FOODBORNE PATHOGENS AND DISEASE
Volume 10, Number 9, 2013

DOI: 10.1089/fpd.2013.1492

Coagulase-Positive Staphylococci Isolated from Chicken Meat: Pathogenic Potential and Vancomycin Resistance

Paula Dalcin Martins,[1] Taiana Trindade de Almeida,[1] Ana Paula Basso,[1] Tiane Martin de Moura,[1] Jeverson Frazzon,[1,2] Eduardo César Tondo,[1,2] and Ana Paula Guedes Frazzon[1,3]

Abstract

Coagulase-positive staphylococci (CPS) cause staphylococcal food poisoning. Recently, these bacteria have received increasing attention due to their potential role in the dissemination of antibiotic resistance markers. The present study aimed to evaluate coagulase-positive staphylococci counts, species distribution, enterotoxin genes prevalence, and the antibiotic resistance profile of CPS isolated from *in natura* chicken meat. Fifteen frozen and 15 chilled industrialized, uncooked chicken parts or entire carcasses were used. Staphylococcal counts revealed that frozen chicken meat samples displayed the lowest CPS count compared with chilled chicken meat samples ($p < 0.01$). *Staphylococcus aureus* (62%) was the most common species, followed by *S. intermedius*, *S. delphini*, and *S. schleiferi* subsp. *coagulans* (10% each) and *S. hyicus* (8%). The polymerase chain reaction identification of *sea, seb, sec, sed*, and *see* genes revealed that 70% of the isolates harbored at least one enterotoxin gene, with *sea* and *sed* being the most frequently encountered ones. Two of the 50 investigated strains harbored three different enterotoxin genes. A high frequency of isolates resistant to penicillin, teicoplanin, oxacillin, and clindamycin was observed, and 80% of CPS were found to be resistant to at least one of the 11 tested antimicrobials. Vancomycin-resistant *S. aureus* and *S. intermedius* showed minimum inhibitory concentrations of 512 and 64 μg/mL, respectively. These isolates might indicate the dissemination of vancomycin resistance in the community and imply food safety hazards.

Introduction

Coagulase-positive staphylococci (CPS) are a group of Gram-positive bacteria comprised of seven species (Euzéby, 2012), with *Staphylococcus aureus* being the most common species involved in illnesses. Staphylococcal food poisoning is a foodborne disease characterized by vomiting, abdominal pain, and diarrhea, and is engendered by the ingestion of staphylococcal enterotoxins (Holmberg and Blake, 1984). The Center for Disease Control and Prevention estimated that more than 185,000 cases of staphylococcal food poisoning occur in the United States per year (Mead *et al.*, 1999). Milk, dairy products, and meats are the foods that are most frequently associated with outbreaks (Asao *et al.*, 2003; Do Carmo *et al.*, 2004). Staphylococci in foods not only cause diseases, but also can serve as a reservoir of resistance genes, favoring the transference of such determinants from nonpathogenic to pathogenic and opportunist bacteria (Sorum and L'Abee-Lund, 2002; Gundogan *et al.*, 2005).

Staphylococci isolated worldwide have shown high rates of antibiotic resistance (Lowy, 2003), which is particularly alarming in relation to hospital-acquired infections. Methicillin-resistant *S. aureus* (MRSA) has received increasing attention because its treatment is more challenging and its frequency of isolation in hospitals is high (Tice and Rehm, 2010; Crivellaro *et al.*, 2011). In recent years, vancomycin-resistant *S. aureus* (VRSA) has been recovered from human infections (Sievert *et al.*, 2008); these isolates harbor the *vanA* gene, which is a genetic determinant from enterococci that provides vancomycin resistance. Since vancomycin is the drug of choice for the treatment of MRSA infections, the emergence of VRSA should be closely monitored in order to avoid the dissemination of these microorganisms (Foucault *et al.*, 2009).

The lack of data regarding the pathogenic potential of CPS species in uncooked poultry meat warrants more studies to establish whether or not safety hazards related to CPS exist in this type of food. The present study aims to evaluate CPS counts, species distribution, enterotoxin genes prevalence,

[1]Graduate Program in Environmental Microbiology, [2]Department of Food Science, and [3]Department of Microbiology, Food Science Institute, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

and the antibiotic resistance profile of CPS isolated from *in natura* chicken meat in southern Brazil.

Materials and Methods

Staphylococcal quantification and coagulase tests

From March to April 2011, 15 frozen and 15 chilled industrialized, uncooked chicken parts or entire carcasses were purchased in supermarkets in the cities of State of Rio Grande do Sul, in southern Brazil. Frozen chickens were maintained at −12°C while chilled chickens were kept at a temperature between −1 and 4°C. In both cases, the meat was within biological safety expiration dates. After being purchased, both types of chicken meat were immediately transported to the Microbiology Laboratory of Federal University of Rio Grande do Sul under refrigeration (<7°C) for analysis. Only one sample of meat was taken from each chicken part or carcass. Under aseptic conditions, each of the 30 samples was further processed into pieces. Those were combined so that a total of 25 g of each sample was used for the analysis. Baird Parker Agar (Himedia) was used for the detection and enumeration of staphylococci. Positive strains were confirmed by the coagulase test. All assays were performed according to the Bacteriological Analytical Manual of the U.S. Food and Drug Administration (Bennett and Lancette, 2001). Coagulase-positive colonies were stored in brain heart infusion (Himedia) broth 20% glycerol at −20°C. Statistical tests (*t*-test) were performed in order to evaluate chilled and frozen sample counts (Rosner, 2011).

Biochemical identification

Coagulase-positive staphylococci were characterized by Gram staining, catalase production, and salt mannitol agar (Himedia) growth. The trial assay included the following: the Voges-Proskauer test (VM VP; Himedia), the maltose and trehalose fermentation test, and the susceptibility to polymyxin B test (300 μg, NewProv) (MacFaddin, 2000). ATCC 25923 and ATCC 19095 were used as controls.

Antibiotic susceptibility test and minimum inhibitory concentrations

Antibiotic susceptibility was determined according to the recommendations of Clinical and Laboratory Standards Institute (CLSI, 2011) by the disc-diffusion method (Bauer *et al.*, 1966). Eleven antimicrobials (NewProv) that are commonly used in the treatment of clinical infections and in broiler production were tested: penicillin 10 U, gentamicin 10 μg, clindamycin 2 μg, ciprofloxacin 5 μg, trimethoprim–sulfamethoxazole 25 μg, cephalothin 30 μg, rifampin 5 μg, chloramphenicol 30 μg, oxacillin 1 μg, vancomycin 30 μg, and teicoplanin 30 μg. ATCC 25923 was used as a control, and all tests were repeated three times. The minimum inhibitory concentrations (MICs) were determined by the broth microdilution method, also in accordance with CLSI guidelines, in order to confirm vancomycin resistance. Vancomycin concentrations of 512 μg/mL and subsequent 1:1 serial dilutions were tested. These tests were also repeated three times.

PCR assays

DNA extractions were performed as previously described (Fredricks and Relman, 1998). Polymerase chain reaction (PCR) assays were carried out in order to detect the presence of the coagulase *coa* gene (Hookey *et al.*, 1998), staphylococcal enterotoxin (SE) *sea*, *seb*, *sec*, *sed*, and *see* genes (Moura *et al.*, 2012), vancomycin resistance *vanA* and *vanB* genes, (Depardieu *et al.*, 2004), *vanC1* (Dutkamalen *et al.*, 1995) and *vanC2/3* (Satake *et al.*, 1997), and the 16S-rDNA region of methicillin-resistant *S. aureus* (Forsman *et al.*, 1997). ATCC 13565, ATCC 23235, ATCC 14458, ATCC 19095, and ATCC 27664 were used as controls.

Results

Staphylococcal isolation and characterization in Southern Brazilian chicken meat samples

The presence of staphylococcal species was determined in 15 frozen and 15 chilled industrialized, uncooked chicken parts or entire carcasses, originated from the cities of Cruz Alta and Porto Alegre (State of Rio Grande do Sul, southern Brazil). Staphylococcal counts revealed that frozen chicken meat samples displayed the lowest counting mean, 348 colony-forming units (CFU)/g (standard deviation [SD] of 295 CFU/g), when compared with chilled chicken meat samples, which displayed a mean of 5315 CFU/g (SD of 5670 CFU/g) ($p < 0.01$). It was possible to recover a total of 50 CPS from 20 chicken meat samples. Only CPS were present in the remaining 10 chicken meat samples. A maximum of three different species was recovered per sample. *Staphylococcus aureus* was isolated from 13 of 20 samples. The *coa* gene was not identified by PCR in 2 of the 50 phenotypically CPS, while the other strains were positive for *coa* PCR amplification. The isolates were morphologically and biochemically characterized and classified as follows: 31 (62%) *S. aureus*, 5 (10%) *S. intermedius*, 5 (10%) *S. delphini*, 5 (10%) *S. schleiferi* subsp. *Coagulans,* and 4 (8%) *S. hyicus*. All species were homogeneously distributed chilled and frozen samples, except *S. hyicus*, which was encountered only in chilled chicken meats. Twenty-two CPS were isolated from frozen chicken meat samples, whereas only 28 CPS were isolated from chilled samples.

Widespread distribution of enterotoxin genes among staphylococcal isolates

Table 1 shows the distribution of enterotoxin genes among CPS species. Thirty-five (70%) of the 50 strains were positive for one or more enterotoxin-encoding genes. The *sea* gene was the most prevalent (64%), followed by *sed* (26%), *seb* (6%), and *sec* (4%); none of the isolates harbored *see*. While *sea* was homogeneously present among the five species, *seb* was encountered only in *S. hyicus* and *S. delphini*, *sec* only in *S. aureus,* and *S. intermedius* and *sed* in all staphylococcal species except *S. delphini*. Eleven strains (22%) exhibited two or more genes. One *S. aureus* was positive for *sea*, *sec*, and *sed* and one *S. hyicus* harbored *sea*, *seb*, and *sed* genes. No statistically significant difference in the prevalence of enterotoxin genes was encountered between frozen and chilled samples.

Antibiotic resistance

Antibiotic susceptibility of staphylococci strains is shown in Table 2. In the present study, a high prevalence of resistance to penicillin (72%), teicoplanin (30%), oxacillin (18%),

Table 1. Distribution of Staphylococcal Enterotoxin Genes Among Coagulase-Positive Staphylococci (CPS) Species Isolated from Chicken Meat Samples in South Brazil

	Number of isolates							
CPS species	a	c	d	ab	ad	abd	acd	*None*
Staphylococcus aureus	15	—	2	—	4	—	1	10
S. schleiferi subsp. *coagulans*	—	—	—	—	3	—	—	1
S. hyicus	—	—	—	—	1	1	—	2
S. delphini	1	—	—	2	—	—	—	2
S. intermedius	3	1	—	—	1	—	—	—

a, Staphylococcal enterotoxins A; b, staphylococcal enterotoxins B; c, staphylococcal enterotoxins C; d, staphylococcal enterotoxins D.

and clindamycin (16%) was observed. Twenty percent of the isolates were sensitive to all antibiotics, while 40% were resistant to one class of antibiotics, 28% to two classes, and 12% to three, four, or five classes. Staphylococci resistant to three or more classes of antibiotics were classified as multiresistant.

All *S. aureus* isolates were susceptible to gentamicin, and *S. intermedius* presented resistance to penicillin, gentamicin, clindamycin, rifampin, oxacillin, vancomycin, and teicoplanin. *S. delphini* and *S. scheiferi* subsp. *coagulans* strains were resistant to penicillin, oxacillin, and trimethoprim–sulfamethoxazole, while *S. hyicus* was resistant to penicillin and teicoplanin. Nine isolates were resistant to oxacillin: two *S. delphini*, two *S. scheiferi* subsp. *coagulans*, two *S. intermedius*, and three *S. aureus*. Only one isolate recovered from chilled chicken meat was identified by PCR as methicillin-resistant *S. aureus* (MRSA).

This study unexpectedly identified three vancomycin-resistant staphylococci (VRS) belonging to two species recovered from different chilled chicken meat samples. These VRS were submitted to broth microdilution, and two vancomycin-resistant *S. intermedius* (VRSI) isolates presented an MIC of 64 μg/mL, while the MRSA and VRSA isolate presented an MIC of 512 μg/mL. All three of the VRS carried *vanA*, *vanB*, and *vanC2/3* genes. No isolates were positive for the *vanC1*gene.

The VRSA isolate was also resistant to penicillin, oxacillin, clindamycin, rifampin, cephalothin, and teicoplanin and did not harbor any classical enterotoxin genes. Both VRSI harbored the *sea* gene and presented resistance to penicillin, gentamicin, clindamycin, oxacillin, and teicoplanin, differing only in rifampin resistance.

Discussion

Staphylococcal isolation, quantification, and biochemical characterization

To evaluate CPS counting, we performed isolation from frozen and chilled samples. As expected, the difference in counting means between frozen and chilled samples was observed, since lower temperatures more efficiently inhibit bacterial growth (Al-Jasser, 2012). These counts show that the presence of staphylococci in chicken meat is significant.

S. aureus was the most abundant species in both frozen and chilled chicken meat samples, corroborating previous results described by other authors (Aarestrup *et al.*, 2000; Citak and Duman, 2011). The presence of *S. aureus* in poultry meat might indicate contamination by handlers or even failures during meat processing. Studies have found that *S. aureus* isolated from food-poisoning sources, healthy nasal carriers, and infected samples shared the same staphylococcal protein A type (Wattinger *et al.*, 2012), which may represent a major focus of contamination, since *S. aureus* was found to colonize the nasal mucosa of 30% of the food handlers investigated (Acco *et al.*, 2003). In Turkey, Altay *et al.* (2003) reported the presence of CPS species in chicken meat; of the 46 CPS isolated, 60.9% were *S. aureus*, 19.6% were *S. delphini*, 6.5% were *S. intermedius*, 4.3% were *S. aureus* subsp. *anaerobius*, 2.2% were *S. schleiferi* subsp. *coagulans*, 2.2% were *S. hyicus*, and 4.3% remained unidentified.

Table 2. Resistance Profile of Coagulase-Positive Staphylococci (CPS) Species Isolated from Chicken Meat Samples in South Brazil

	Antibiotic and number of CPS resistant											
CPS species	*PEN*	*GEN*	*CLI*	*CIP*	*SUT*	*CFL*	*RIF*	*CLO*	*OXA*	*VAN*	*TEC*	*None*
Staphylococcus aureus ($n=31$)	27	—	5	1	1	1	2	1	3	1	10	3
S. delphini ($n=5$)	2	—	—	—	1	—	—	—	2	—	—	3
S. schleiferi subsp. *coagulans* ($n=5$)	2	—	—	—	2	—	—	—	2	—	—	2
S. intermedius ($n=5$)	2	2	3	—	—	—	1	—	2	2	2	2
S. hyicus ($n=4$)	3	—	—	—	—	—	—	—	—	—	3	—
Total	36	2	8	1	4	1	3	1	9	3	15	10
(%)	(72)	(4)	(16)	(2)	(8)	(2)	(6)	(2)	(18)	(6)	(30)	(20)

PEN, penicillin 10 UI; GEN, gentamicin 10 μg; CLI, clindamycin 2 μg; CIP, ciprofloxacin 5 μg; SUT, trimethoprim–sulfamethoxazole 25 μg; CFL, cephalothin 30 μg; RIF, rifampin 5 μg; CLO, chloramphenicol 30 μg; OXA, oxacillin 1 μg; VAN, vancomycin 30 μg; TEC, teicoplanin 30 μg.

Another important set of data described in the present study demonstrated that the coagulase-enzyme phenotype was not correlated with the *coa* gene, since two isolates with coagulase activity did not carry the gene. The absence of amplicons in these two isolates might have been engendered either by mutations in the primer annealing region or by the presence of pseudocoagulase genes (Chomarat and Flandrois, 1984; Bulanda *et al*., 1988). Moura *et al.* (2012) also found staphylococci that were positive to the coagulase test but were negative to identification of the *coa* gene.

SE prevalence

The enterotoxigenicity of *S. aureus* isolated from diverse foods has been described by several authors. However, few studies have reported the presence of enterotoxigenic non-*S. aureus* CPS recovered from uncooked chicken meat. Kitai *et al.* (2005) analyzed enterotoxigenic *S. aureus* in retail uncooked chicken meat in Japan. Among them, *seb* was the most prevalent gene, followed by *sea* and *sec*. In the present study, the most prevalent genes were *sea* and *sed*. This fact is remarkable because, among the described SEs and SE-like proteins, SEA and SED are the enterotoxins that are most typically involved in foodborne outbreaks (Casman, 1965; Genigeorgis, 1989). Moreover, in our analysis, 70% of the isolates harbored at least one enterotoxin gene. Similarly, other studies also found a high prevalence of enterotoxin genes among *S. aureus* isolates, such as 60.1% (Guven *et al*., 2010), 67% (Morandi *et al*., 2007), 68.4% (Rall *et al*., 2008) and 69% (Pereira *et al*., 2009); *sea* was the most prevalent gene in the latter two studies.

Regarding non-*S. aureus* CPS, *S. intermedius* was responsible for an outbreak of food poisoning in the United States (Khambaty *et al.*, 1994), and *S. hyicus* was capable of inducing emetic activity in monkeys (Adesiyun *et al.*, 1984). *S. delphini* was the etiological agent of a diarrhea outbreak on a commercial mink farm in the United States, causing the death of 2000 animals (Sledge *et al.*, 2010). However, one study reported that *S. schleiferi* subsp. *coagulans* isolated from bovine and ovine mastitis was not enterotoxigenic (Almeida, 2009).

Antibiotic resistance

Antibiotic resistance rates were high, especially in relation to penicillin, teicoplanin, oxacillin, and clindamycin. Similar results were found by other authors (Guven *et al.*, 2010; Pu *et al.*, 2011). On the other hand, only one MRSA was recovered. The isolation of MRSA from raw meats was rare in both the present study and in others (Lee *et al.*, 2008; Pereira *et al.*, 2009). The spread of antibiotic resistance among broilers might occur due to the use of antimicrobials for therapeutics and growth promotion in chicken breeding (Butaye *et al.*, 2003).

In foods, resistant bacteria have very often been encountered (Valsangiacomo *et al.*, 2000), and antibiotic-resistant specimens in foods are a safety hazard for many reasons. First, resistant bacteria in foods can cause diseases. For example, in a community-acquired MRSA case, a hospital patient was infected by ingesting a banana containing nasal carriage of MRSA. It was later discovered that the person who prepared the hospital meals was an asymptomatic carrier of MRSA (Kluytmans *et al.*, 1995). Another case involved an outbreak caused by the ingestion of baked pork meat contaminated with MRSA by the food handler (Jones *et al.*, 2002). Second, foods may be a reservoir of resistant bacteria. Gundogan *et al.* (2005) analyzed raw veal and lamb samples as well as uncooked chicken parts. Of 80 *S. aureus* isolates, 67.5% were resistant to methicillin, 53.8% to penicillin, and 87.5% to bacitracin. Finally, nonpathogenic bacteria can transfer resistance genes to pathogenic and opportunistic bacteria (Sorum and L'Abee-Lund, 2002). Nawaz *et al.* (2000) suggested that the transfer of genetic determinants from poultry staphylococci to human microbiota is possible.

To our knowledge, this is the first report on VRSA isolated from foods. In addition, this is also the first case of VRSI reported in this species. The findings of VRSA and VRSI in chicken meats are a motive of concern, as they may suggest the spread of such microorganisms or their genetic material outside clinical boundaries. The spread of staphylococcal antimicrobial resistance in foods constitutes a potential risk for the community. Furthermore, the possible correlation between strains present in hospitals and those encountered in foods must be considered. MRSA represents a challenge for the treatment of frequent *S. aureus* infections acquired in hospitals. In particular, vancomycin resistance stands out rather prominently in this scenario (Boucher *et al.*, 2010).

Conclusions

Coagulase-positive staphylococci isolated from uncooked chicken meat might represent a food safety hazard. The finding of VRSA and VRSI harboring the *vanA*, *vanB*, and *vanC* genes and presenting MICs of 512 and 64 μg/mL, respectively, is particularly alarming, given the difficulties in the treatment of vancomycin-resistant staphylococci and the potential spread of these genetic determinants. However, not only should *S. aureus* species receive attention from public health authorities, but other CPS species should also be monitored. In the present study, non–*S. aureus* species demonstrated remarkable enterotoxigenicity and antibiotic resistance. Many studies do not properly distinguish *S. aureus* from other CPS species because their protocol for *S. aureus* identification is based on tests for which all CPS are positive. Therefore, we ask whether security issues regarding other CPS species have been underestimated due to the mistaken attribution of enterotoxin genes, antibiotic resistance, and other virulence factors to *S. aureus* instead of CPS. More studies are necessary to determine the risks that CPS species imply for food safety. Currently, Brazil and the United States are world leaders in chicken meat exportation (Brazil provides chicken meat for over 180 countries). In addition, most of the slaughterhouses in Brazil are located in the southern region. Therefore, these data must be considered.

Acknowledgments

We would like to thank the National Council of Technological and Scientific Development of Brazil (CNPq) for sponsoring this research (JF grant #300912/2012-9).

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Jeverson Frazzon, PhD
Graduate Program in Environmental Microbiology
Food Science Institute
Federal University of Rio Grande do Sul
Av. Bento Gonçalves 9500
Building 43212/Room 208
Porto Alegre, RS, CEP 91501-970, Brasil

E-mail: jeverson.frazzon@ufrgs.br