isolate obtained from "requeijão light" (brand C and lot 2) and another isolate obtained from "especialidade láctea type requeijão light" (brand C and lot 2) were considered as positive. The prevalence of methicillin-resistant coagulase-negative *Staphylococcus* spp. in evaluated samples was 3.70% among the 54 isolates. This two isolates showed resistance to oxacillin, penicillin G and

erythromycin but did not to cefepime, rifampicin, chloramphenicol, clindamyicin, sulfatrim, tetracycline, gentamicin and ciprofloxacin antimicrobials. Resistance to certain non-beta-lactam (as erythromycin) in *Staphylococcus* spp. isolates carrying *mecA* gene was already reported (HUBER et al., 2011).

NORMANNO et al. (2007) established 3.75% as the prevalence of methicillin-resistant *Staphylococcus* spp. in milk and dairy products in Italy highlighting the role of dairy products in transmission of antimicrobial-resistant bacteria to humans. Furthermore, some authors isolated strains of *Staphylococcus* spp. methicillin-resistant from some food such as: cow's milk, pecorino and mozzarella cheeses (NORMANO et al., 2009) and raw meat (BOER et al., 2009). However, the occurrence of methicillin-resistant *Staphylococcus* spp. in "requeijão" and "especialidade láctea", or even in any other heat-treated dairy product, was never reported in Brazil, until this paper.

"Requeijão" is one of the most consumed cheeses in Brazil and it is usually consumed raw (ZACARCHENCO et al., 2012). Presence of coagulase-negative methicillin-resistant *Staphylococcus* spp. strains in these products present a risk to the country's public health. Thus, considering the potential implications in food production chain, there is a need to be constantly monitoring the employers' health and hygiene, once humans can be colonized by contact during food processing or consumption of contaminated food (KLUYTMANS, 2009).

Moreover, nowadays coagulase-negative *Staphylococcus* spp. is considered as able on transferring antimicrobial resistance genes to other S*taphylococcus* spp. strains (TULINSKI et al., 2012). Human infections caused by methicillin-resistant S*taphylococcus* spp. are increasing (MARTINS & CUNHA, 2007), even in animals (CUNY et al., 2010), being considered an emergent problem in veterinary medicine and public health (HUBER et al., 2011). Animals usually are infected and colonized during contact with humans and vice-versa (GRAVELAND et al., 2010). Consequently, prophylactic measures to avoid methicillin-resistant S*taphylococcus* spp. contamination in foods and infections in humans and animals are required in order to reduce risk in Brazil.

CONCLUSION

In conclusion, processing technology ensures the safety of these products categories; however, they may be carriers of methicillin resistant *Staphylococcus* spp. strains. Results served as a warning to Brazilian public sanitary authorities to control multidrug-resistant strains in veterinary and human medicine.

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