



Evaluation of Selected Toxigenic Genes and Antimicrobial Agent Susceptibility in *Staphylococcus* Spp Isolated from Foods Purchased from North Dakota Grocery Stores

Aye R^{1*}, Gautam A³, Reyaz A¹, Vinson H¹, Gibbs PS^{1,2} and Barigye R^{1,2}

Abstract

The present study was done to assess the incidence of five superantigenic toxin genes and toxin expression; and the antimicrobial susceptibility in food-derived coagulase-negative-*Staphylococcus* (CNS). Sixty-two (16.1%) *Staphylococcus* isolates from 385 food samples were tested by polymerase chain reaction (PCR) for five superantigenic toxin genes including four classical enterotoxins (*sea*, *seb*, *sec*, *sed*, and *see*), and toxic shock syndrome toxin-1 (*tsst-1*). The PCR-positive isolates were further tested by immunoblotting for toxin expression and susceptibility patterns to antimicrobial agents assessed for all isolates. Of all study isolates, 49/62 (79.0%) were CNS and 13/62 (21.0%) were coagulase-positive *Staphylococcus* spp (CPS). A *S. lugdunensis* isolated from strawberries (1.4% of CNS) was positive for the *sec* gene but negative for SEC toxin. Two *S. aureus* isolates from steak (15.4% of CPS) were positive for both the *sec* gene and SEC toxin. Overall, CNS isolates were generally susceptible to β -lactam antimicrobial agents and 80% of the CPS isolates were resistant to penicillin and amoxicillin. This data demonstrates a low incidence of toxigenic genes among food-derived CNS hence the potential role CNS may play in the epidemiology of foodborne illnesses is minimal. The data further suggests that other mechanisms of drug resistance are likely to be more frequent among food-borne staphylococci.

Keywords

Coagulase-negative *Staphylococcus*; Enterotoxins; Toxic shock syndrome toxin

Abbreviations: CNS: Coagulase Negative Staphylococcus; CPS: Coagulase Positive Staphylococcus; SE: Staphylococcal Enterotoxins; SFP: Staphylococcal Food Poisoning; TSST-1: Toxic Shock Syndrome Toxin-1

Introduction

Staphylococcus spp are broadly divided into two groups, coagulase-positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS) sub-groups. *Staphylococcus aureus*, the prototype of the CPS sub-group, is responsible for a number of human diseases including but not limited to food poisoning, skin infections, toxic shock syndrome and sepsis [1,2]. Staphylococcal food poisoning (SFP), which is widely attributed to toxigenic *S. aureus*, occurs following ingestion of at least 1.0 μ g of preformed enterotoxin in food [3]. Humans and animals serve as primary reservoirs of this agent and *Staphylococcus* spp are widely found as normal flora in more than 50% of healthy individuals [3]. The FDA report cites food handlers as the main source of food contamination in majority of the SFP outbreaks. To a lesser extent, however, equipment and environmental surfaces can also be sources of contamination [3]. Another important public health problem associated with *S. aureus* is methicillin resistance [4,5]. While majority of the methicillin resistant *S. aureus* (MRSA) cases have been associated with nosocomial and community acquired infections [6], current evidence suggests that food may be an important transmission vehicle [7-9]. Recently, a *Staphylococcus* strain associated with a food poisoning outbreak was found to be resistant to methicillin [10].

Whereas the pathogenicity of CPS and *S. aureus* in particular, is well documented, most CNS are believed to be non-pathogenic. However, a number of studies have provided evidence that suggests that some CNS strains may be important nosocomial pathogens [11,12]. In fact, a number of such strains have been recognized for ability to elaborate superantigenic toxins such as staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), as well as other toxins like hemolysins, coagulase [13-15], and exfoliative toxins [16]. Quite interestingly, a number of workers have recently reported enterotoxin and other toxigenic genes in CNS cultured from foods [6,17] underscoring the potential role CNS might play in the epidemiology of foodborne illnesses [18]. Although mainly linked to *S. aureus*, few reports have documented CNS-associated methicillin resistance [18]. The objectives of this research therefore were to elucidate the incidence of selected superantigenic toxin genes and the corresponding toxins in food-derived staphylococci. In addition, the investigation also assessed the antimicrobial susceptibility of food-derived staphylococci.

Materials and Methods

Staphylococcus isolates and positive controls

Study bacterial isolates were cultured from 385 food samples (Table 1) procured from two grocery stores in Fargo, North Dakota (ND), USA during the months of July and August 2009. In the laboratory, the food samples were processed within 12 h of purchase. To isolate *Staphylococcus* spp from food, Giolitti-Cantoni broth (BD Diagnostics, Franklin Lakes, NJ, USA), Baird parker agar (BD Diagnostics), and blood agar plates (TSA with 5% sheep blood) (BD Diagnostics) were used. Food samples were washed with 250 ml sterile phosphate buffered saline, pH 7.4 (PBS) for 10 min and 25 ml of the PBS washings added to 10 ml of Giolitti - Cantoni broth, an enrichment media for the isolation of *Staphylococcus* spp from food. The tubes were incubated at 37°C for 18-24 hours with shaking

*Corresponding author: Aye R, Department of Veterinary and Microbiological Sciences, North Dakota State University, 1523 Centennial Blvd, Fargo, ND 58108, USA, Tel: +254 753 57715; Fax: +251-11 646 1252; E-mail: racheal.aye@gmail.com

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at 200 × rpm. Ten 10 µl of the enriched cultures were streaked on Baird Parker Agar; a *Staphylococcus* selective medium. The plates were incubated at 37°C for 18-24 h after which single colonies were streaked onto blood agar plates and further incubated at 37 °C for 12-18 h. Suspect *Staphylococcus* colonies were identified using the RapID™ STAPH PLUS System (Thermo Fischer Scientific, Lenexa, KS, USA) according to the manufacturer's protocol. For the positive controls, the following *S. aureus* American Type Culture Collection (ATCC) strains (ATCC, Manassas, VA, USA) were used namely: ATCC #13565 (*sea* gene); #14458 (*seb* gene); #19095, (*sec* gene); #23235, (*sed* gene); #27664, (*see* gene); and #51650, (*tsst-1* gene).

DNA extraction

A single colony of each isolate was added to 40 µl of TE buffer with 1% 20 mg/ml proteinase K. Samples were incubated at 55°C for 10 min followed by 10 min incubation at 80°C. DNA was then diluted with 80 µl of sterile water and stored at -20°C for future PCR analysis.

Polymerase chain reaction (PCR)

Oligonucleotide primers were synthesized at Trilink Biologicals Inc. (San Diego, CA, USA) based on nucleotide sequences of the five classical enterotoxin genes (*sea-see*), and toxic shock syndrome toxin (*tsst-1*) genes initially reported by Johnson, et al. [19]. Twenty five µl PCR reactions were set up, each containing 5 µl of 5× buffer, 0.2 µl dNTP, 0.25 µl each forward and reverse primers, 0.125 µl DNA polymerase, and 2 µl DNA. Each PCR reaction was run twice on the DNA Engine thermocycler (Bio-Rad, Hercules, CA, USA) with the following conditions: 94°C for 5 min; 30 cycles of 94°C for 2 min, 55°C for 1 min, and 72°C for 1 min; 72°C for 7 min and 20°C. PCR products were visualized in 1.5% agarose gels stained with ethidium bromide.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

Staphylococcus protein was extracted using SoluLyse™ Bacterial Protein Extraction Reagent (Genlantis, San Diego, CA, USA), followed by determination of protein concentration using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, DE, USA). Twenty µg of extracted *Staphylococcus* protein from each of the isolates were diluted 1:2 in sample buffer (625 mM Tris-HCl, pH 6.8; 10% glycerine; 5% SDS; 0.002% Bromophenol blue) plus 10% β-Mercaptoethanol . The samples were boiled for 4 min at 95°C, cooled and then loaded onto a 12% Tris-HCl precast polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Five µl of Precision Plus Protein Standards,

dual colored molecular weight marker (Bio-Rad Laboratories, Inc., Hercules, CA) were also loaded in a separate lane. SDS-PAGE was carried out in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 150 V (constant voltage) for 2 h and the separated proteins were visualized by staining the gels with Silver Stain Plus (Bio-Rad Laboratories) as described in the manufacturer's protocol.

Immunoblotting

All the isolates that tested positive for toxigenic genes were further tested by western immunoblotting. Following SDS-PAGE, the *Staphylococcus* proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using a wet transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA) using transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 100 V for 1 h at room temperature (rT). The PVDF membrane was incubated overnight at 4 °C in a blocking solution (5% nonfat dry milk, 10% TBS with 0.2 M Tris, 1.37 M NaCl pH 7.6) with gentle agitation. The following day, the membrane was washed in 10% TBS (0.2 M Tris, 1.37 M NaCl, pH 7.6) plus 0.2% Tween 20 (TBS-Tween), and then reacted with diluted primary antibody (anti-*Staphylococcus* enterotoxin mouse *mAb* diluted 1:80,000; anti-*Staphylococcus* TSST-1 mouse *mAb* diluted 1:1600). The membrane was then washed with TBS-Tween and reacted with diluted secondary antibody (goat anti-mouse IgG ALP 1; 10,000 [Abcam, Inc., Cambridge, MA]) at rT with gentle agitation. After incubation with secondary antibody, the membrane was developed with premixed BCIP/NBT substrate solution (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Susceptibility patterns of the 62 *Staphylococcus* isolates were assessed against 21 antimicrobial agents. The antimicrobial panel contained Amikacin, Amoxicillin/Clavulanic acid, Ampicillin, Cefazolin, Cefoxitin, Cefpodoxime, Ceftiofur, Chloramphenicol, Clindamycin, Enrofloxacin, Erythromycin, Gentamycin, Imipenem, Marbofloxacin, Orbifloxacin, Oxacillin + 2% NaCl, Penicillin, Rifampin, Ticarcillin, Ticarcillin/Clavulanic acid, and Trimethoprim/Sulphamethoxazole (COMEQ3F, Trek Diagnostic Systems Inc, Cleveland, OH, USA) . The antimicrobial susceptibility testing was done using the Sensititre system (Trek Diagnostic Systems Inc, Cleveland, OH, USA) according to the manufacturer's protocol.

Results

Staphylococcus spp was cultured from 62 (18.0%) of the 385 foods tested including foods of plant, fungal and animal origin (Table 1).

Table 1: The different *Staphylococcus* spp cultured from foods of plant and animal origin (n=385).

Food type	<i>Staphylococcus</i> negative samples	<i>Staphylococcus</i> positive samples	<i>Staphylococcus</i> isolates
Cheese	25 (89.3%)	3 (10.7%)	<i>S. saprophyticus</i> (3)
Chicken	18 (62.1%)	11 (37.9%)	* <i>S. aureus</i> (4); <i>S. hominis</i> (3); <i>S. xylosus</i> (1); <i>S. hyicus</i> (3).
Beef	18 (62.1%)	11 (37.9%)	* <i>S. aureus</i> (4); <i>S. warneri</i> (3); <i>S. ureolyticus</i> (1); <i>S. epidermidis</i> (3)
Pork	16 (59.3%)	11 (40.7%)	* <i>S. aureus</i> (3); <i>S. warneri</i> (4); <i>S. mitis</i> (2); <i>S. hominis</i> (1); <i>S. scheleferi</i> (1)
Sausage	27 (90%)	3 (10%)	<i>S. warneri</i> (1); <i>S. hyicus</i> (1); <i>S. saprophyticus</i> (1)
Salad	32 (94.1%)	2 (5.9%)	<i>S. heamoliticus</i> (1); <i>S. hyicus</i> (1)
Grapes	56 (93.3%)	4 (6.6%)	* <i>S. aureus</i> (2); <i>S. warneri</i> (1); <i>S. xylosus</i> (1)
Carrots	27 (96.4%)	1 (3.6%)	<i>S. hyicus</i> (1)
Mushrooms	16 (55.2%)	13 (44.8%)	<i>S. epidermidis</i> (1); <i>S. hyicus</i> (2); <i>S. sciuri</i> (5); <i>S. xylosus</i> (5)
Strawberries	27 (93.1%)	2 (6.9%)	<i>S. lugdunensis</i> (1); <i>S. saprophyticus</i> (1)
Salsa	61 (98.4%)	1 (1.6%)	<i>S. warneri</i> (1)

*Coagulase-positive *Staphylococcus*.

Table 2: Antimicrobial agent susceptibility patterns of the *Staphylococcus* spp to β -lactam antibacterial agents (n=62).

Drug	CNS (n= 49)				CPS (n= 13)			
	R (%)	S (%)	N (%)	I (%)	R (%)	S (%)	N (%)	I (%)
Amoxicillin/ Clavulanic acid	36	64	0	0	50	50	0	0
Ampicillin	35	11	54	0	70	10	20	0
Cefazolin	36	64	0	0	50	50	0	0
Cefoxitin	29	71	0	0	50	50	0	0
Cefpodoxime	35	65	0	0	50	50	0	0
Imipenem	33	67	0	10	40	45	0	5
Oxacillin + 2% Nacl	33	77	0	0	47	53	0	0
Penicillin	42	58	0	0	80	5	15	0
Ticarcillin	39	61	0	0	50	30	20	0
Ticarcillin/ Clavulanic acid	39	61	0	0	50	45	5	0

R= Resistant; S= Sensitive; N= No interpretation possible; I= Inhibitory.

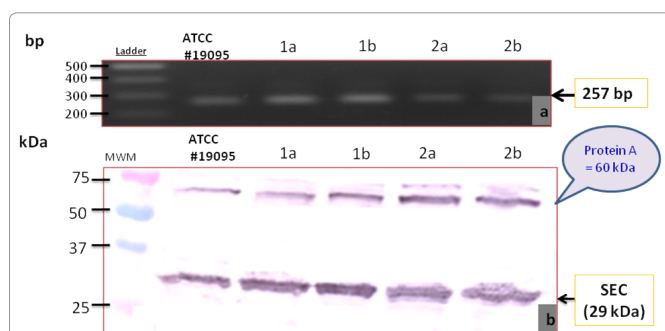


Figure 1: Presence of *sec*-gene and SEC toxin in two isolates of *S. aureus* cultured from steak. Fig 1a shows expression of the *sec* gene with *S. aureus* ATCC #19095 as a positive control and duplicate samples of isolates 1 and 2. 1a shows that both isolates expressed the corresponding SEC toxin when probed with anti-enterotoxin SEC monoclonal antibody on western blot. Size standards were in Fig 1a Promega DNA ladders #G210A and in 1b Bio-rad # 161-0374 (Precision plus protein standard dual color).

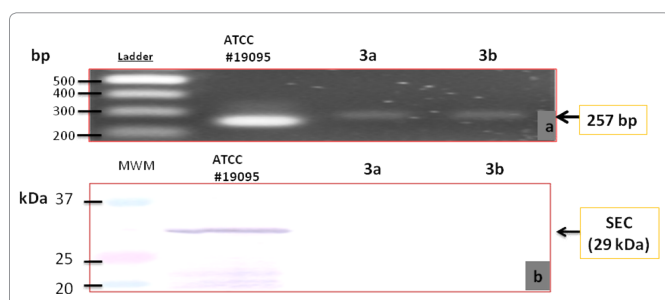


Figure 2: Presence of *sec*-gene but absence of the corresponding SEC-toxin in *S. lugdunensis* cultured from strawberries. 2a shows the expression of *sec*-gene with *S. aureus* ATCC #19095 as the positive control and duplicates of isolate 3. 2b shows absence of the corresponding SEC toxin when probed with anti-enterotoxin SEC monoclonal antibody on western blot. Size standards were in Fig 1a Promega DNA ladders #G210A and in fig 1b Bio-rad # 161-0374 (Precision plus protein standard dual color).

Of the 143 animal-derived food samples tested, 27.3% (39/143) were positive for *Staphylococcus* isolates compared to only 9.5% (23/242) of the plant/fungal foods (Table 1). Of the 62 *Staphylococcus* cultured from the food samples, 13 isolates were CPS and 49 were CNS. Of the plant/fungal-derived food tested, mushrooms (mushrooms are fungi but were grouped under plant-derived foods for convenience) yielded the highest number [21.0% (13/62)] of all *Staphylococcus* isolates.

Of the 13 CPS isolates, two *S. aureus* isolates cultured from steak

were positive for both the *sec* gene (257 bp) (Figure 1a) and SEC toxin (29 kDa) (Figure 1b). In addition to the SEC protein, the 2 *S. aureus* isolates also expressed the non-specific protein A (Figure 2b). Of the 49 CNS, one *S. lugdunensis* isolate cultured from strawberries tested positive for the *sec* gene (257 bp) by PCR (Figure 2a) but was negative for the SEC toxin (29 kDa) by immunoblotting (Figure 2b). All the other study *Staphylococcus* isolates tested negative for the classical enterotoxins and *tsst-1* genes.

The CNS isolates were generally susceptible to most of the antimicrobial agents tested while up to 80% of the CPS isolates were resistant to the older β -lactam antibacterial agents (Table 2). However, when compared to newer β -lactam and non β -lactam antibacterial agents, the CPS isolates were generally susceptible.

Discussion

Of the 143 animal-derived food samples tested, 27.3% (39/143) were *Staphylococcus*-positive compared to only 9.5% (23/242) of the plant/fungal foods (Table 1). As would have been expected, this data indicates that animal-derived foods have a higher *Staphylococcus* contamination rate compared to those of plant origin. It is widely documented in the literature that animals and humans are the main reservoirs of *Staphylococcus* with $\geq 50\%$ of healthy individuals being colonized by the organism [3]. Supported by literature, therefore, the present data imply that foods of animal origin might be contaminated by the animal itself. It is also widely believed that food handlers are a major source of contamination of table foods [3] including those that were tested during this study. However, testing of humans that came into contact with the study foods was not an objective of this study and therefore was never attempted.

Among the foods of plant and fungal origin, mushrooms yielded the highest number of *Staphylococcus* isolates. Previously, other researchers have also reported a surprisingly high incidence of *Staphylococcus* spp in mushrooms [20,21]. Based on the reports by these researchers, the finding in the present study would appear not to be a random event but rather underscores the high *Staphylococcus* contamination rate of mushrooms. Consumers need to be aware of this as it could have food safety implications. Interestingly, none of the previous studies have given a satisfactory explanation for a high *Staphylococcus* recovery rate from mushrooms. Subject to further investigations, the anatomy, and probably other biological features unique to fungal foods and mushrooms in particular, may facilitate growth and survival of this agent.

Of the various CNS isolates tested, an isolate of *S. lugdunensis* cultured from strawberries was positive for the *sec* gene but not the

corresponding SEC toxin. The pathogenicity of CNS has been reported by a number of researchers [18,22,23] but also questioned by others [6,24]. Given the fact that the conditions under which *Staphylococcus* spp produce toxins are variable [25,26], the potential of such an isolate to cause food poisoning cannot be disputed since it carried the *sec* gene. To definitively determine toxin producing ability, the isolate should be experimentally subjected to variable growth conditions that favor SE production including but not limited to exposure to various amino acids [27], and neutral pH [28]. Likewise, the production of SE is inhibited by glucose [29], low pH [23,28] and salt concentrations above 12% [26]. A number of researchers have previously reported enterotoxigenic CNS [30-32] even when some literature indicates that *S. intermedius* is the only CNS species that has been definitively linked to SFP outbreaks [23]. A number of workers have also reported that CNS may produce *tsst-1* alone or in combination with other toxins [33]. Based on data obtained in the present study, none of the CNS tested positive to *tsst-1*. A study by Valle, et al. [24] reported up to 16% of CNS that were positive for the *tsst-1* gene.

During the study, all *Staphylococcus* isolates were screened for selected toxigenic genes of food safety importance and the PCR results were further corroborated by immunoblotting analysis. As presented above, two isolates of *S. aureus* (15.4% of the CPS) cultured from steak were positive for both the *sec* gene (257 bp) and SEC toxin (25 kDa) but not any other classical enterotoxins. Staphylococcal food poisoning in humans normally follows ingestion of food containing about 100,000 bacterial cells/g of food [3]. Since enterotoxins are heat stable, it is plausible to conclude that the two *sec*/*SEC*-positive-*S. aureus* isolates from the steak could have potentially caused SFP in the consumer. In 2005, an incident was reported in Kansas in which SFP occurred in persons that ate contaminated ham [34]. That report provides evidence that meat products can be a source of clinically important food poisoning caused by toxigenic *Staphylococcus*. Per the immunoblotting data obtained during this study, all the *S. aureus* isolates expressed a 60 kDa protein which was on the basis of electrophoretic mobility and molecular weight consistent with protein A. This protein which was variably expressed by the study *Staphylococcus* isolates (Figure 1). Protein A binds the Fc portion of the immunoglobulin G (IgG) molecule and the resulting steric interference disrupts the processes of opsonisation and phagocytosis both crucial events in innate and adaptive immunity. This phenomenon may increase the virulence of isolates that express the protein by acting as an immunologic disguise and preventing phagocytosis [35].

Similar to data reported by the CDC, 80% of the CPS [36] and less than 50% of the CNS isolates, in the present study, were resistant to penicillin. When penicillin was introduced in 1944, 94% of *Staphylococcus* spp were susceptible to the drug [35]. At present, a number of studies have shown that 80-90% of staphylococci are resistant to penicillin [36,37].

In summary 1/49 (2.0%) CNS isolates were positive for toxigenic genes providing evidence that CNS of food origin may potentially play a role in the epidemiology of foodborne illnesses. Two *S. aureus* isolates from steak (15.4% of the CPS) were positive for both the *sec* gene and SEC toxin corroborating other studies that have implicated CPS in food poisoning. The study CNS isolates were generally susceptible to β -lactam antimicrobial agents, and majority of the CPS were resistant to penicillin and amoxicillin. The toxin-gene-positive but toxin/protein-negative *S. lugdunensis* from strawberries should be further investigated for ability to secrete the corresponding enterotoxins.

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Author Affiliation

Top

¹Department of Veterinary and Microbiological Sciences, North Dakota State University, USA

²Department of Veterinary Diagnostic Services, North Dakota State University, USA

³Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, USA

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