Revista da Sociedade Brasileira de Medicina Tropical 45(5):579-585, Sep-Oct, 2012

Major Article

Prevalence of enterotoxin-encoding genes and antimicrobial resistance in coagulase-negative and coagulase-positive *Staphylococcus* isolates from black pudding

Tiane Martin de Moura[1, 2], Fabrício Souza Campos[3], Pedro Alves d'Azevedo[4], Sueli Teresinha Van Der Sand[1, 2], Ana Cláudia Franco[1, 3], Jeverson Frazzon[5] and Ana Paula Guedes Frazzon[1, 2]

[1]. Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente, Departamento de Microbiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS. [2]. Laboratório de Bacteriologia, Departamento de Microbiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS. [3]. Laboratório de Virologia, Departamento de Microbiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS. [4]. Laboratório de Cocos Gram-Positivos, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS. [5]. Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

ABSTRACT

Introduction: Staphylococcal species are pathogens that are responsible for outbreaks of foodborne diseases. The aim of this study was to investigate the prevalence of enterotoxin-genes and the antimicrobial resistance profile in staphylococcus coagulase-negative (CoNS) and coagulase-positive (CoPS) isolates from black pudding in southern Brazil. **Methods:** Two hundred typical and atypical colonies from Baird-Parker agar were inoculated on mannitol salt agar. Eighty-two mannitol-positive staphylococci were submitted to conventional biochemical tests and antimicrobial susceptibility profiling. The presence of coagulase (*coa*) and enterotoxin (se) genes was investigated by polymerase chain reaction. **Results:** The isolates were divided into 2 groups: 75.6% (62/82) were CoNS and 24.4% (20/82) were CoPS. The biochemical tests identified 9 species, of which *Staphylococcus saprophyticus* (37.8%) and *Staphylococcus carnosus* (15.9%) were the most prevalent. Antimicrobial susceptibility tests showed resistance phenotypes to antibiotics widely administered in humans, such as gentamicin, tetracycline, chloramphenicol, and erythromycin. The *coa* gene was detected in 19.5% (16/82) of the strains and 4 polymorphic DNA fragments were observed. Five CoNS isolates carrying the *coa* gene were submitted for 16S rRNA sequencing and 3 showed similarity with CoNS. Forty strains were positive for at least 1 enterotoxin-encoding gene, the genes most frequently detected were *sea* (28.6%) and *seb* (27.5%). **Conclusions:** The presence of antimicrobial resistant and enterotoxin-encoding genes in staphylococci isolates from black pudding indicated that this fermented food may represent a potential health risk, since staphylococci present in food could cause foodborne diseases or be a possible route for the transfer of antimicrobial resistance to humans.

Keywords: Staphylococcal enterotoxin. Coagulase. Antimicrobial-resistance.

INTRODUCTION

Black pudding or blood sausage is a type of sausage, made from the blood, fat, and skin of cattle or pig, stuffed into natural or synthetic casing, and tied manually. This kind of sausage is very popular in south Brazil, Argentina, and Uruguay. Animal products are susceptible to microorganism contamination, and bacteria present in food could cause foodborne disease or be a possible route for the transfer of antimicrobial resistance to humans[1,2].

Members of the genus *Staphylococcus* are gram-positive cocci, and are natural inhabitants of the skin and mucous membranes of humans and animals. Currently, according to literature[3], this genus comprises 45 species, which are divided into 2 groups: coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS), based on the ability to coagulate rabbit plasma. On the one hand, some CoNS species are components of the natural microbiota of food, and play an important role in the manufacturing processes of diverse meat-derived products; in particular, in dry fermented sausages, they act as starters to ensure the quality and safety of the final products[4]. On the other hand, CoPS and CoNS species, such as *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *S. saprophyticus,* are well known for their implications in human health and disease. *S. aureus* is considered to be one of the most common pathogens responsible for the outbreaks in 1994 and 1998 in São Paulo (Brazil); in general, 51.5% of the outbreaks were caused by *S. aureus*[5]. In addition, the incidence of nosocomial infections caused by CoNS has increased in the last few years. In Brazil[6] and the United States of America (USA), CoNS are the most common cause of nosocomial infections in the intensive care nursery. They are responsible for blood stream infections in neonates, also causing infections of the urinary tract, wounds, bloodstream, and the endocardium in immunocompetent individuals, where *S. saprophyticus* is the most prevalent species[7]. A prospective study was conducted from June 2001 to May 2002 in a hospital burn unit, with 252 patients; 49 (19.4%) of these developed clinically and microbiologically proven sepsis and the most prevalent bacteria were *S. aureus* and CoNS[8].

Perhaps the most notable virulence factors associated with staphylococci are the heat-stable enterotoxins (SEs) produced by certain strains. These toxins are a leading cause of gastroenteritis, including vomiting, abdominal cramping, diarrhea, and malaise, in 3–10 h following the consumption of preformed toxin by susceptible individuals[9]. The SEs are classified into 5 classical serological types: SEA, SEB, $SEC_{1,2,3}$, SED, and SEE, but recently other enterotoxins were described in the literature, including SEG, SEH, SEI, SER, SES, SET and the enterotoxin-like proteins SElJ, SElK, SElL, SElM, SElN, SElO, SE1P, SE1Q, and SElU[10]. Among the CoPS, *S. aureus* is frequently responsible for outbreaks of food poisoning, due to its ability to express 7 different toxins. However, other CoPS, such as *S. intermedius* and *Staphylococcus hyicus*

Address to: Dra. Tiane Martin de Moura. PPGMAA/Deptº Microbiologia/UFRGS. Av. Sarmento Leite 500/sala 209, 90050-170 Porto Alegre, RS, Brasil.
Phone: 55 51 3308-3935; Fax 55 51 3308-4111
e-mail: tianedemoura@gmail.com
Received in 12/09/2011
Accepted in 13/03/2012

can also express enterotoxins[11]. During the period from 1999 to 2009, 6,349 outbreaks of foodborne diseases were reported in Brazil, and 20.5% of these cases were caused by *Staphylococcus* spp. Furthermore, enterotoxigenic CoNS have also been isolated from the hands of food handlers and food, demonstrating the importance of CoNS in public health[12,13].

The use of antimicrobial agents in animal husbandry, as a growth promoter, has a selective effect in the emergence and maintenance of resistant bacteria in animals, animal products, and the environment[2]. *Staphylococcus* spp. have been isolated from poultry-processing plants, chicken carcasses, milk, and dairy products[14-17]. Evidence suggests that resistant microorganisms or their antibiotic resistance genes can be transferred from food, animals, or the environment to humans[1].

So far, in Brazil, there have been no studies examining the presence of enterotoxin-encoding genes and antimicrobial resistance in staphylococcal isolates from black pudding. Therefore, the aim of the present study was to investigate the prevalence of enterotoxin-encoding *sea*, *seb*, *sec, sed,* and *see* genes by polymerase chain reaction (PCR) and antimicrobial resistance profiling in coagulase-negative and coagulase-positive isolates from black pudding in southern Brazil.

METHODS

Bacterial isolates and biochemical characterization

Twenty samples of black pudding purchased in a public market in Pelotas in southern Brazil, in the State of Rio Grande do Sul (RS), during March to November 2008, were analyzed. The first isolation step was to inoculate 25 g of black pudding into sterile buffered peptone water (225ml) with subsequent 10-fold serial dilutions. One milliliter of each suspension was spread over the surface of each of 3 plates containing Baird-Parker agar (BPA) (Merck, Darmstadt, Germany) supplemented with 5% egg yolk-tellurite emulsion. The plates were incubated for 45-48h at 35°C. Two hundred typical circular and atypical colonies of *S. aureus* were randomly selected from BPA and inoculated on mannitol salt agar (MSA) (Hi-Media, Mumbai, India). Typical colonies were smooth, convex, moist, 2-3 mm in diameter, gray to jet-black, often with a light (off-white)-colored margin, surrounded by an opaque zone, and frequently with a clear outer zone. Eighty-two mannitol-positive isolates were identified to the genus level by gram staining and catalase production. The biochemical characterization of *Staphylococcus* species was carried out following the method proposed in the literature[2]. The enzymatic activity of staphylocoagulase (free coagulase) was determined in a tube with rabbit plasma (Laborclin-Pinhais, Brazil) according to the manufacturer. The *S. aureus* strains ATCC 25923 and ATCC 19095 were used as positive controls, and negative controls were inoculated with sterile water instead of bacteria.

Antimicrobial susceptibility test

The antimicrobial susceptibility test was performed by the disk-agar diffusion method as recommended by the Clinical and Laboratory Standards Institute[18]. Five antimicrobials commonly used in the treatment of clinical infection and agricultural procedures were tested (concentrations are expressed in μg ml^{-1}): erythromycin (ERY; 15μg), tetra-cycline (TET; 30μg), gentamicin (GEN; 10μg), vancomycin (VAN; 30μg), and chloramphenicol (CLO; 30μg). The strain *S. aureus* ATCC 25923 was used as a control.

Polymerase chain reaction (PCR) amplification of the coagulase (coa) gene

Genomic deoxyribonucleic acid (DNA) extraction and PCR amplification of the *coa* gene were performed based on previously described protocols [19,20] **(Table 1)**. Reactions were performed in the Eppendorf Mastercycler Thermal Cycler under the following cycle conditions: 5 min at 94°C; followed by 40 cycles of 1 min at 94°C, 1 min at 56.7°C, and 1 min at 72°C; followed by 5 min at 72°C. *Staphylococcus aureus* strains ATCC 13565, ATCC 14458, ATCC 19095, ATCC 23235, ATCC 25923, and ATCC 27664 were used as positive controls and were kindly provided by *Instituto Oswaldo Cruz*, Rio de Janeiro, Brazil.

TABLE 1 - Nucleotide sequences and annealing temperatures of the primers used for the amplification of staphylococci genes.

Gene	Nucleotide sequence (52 - 32)	Annealing temperature (°C)	Amplicon (bp)	References
Enterotoxins				
SEA[a]	CCT TTG GAA ACG GTT AAA ACG CTG AAC CTT CCC ATC AAA AAC	54	126	This study
SEB[b]	GGT ACT CTA TAA GTG CCT GC TTC GCA TCA AAC TGA CAA ACG	55	475	This study
SEC[c]	AGA ACT AGA CAT AAA AGC TAG G TCA AAA TCG GAT TAA CAT TAT CC	55	267	This study
SED[d]	TTT GGT AAT ATC TCC TTT AAA CG CTA TAT CTT ATA GGG TAA ACA TC	54	309	This study
SEE[e]	CCT ATA GAT AAA GTT AAA ACA AGC TAA CTT ACC GTG GAC CCT TC	55	173	This study
Coagulase				
coa	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	56.7	840	Goh et al.
16S rRNA				
16S	AGA GTT TGA TCC TGG CTC AG CCG CGG CTG CTG GCA CGT A	58	530	Gontang et al.

sea: staphylococcal enterotoxin A; **seb**: staphylococcal enterotoxin B; **sec**: staphylococcal enterotoxin C; **sed**: staphylococcal enterotoxin D; **see**: staphylococcal enterotoxin E; **coa**: coagulase; **rRNA**: ribosomal ribonucleic acid. *Staphylococcus aureus* strains [a]ATCC 13565; [b]ATCC 14458; [c]ATCC 19095; [d]ATCC 23235, and [e]ATCC 27664 were used as positive controls

Amplification of *16S rRNA* gene and sequencing

Isolates classified as CoNS and *coa*-positive were submitted for 16*S* ribosomal ribonucleic acid (rRNA) gene amplification and sequencing. The primers and PCR reaction followed the protocols previously described[21]. A 520-bp DNA fragment was purified using the Illustra GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK) and analyzed in an Applied Biosystems (ABI) sequencer model

3130 using the polymer pop6 and the Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA). The nucleotide sequence was compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR for the detection of enterotoxin-encoding A (*sea*) and D (sed) genes

All strains were tested for the presence of *sea* and *sed* genes (using the primers described in **Table 1**). The PCR reactions were performed in a final volume of 25µl containing 1mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 10 pMol of each primer (Integrated DNA Technologies; IDT, Coralville, IA), 1U *Taq* DNA polymerase (Invitrogen), 1× PCR buffer (Invitrogen), 200µM deoxynucleoside triphosphates (ABgene, Epsom, UK), and deionized water (Milli Q plus, Millipore, Billerica, MA). Reactions were performed in an Eppendorf Mastercycler Thermal Cycler under the following cycle conditions: 5 min at 94°C; followed by 30 cycles of 45 s at 94°C, 45s at 54°C, and 45 s at 72°C; followed by 5 min at 72°C. The *S. aureus* ATCC 13565 and ATCC 23235 strains were used as positive controls for the *sea* and *sed* genes, respectively.

Multiplex PCR for detection of the enterotoxin-encoding genes B (*seb*), C (*sec*), and E (*see*)

Multiplex PCR was used to determine the frequency of the *seb*, *sec,* and *see* genes in all isolates. The nucleotide sequences of the primers are described in **Table 1**. The PCR reactions (25µl) contained 1mM $MgCl_2$ (Invitrogen), 5pMol of each primer (IDT), 1U *Taq* DNA polymerase (Invitrogen), 1× PCR buffer (Invitrogen), 300µM deoxynucleoside triphosphates (ABgene), and deionized water (Milli Q plus, Millipore) per reaction. Reactions were performed in an Eppendorf Mastercycler Thermo Cycler under the following conditions: 5 min at 94°C; followed by 35 cycles of 45s at 94°C, 45s at 55°C, and 45s at 72°C; followed by 5 min at 72°C. The *S. aureus* ATCC 14458, ATCC 19095, and ATCC 27664 strains were used as positive controls for the *seb, sec,* and *see* genes, respectively.

RESULTS

Isolation and identification of coagulase-negative and coagulase-positive staphylococci from black pudding

A total of 200 colonies from BPA were inoculated on MSA agar. Eighty-two colonies showing mannitol fermentation were selected and divided into 2 groups based on the coagulase test: 75.6% (62/82) CoNS and 24.4% (20/82) CoPS. All isolates were tested for the presence of the *coa* gene by PCR and 19.5% (16/82) were *coa*-positive **(Table 2)**. Four polymorphic DNA fragments of the *coa* gene were observed in the strains **(Table 2)**. Among the 16 *coa* positives, 5 were identified as coagulase-negative and submitted for *16S* rRNA gene amplification and sequencing. Three strains showed 99%, 97%, and 90% similarity with *Staphylococcus vitulinus* (GenBank: AM062694.1), *Staphylococcus cohnii* (GenBank: HQ154559.1), and *Staphylococcus equorum* (GenBank: DQ232735.1), respectively. To the best of our knowledge, this is the first time that the *coa* gene was detected in these CoNS species. Two CoNS isolates showed similarity to *Staphylococcus pseudintermedius* and *S. aureus*, and were reclassified as CoPS species.

Table 2 shows the overall distribution of mannitol-fermenting CoNS and CoPS species identified from black pudding. Nine different species were identified; *S. saprophyticus* was the most prevalent (37.8%), followed by *Staphylococcus carnosus* (15.9%), *S. vitulinus* (8.5%), *S. pseudintermedius* (7.3%), *S. cohnii* (6.1%), *S. aureus* (3.7%), *S. equorum* (2.4%), *S. schleiferi* (1.2%), and *S. intermedius* (1.2%). Thirteen

TABLE 2 - Phenotype and genotype analysis of coagulase in *Staphylococcus* spp. isolates from morcilla.

Phenotype	Isolate		Size of *coa*	
	n	%	Species	gene by PCR[a]
CoPS	1	0.2	*Staphylococcus aureus*	900
CoNS	1	1.2	*Staphylococcus cohnii*[b]	900
CoPS	2	2.4	*Staphylococcus aureus*	700
CoPS	1	1.2	*Staphylococcus* spp.	700
CoPS	1	1.2	*Staphylococcus schleiferi*	700
CoNS	1	1.2	*Staphylococcus vitulinus*[b]	650
CoPS[c]	1	1.2	*Staphylococcus* spp.	550
	1	1.2	*Staphylococcus* spp.	550
CoNS	1	1.2	*Staphylococcus equorum*[b]	550
CoPS[c]	6	7.3	*Staphylococcus pseudintermedius*[b]	550
CoPS	1	1.2	*Staphylococcus intermedius*	550
CoPS	9	11.0	*Staphylococcus* spp.	negative
CoNS	31	37.8	*Staphylococcus saprophyticus*	negative
CoNS	13	15.9	*Staphylococcus carnosus*	negative
CoNS	4	9.0	*Staphylococcus cohnii*	negative
CoNS	6	7.3	*Staphylococcus vitulinus*	negative
CoNS	1	1.2	*Staphylococcus equorum*	negative
CoNS	2	2.4	*Staphylococcus* spp.	negative
	82	**100.0**		

coa: coagulase; **PCR:** polymerase chain reaction; **CoPS:** coagulase-positive staphylococci. **CoNS**: coagulase-negative staphylococci. [a]approximated values in base pairs; [b]identified by sequencing; [c]strains originally identified as CoNS based on the coagulase test.

TABLE 3 - Distribution of resistant coagulase-negative and coagulase-positive staphylococci isolates from black pudding in southern Brazil.

Coagulase phenotype	Species	Number of antimicrobial-resistant isolates ERY (15µg)*	TET (30µg)*	GEN (10µg)*	CLO (30µg)*	VAN (30µg)*
CoNS						
	Staphylococcus saprophyticus	6	9	1	2	0
	Staphylococcus carnosus	0	1	1	2	0
	Staphylococcus vitulinus	2	2	1	0	0
	Staphylococcus cohnii	3	1	2	0	0
	Staphylococcus spp.	1	1	0	0	0
	Staphylococcus equorum	1	1	0	0	0
CoPS						
	Staphylococcus spp.	5	2	1	1	0
	Staphylococcus pseudintermedius	0	1	1	0	0
	Staphylococcus aureus	2	1	0	0	0
	Staphylococcus schleiferi	1	0	0	0	0
Total: n/%		**21/25.6**	**19/23.2**	**7/8.5**	**5/6.1**	**0/0.0**

*concentrations are expressed in µg ml-1; **ERY:** erythromycin; **TET:** tetracycline; **GEN:** gentamicin; **CLO:** chloramphenicol; **VAN:** vancomycin; **CoNS**: coagulase-negative staphylococci; **CoPS:** coagulase-positive staphylococci.

TABLE 4 - Distribution of enterotoxin-encoding genes in coagulase-negative and coagulase-positive staphylococci isolates from black pudding in southern Brazil.

Species	Numbers of isolates positive for the enterotoxin-encoding genes by PCR sea	seb	sec	sed	see	Total
CoNS						
Staphylococcus carnosus	2	1	4	0	0	**7**
Staphylococcus vitulinus	2	1	1	0	0	**4**
Staphylococcus cohnii	2	1	0	0	0	**3**
Staphylococcus saprophyticus	2	4	1	1	3	**11**
Staphylococcus equorum	1	0	1	0	0	**2**
CoPS						
Staphylococcus spp.	1	2	1	0	2	**6**
Staphylococcus pseudintermedius	2	2	0	1	1	**6**
Staphylococcus schleiferi	0	0	0	0	1	**1**
Total: n/%	**12/28.6**	**11/27.5**	**8/20.0**	**2/5.0**	**7/17.5**	**40/100.0**

PCR: polymerase chain reaction; **sea**: staphylococcal enterotoxins A; **seb**: staphylococcal enterotoxins B; **sec**: staphylococcal enterotoxins C; **sed**: staphylococcal enterotoxins D; **see**: staphylococcal enterotoxins E; **CoNS**: coagulase-negative staphylococci; **CoPS:** coagulase-positive staphylococci.

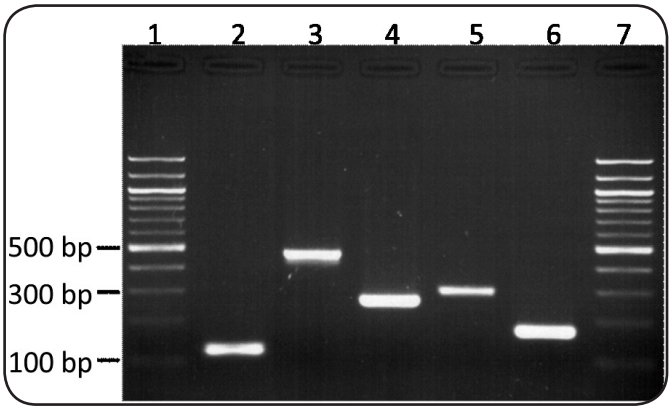

FIGURE 1 - PCR analysis of genes encoding enterotoxins A, B, C, D, and E in *Staphylococcus aureus* positive controls. **Lanes 1** and **7**: 100-bp molecular weight marker ladder (Fermentas, Milpitas, CA); **Lane 2:** sea (126 bp); **Lane 3:** seb (475 bp); **Lane 4:** sec (276 bp); **Lane 5:** sed (309 bp); **Lane 6:** see (173 bp).

isolates (15.9%) could not be identified to the species level and were classified as *Staphylococcus* spp.

Antimicrobial susceptibility testing

In the present study, 63.4% of coagulase-negative and coagulase-positive staphylococci strains from black pudding showed antibiotic resistance **(Table 3)**. Resistance to erythromycin (25.6%) and tetracycline (23.2%) were the most frequent profiles detected. All strains were susceptible to vancomycin, 93.9% to chloramphenicol, and 91.5% to gentamycin. Thirteen (15.9%) strains exhibited multidrug resistance, and erythromycin/tetracycline resistance was the profile most commonly observed.

Prevalence and distribution of enterotoxin-encoding genes (*se*) in coagulase-negative and coagulase-positive staphylococci

Forty (48.8%) of 82 strains were positive for 1 or more enterotoxin-encoding genes **(Table 4)**; amongst these, 67.5% (27/40) were coagulase-negative and 32.5% (13/40) were coagulase-positive. The *sea* gene was the most frequently detected (28.6%), followed by *seb* (27.5%), *sec* (20%), *see* (17.5%), and *sed* (5%). The **Figure 1** shows the amplification profile of the enterotoxin-encoding *sea*, *seb, sec, sed,* and *see* genes in the control. Five strains (3 CoNS and 2 CoPS) exhibited more than 1 gene.

DISCUSSION

Isolation and identification of coagulase-negative and coagulase-positive staphylococci from black pudding

In this study, we found no correlation between the presence of the *coa* gene and the detection of coagulase activity among the staphylococci isolates studied, suggesting that the presence of the coagulase gene is not necessarily associated with its expression. Nine CoPS strains showed *coa*-negative amplification. This behavior can be explained by (1) the production of pseudocoagulase, which has been described in CoNS[22] and could lead to false-positive coagulase activity, or (2) the presence of various numbers of degenerate repeat sequences in the *coa* gene, which gives a polymorphic characteristic in number and sequence, and could generate a mutation in the target DNA region of the *coa* gene[20]. On the other hand, 5 CoNS strains identified by the coagulase test carried the *coa* gene. These strains were sequenced, and the DNA sequence was analyzed against the National Center for Biotechnology Information (NCBI**)** database for similarity with *S. vitulinus*, *S. cohnii,* and *S. equorum*. Previous studies also observed the presence of the *coa* gene in CoNS isolated from cheese[11]. Other authors have suggested that the presence of *coa* gene is not necessarily associated with the phenotypic expression of the enzyme[23]. The coagulase test, though specific and sensitive, is subjected to variability in sample conditions. Factors like nutrient availability, environment,

and intrinsic physiological conditions of the bacteria can be associated with the non-expression of this gene[24]. The expression of the *coa* gene usually occurs during the exponential growth phase, and it can be repressed by an accessory gene regulator (*agr*). Another locus of regulation, called the *Staphylococcus* accessory regulator (*sar*), also affects the expression of exoproteins in *S. aureus*, and mutants at this locus produce low amounts of coagulase[25]. A mutation in one of these *loci* could theoretically result in a coagulase-negative phenotype, since a small amount of this enzyme does not cause clotting of plasma. PCR amplification of the *coa* gene showed DNA fragments of 550-900bp between the strains. The same polymorphic DNA fragments have been observed in the *coa* gene in other studies[20,26].

The *Staphylococcus* species identified in the current study have also reported by other authors[27,28] in artisanal morcilla and fermented meat products. *S. saprophyticus* is considered to be a frequent contaminant of fermented sausages and raw meats and also has been isolated from rectal swabs of cattle carcasses and pigs. In humans, the main reservoir of *S. saprophyticus* is the gastrointestinal tract[7,10]. The frequency of *S. carnosus* (15.9%) was relatively elevated in the present study, compared to other studies[27,28]. The presence of *S. carnosus* in black pudding samples can be justified because this species is often described as a common commercial starter culture for manufacturing sausages[29]. *Staphylococcus vitulinus* is a member of the Staphylococcus *sciuri* group and can be isolated from animals and various food products of animal origin, like cheeses and sausages[10,30,31]. The occurrence of different species of staphylococci in black puddings can be explained by the fact that some species are common contaminants of food and others are associated with a lack of hygiene during food manipulation.

The lower prevalence of *S. aureus* (3.7%) detected in black pudding samples in the present study agree with previously described results[32], in which *S. aureus* was not isolated from samples of black pudding analyzed in Buenos Aires, Argentina. The low occurrence of *S. aureus* demonstrated here, compared to other studies, can be explained by the fact that some laboratories in developing countries screen for presumptive *S. aureus* based on growth on MSA and/or DNase tests. The MSA medium was developed for the presumptive isolation of *S. aureus* in a single step for clinical samples, which is convenient for diagnostic laboratories. However, misclassification of the species could occur when the classification is based on mannitol fermentation only.

Antimicrobial susceptibility test in staphylococci strains from black pudding

Antimicrobial resistant staphylococci isolated from food have been identified in previous studies[14-17]. None of the strains were resistant to vancomycin, consistent with previous studies[14-16,33]. In the current study, a high prevalence of erythromycin and tetracycline resistance was observed. One possible reason for this phenotype in staphylococcal isolates from black pudding could be that tetracycline and erythromycin are drugs used in veterinary medicine and as growth promoters[34,35]. Although tetracycline is an antimicrobial that is not approved by the European Union as a food supplement for animals, therapeutic and prophylactic use in veterinary medicine is common, and *Staphylococcus* spp. resistant to tetracycline are frequently found in cured ham, fermented fish, hard and soft cheese, meat starter cultures, and sausage[14,16,17]. Multidrug-resistant staphylococcal isolates were detected in the present study, consistent with other studies that have isolated multidrug-resistant *Staphylococcus* spp. from food[14,16,17]. Resistant bacteria isolated from food are an important problem, since food may act as a vehicle for the transfer of antibiotic resistant microorganisms to humans[1,2].

Prevalence and distribution of enterotoxin-encoding genes (*se*) in coagulase-negative and coagulase-positive staphylococci

In the present research, 40.2% of the coagulase-negative and coagulase-positive staphylococci isolates from black pudding were positive for one or more enterotoxin-genes. Coagulase-positive staphylococci are important with regard to food hygiene, because of their ability to express enterotoxins. Many studies are conducted to evaluate enterotoxin-encoding genes in CoPS isolated from food or raw materials[13]. The CoPS isolated from black pudding in the present study were positive for enterotoxin-encoding genes. The species *Staphylococcus schleiferi* has been described as enterotoxigenic in refrigerated raw milk, but enterotoxigenic *S. pseudintermedius* was not observed in food samples, and was only isolated from dogs[36,37]. Enterotoxin-encoding genes were not identified in 3 isolates of *S. aureus*. The high prevalence of CoNS (67.5%) that were positive for enterotoxin-encoding genes is an important result. The enterotoxigenicity of CoNS has been described by other authors[10,12,13], but few studies have been conducted to determine the presence of enterotoxigenic CoNS in food products. Until today, in Brazil, there have been few studies investigating the occurrence of enterotoxin-encoding genes in coagulase negative strains; this could be explained by the fact that the Brazilian legislation regarding food contamination does not require the determination of CoNS in animal products. In Minas Gerais, Brazil[13], evaluation of CoNS isolated from dairy products responsible for outbreaks of food poisoning revealed that some strains carry enterotoxin-encoding genes. The presence of staphylococcal enterotoxin *sea* and *sec* genes have also been observed[38] in samples of CoNS isolated from food. These data demonstrate the toxigenic potential of CoNS isolated from black pudding.

In the current study, the *sea* gene was the most prevalent, followed by the *seb*, *sec*, *see,* and *sed* genes. This result agrees with previous observations that the staphylococcal enterotoxins SEA, SEB, and SEC are the toxins frequently identified in foodborne outbreaks, and that the staphylococcal enterotoxins SED and SEE are less common[11]. The SEA and SEB toxins are known to occupy the same locus on the chromosome, which may explain why these enterotoxins are commonly found together in outbreaks of food poisoning[39].

Conclusions

The present study showed that staphylococci isolated from black pudding are predominantly CoNS, and that *S. saprophyticus* and *S. carnosus* were the most prevalent isolates. The detection of enterotoxin-encoding genes and resistance in staphylococcal isolates from black pudding indicated that this fermented food may represent a potential health risk, since staphylococci present in food could cause foodborne diseases or be a possible route for the transfer of antimicrobial resistance to humans. This is the first study that describes the detection of antibiotic-resistant staphylococci and the presence of enterotoxin-encoding genes in staphylococcal isolates from black pudding in southern Brazil.

ACKNOWLEDGMENTS

The authors would like to thank the laboratories of *Universidade Federal de Pelotas* (UFPEL), *Universidade Federal do Rio Grande do Sul* (UFRGS), and *Universidade Federal de Ciências da Saúde de Porto Alegre* (UFCSPA) for partnership and for providing infrastructure and resources.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This work was supported by *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq).

ABSTRACT IN PORTUGUESE

Prevalência de genes codificadores de enterotoxinas e resistência antimicrobiana em estafilococos coagulase-negativo e coagulase-positivo isolados de morcilhas no sul do Brasil

Introdução: Estafilococos são patógenos responsáveis por surtos de doenças transmitidas por alimentos. O estudo investigou a prevalência de genes de enterotoxinas e o perfil de resistência aos antimicrobianos em estafilococos coagulase-negativo (CoNS) e estafilococos coagulase-positivo (CoPS) isolados de morcilhas no sul do Brasil. **Métodos**: Duzentas colônias típicas e atípicas do ágar Baird-Parker foram inoculadas em ágar sal-manitol. Oitenta e dois estafilococos manitol-positivos foram submetidos a testes bioquímicos e perfil de susceptibilidade antimicrobiana. A presença dos genes da coagulase (*coa*) e enterotoxinas (se) foi investigada por reação em cadeia da polimerase (PCR). **Resultados**: Os isolados foram divididos em dois grupos: 75,6% (62/82) CoNS e 24,4% (20/82) CoPS. Através dos testes bioquímicos, 9 espécies foram determinadas, *Staphylococcus saprophyticus* (37,8%) e *Staphylococcus carnosus* (15,9%) foram as mais prevalentes. Testes de susceptibilidade demostraram fenótipos de resistência aos antibióticos administrados em humanos, como gentamicina, tetraciclina, cloranfenicol e eritromicina. O gene *coa* foi detectado em 19,5% (16/82) das cepas e quatro fragmentos de DNA polimórficos foram observados. Cinco CoNS contendo o gene *coa* foram submetidos ao sequenciamento do 16S rRNA e três mostraram similaridade com CoNS. Quarenta amostras foram positivas para pelo menos um gene *se*, os mais frequentes foram *sea* (28,6%) e *seb* (27,5%). **Conclusões**: A presença de resistência aos antimicrobianos e de genes *se* nos isolados de morcilha indicou que este alimento pode representar um risco potencial à saúde, já que a presença nos alimentos pode causar doenças de origem alimentar ou ser uma possível rota de transferência de estafilococos resistentes aos humanos.

Palavras-chaves: Enterotoxina estafilocócica. Coagulase. Resistência antimicrobiana.

REFERENCES

1. Muhammad G, Hoblet KH, Jackwood DJ, Bech-Nielsen S, Smith KL. Interspecific conjugal transfer of antibiotic resistance among staphylococci isolated from bovine mammary gland. Am J Vet Res 1993; 54:1432-1440.
2. Bannerman TL, Peacock SJ. *In*: Murray P, Baron E, Jorgensen JH, Landry ML, Pfaller M, editors. *Staphylococcus*, *Micrococcus* and other catalase-positive cocci. Manual of Clinical Microbiology. 9th ed. Washington (DC): American Society for Microbiology; 2007. p. 390-404.
3. Euzéby JPM. List of Prokaryotic names with Standing in Nomenclature (LPSN). [Internet] France [Accessed 2011 Mar 1]. Available from: http://www.bacterio.cict.fr/s/staphylococcus.html/.
4. Jay JM, Loessner MJ, Golden DA. Processed Meats and Seafoods. *In*: Heldman DR, editor. Modern Food Microbiology. 7th ed. Springer Science; 2005. p.101-124.
5. Ministério da Saúde. Análise Epidemiológica dos Surtos de Doenças Transmitidas por Alimentos no Brasil, 1999-2009. Brasília: Secretaria de Vigilância em Saúde. [Internet]. 2009 Sept 4 [Accessed on 2010 Nov 8]. Available from: http://portal.saude.gov.br/portal/arquivos/pdf/analise_ep_surtos_dta_brasil_2009.pdf/.
6. Brito DVD, Brito CS, Resende DS, do Ó JM, Abdallah VOS, Gontijo Filho PP. Nosocomial infections in a Brazilian neonatal intensive care unit: a 4-year surveillance study. Rev Soc Bras Med Trop 2010; 43:633-637.
7. Piette A, Verschraegen G. Role of coagulase-negative staphylococci in human disease. Vet Microbiol 2008; 134:45-54.
8. Macedo JLS, Rosa SC, Castro C. Sepsis in burned patients. Rev Soc Bras Med Trop 2003; 36:647-652.
9. Balaban N, Rasooly A. Staphylococcal enterotoxins. Int J Food Microbiol 2000; 61:1-10.
10. Blaiotta G, Pennacchia C, Villani F, Ricciardi A, Tofalo R, Parente E. Diversity and dynamics of communities of coagulase-negative staphylococci in traditional fermented sausages. J Appl Microbiol 2004; 7:271-284.
11. Jett M, Ionin B, Das R, Neill R. The staphylococcal enterotoxins. *In*: Sussman M, editor. Molecular medical microbiology. Salt Lake City (USA): Academic Press Salt Lake City; 2001. p. 1089-116.
12. Udo EE, Al-Bustan MA, Jacob LE. Enterotoxin production by coagulase-negative staphylococci in restaurant works from Kuwait city may be a potential cause of food poisoning. J Med Microbiol 1999; 48:819-823.
13. Veras JF, Santos DA, Carmo LS, Fernandes TMG, Azalim CC, Silva MCC, et al. Levantamento de surtos de toxinfecção envolvendo leite e produtos derivados no estado de Minas Gerais, Brasil. Hig Alim 2003; 17:218-222.
14. Perreten V, Giampa N, Schuler-Schmid U, Teuber M. Antibiotic resistance genes in coagulase-negative staphylococci isolated from food. Syst Appl Microbiol 1998; 21:113-120.
15. Pesavento G, Ducci B, Comodo N, Lo Nostro A. Antimicrobial resistance profile of *Staphylococcus aureus* isolated from raw meat: A research for methicillin resistant *Staphylococcus aureus* (MRSA). Food Control 2007; 18:196-200.
16. Resch M, Nagel V, Hertel C. Antibiotic resistance of coagulase-negative staphylococci associated with food and used in starter cultures. Int J Food Microbiol 2008; 127:99-104.
17. Pereira V, Lopes C, Castro A, Silva J, Gibbs P, Teixeira P. Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. Food Microbiol 2009; 26:278-282.
18. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. CLSI document M100-S15 (ISBN 1-56238-556-9). Wayne, PA: CLSI; 2005.
19. Sambrook, J, Russell DW. Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press; 2001.
20. Goh SH, Byrne SK, Zhang JL, Chow AW. Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphism. J Clin Microbiol 1992; 30:1642-1645.
21. Gontang EA, Fenical W, Jensen PR. Phylogenetic Diversity of Gram-Positive Bacteria Cultured from Marine Sediments. Appl Environ Microbiol 2007; 73:3272-3282.
22. Wegrzynowicz Z, Heczko PB, Jeljaszewicz J, Neugebauer M, Pulverer G. Pseudocoagulase activity of staphylococci. J Clin Microbiol 1979, 9:15-19.
23. Cremonesi P, Luzzana M, Brasca M, Morandi S, Lodi R, Vimercati C, et al. Development of a multiplex PCR assay for the identification of *Staphylococcus*

aureus enterotoxigenic strains isolated from milk and dairy products. Mol Cell Probes 2005; 19:299-305.

24. Engels W, Kamps M, Van Boven CPA. Influence of cultivation conditions on the production of staphylocoagulase by *Staphylococcus aureus*. J Gen Microbiol 1978; 109:237-243.
25. Cheung AL, Koomey JM, Butler CA, Projan SJ, Fischetti VA. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (sar) distinct from agr. Proc Natl Acad Sci U S A 1992; 89:6462-6466.
26. Moon JS, Lee AR, Kang HM, Lee ES, Joo YS, Park YH, et al. Antibiogram and Coagulase Diversity in Staphylococcal Enterotoxin Producing *Staphylococcus aureus* from Bovine Mastitis. J Dairy Sci 2007; 90:1716-1724.
27. Coton E, Desmonts MH, Leroy S, Coton M, Jamet E, Christieans S, et al. Biodiversity of Coagulase-Negative Staphylococci in French cheeses, dry fermented sausages, processing environments and clinical samples. Int J Food Microbiol 2010; 137:221-229.
28. Leroy S, Giammarinaro P, Chacornac JP, Lebert I, Talon R. Biodiversity of indigenous staphylococci of naturally fermented dry sausages and manufacturing environments of small-scale processing units. Food Microbiol 2010; 27:294-301.
29. Corbière Morot-Bizot S, Leroy S, Talon R. Monitoring of staphylococcal starters in two French processing plants manufacturing dry fermented sausages. J Appl Microbiol 2007; 102:238-244.
30. Irlinger F. Safety assessment of dairy microorganisms: coagulase-negative staphylococci. Int J Food Microbiol 2008; 126:302-310.
31. Corbière Morot-Bizot S, Leroy S, Talon R. Staphylococcal community of a small unit manufacturing traditional dry fermented sausages. Int J Food Microbiol 2006; 108:210-217.
32. Oteiza JM, Giannuzzi L, Califano A. Thermal inactivation of *Escherichia coli* O157:H7 and *Escherichia coli* isolated from black pudding as affected by composition of the product. Food Res Int 2003; 36:703-712.
33. Leão LSNO, Passos XS, Reis C, Valadão LMA, Silva MRR, Pimenta FC. Fenotipagem de bactérias isoladas em hemoculturas de pacientes críticos. Rev Soc Bras Med Trop 2007; 40:537-540.
34. Tavares W. Bactérias gram-positivas problemas: resistência do estafilococo, do enterococo e do pneumococo aos antimicrobianos. Rev Soc Bras Med Trop 2000; 33:281-301.
35. Phillips I, Casewell M, Cox T, Groot B, Friis C, Jones R, et al. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J Antimicrob Chemother 2004; 53:28-52.
36. Lamaita HC, Cerqueira MMOP, Carmo LS, Santos DA, Penna FAM, Souza MR. Contagem de *Staphylococcus* sp. e detecção de enterotoxinas estafilocócicas e toxina da síndrome do choque tóxico em amostras de leite cru refrigerado. Arq Bras Med Vet Zootec 2005; 57:702-709.
37. Yoon JW, Lee GJ, Lee SY, Yoo JH, Park HM. Prevalence of genes for enterotoxins, toxic shock syndrome toxin 1 and exfoliative toxin among clinical isolates of *Staphylococcus pseudintermedius* from canine origin. Vet Dermatol 2010; 21: 484-489.
38. Cunha MLRS, Peresi E, Calsolari RAO, Araújo Jr JP. Detection of Enterotoxins genes in coagulase-negative Staphylococci isolated from foods. Braz J Microbiol 2006; 37:70-74.
39. Shafer WM, Iandolo JJ. Chromosomal locus for staphylococcal enterotoxin B. Infect Immun 1978; 20:273-278.