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Staphylococci isolated from ready-to-eat meat – Identification, antibiotic resistance and toxin gene profile

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ABSTRACT

The aim of this study was to analyse the staphylococci isolated from ready-to-eat meat products, including pork ham, chicken cold cuts, pork sausage, salami and pork luncheon meat, sliced in the store to the consumer's specifications, along with species identification and determination of antibiotic resistance. Genes encoding staphylococcal enterotoxins, staphylococcal enterotoxin-like proteins, exfoliative toxins, and toxic shock syndrome toxin 1 were also investigated. From the 41 samples, 75 different staphylococcal isolates were obtained. Based on PCR-RFLP analysis of the *gap* gene using *Alu*I and *Hpy*CH4V restriction enzymes, the isolates were identified as *Staphylococcus equorum* (28%), *S. vitulinus* (16%), *S. carnosus* (14%), *S. succinus* (11%), *S. xylosus* (11%), *S. saprophyticus* (9%), *S. warneri* (9%), *S. haemolyticus* (1%) and *S. pasteuri* (1%). The incidence and number of resistances to antimicrobials was found to be species but not source of isolation dependent. All *S. xylosus*, *S. saprophyticus*, *S. haemolyticus* and *S. pasteuri* isolates showed antibiotic resistance. A lower percentage of resistance was recorded for *S. warneri* (71%) and *S. vitulinus* (58%), followed by *S. equorum* (57%), *S. carnosus* (50%) and *S. succinus* (50%). The most frequent resistance was observed to fusidic acid (43%). The *mec*A gene was amplified in 4% of the staphylococci. However, phenotypic resistance to methicillin was not confirmed in any of these isolates. On the other hand, the *mec*A gene was not detected in any of 9% of the isolates resistant to cefoxitin. It was also found that among 75 isolates, 60 (80%) harbored from 1 to 10 out of 21 analyzed superantigenic toxin genes. The most prevalent genes were: *sei* (36% isolates) among enterotoxins, *seln* (32% isolates) among enterotoxin-like proteins and *eta* encoding exfoliative toxin A (37% isolates). The findings of this study further extend previous observations that, when present in food, not only *S. aureus* but also other species of staphylococci could be of public health significance.

1. Introduction

The genus *Staphylococcus* is divided into 52 species and 28 subspecies that are grouped into coagulase-positive (CPS) and coagulase-negative staphylococci (CNS) (http://www.bacterio.net). Most staphylococcal species are harmless and reside normally on the skin and mucous membranes of humans and other organisms (Chajęcka-Wierzchowska et al., 2014; Dubois et al., 2010; Guran and Kahya, 2015; Podkowik et al., 2012, 2016). There are also well known staphylococcal species responsible for a wide variety of diseases of animals and humans (van Duijkeren et al., 2008).

The pathogenic capacity of staphylococci is attributed to a combination of invasive properties, production of extracellular factors and antibiotic resistance. Staphylococcal toxins include toxic shock syndrome toxin 1 (TSST-1), exfoliative toxins (ETA to ETD), staphylococcal enterotoxins (SEs; SEA to SEE, SEG to SEI, SER) with demonstrated emetic activity, and staphylococcal enterotoxin-like (SEl) proteins. All the toxins listed above possess superantigenic activity and were designated as staphylococcal superantigens (SAgs) (Bukowski et al., 2010; Fowoyo and Ogunbanwo, 2016; Stich et al., 2010; Vasconcelos et al., 2011). Staphylococcal food poisoning is an intoxication that results from the consumption of foods containing sufficient amounts of one (or more) of the above-mentioned toxins (Dinges et al., 2000; Le Loir et al., 2003). Enterotoxigenic CNS strains, including organisms responsible for outbreaks of food poisoning have also been described by several authors (Batista et al., 2013; Podkowik et al., 2016; Vasconcelos et al., 2011; Zell et al., 2008). Additionally, it was reported that CNS may be a possible reservoir of enterotoxin genes typically identified in *S. aureus* (Ławrynowicz-Paciorek et al., 2007; Vasconcelos and da Cunha, 2010).

In addition to toxin production, an important factor determining the pathogenicity of staphylococci is associated with their antibiotic resistance. In recent years, a systematic growth in the number of antibiotic-

Abbreviations: CPS, coagulase-positive staphylococci; CNS, coagulase-negative staphylococci; TSST-1, toxic shock syndrome toxin 1; ET, exfoliative toxin; SE, staphylococcal enterotoxin; SEl, staphylococcal enterotoxin-like protein; SAg, staphylococcal superantigen.

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resistant staphylococcal strains in the human environment has been observed. Most research concerning antibiotic resistance of staphylococci isolated from food focuses on *S. aureus*, whereas less attention is paid to other species (Gao et al., 2012). Antibiotic-resistant strains other than *S. aureus* were also found in food (Gardini et al., 2003; Guran and Kahya, 2015; Martin et al., 2006; Podkowik et al., 2012) and genes encoding microbial resistance to tetracycline, erythromycin and β-lactams have been detected in CNS isolated from starter cultures, probiotic bacteria, fermented food and meat (Chajęcka-Wierzchowska et al., 2014; Guran and Kahya, 2015; Simeoni et al., 2008). Also in this case, different species of staphylococci have been suggested as a reservoir of antibiotic resistance genes (Chajęcka-Wierzchowska et al., 2014; Kloos and Bannerman, 1994; Neu, 1992) which can be transferred to *S. aureus*, making it resistant to multiple agents (Al-Masaudi et al., 1991).

Humans are the most important source of staphylococci, especially *S. aureus* and *S. epidermidis* but also *S. hominis*, *S. haemolyticus*, *S. saprophyticus*, *S. capitis*, *S. warneri*, *S. simulans* and *S. cohnii*. Because human skin staphylococci are not indigenous microbiota of raw foods, contamination is mainly associated with improper handling of cooked or processed foods. Food handlers may contaminate raw materials, equipment and finished products via manual contact or through respiratory secretions (Kadariya et al., 2014; Katsaras et al., 1985). Air, dust and food contact surfaces can also serve as vehicles in the transfer of staphylococci to foodstuffs (Bhatia and Zahoor, 2007).

Food-borne diseases are of major concern worldwide (Kadariya et al., 2014). Among the predominant bacteria involved in food-borne diseases, staphylococci (especially *S. aureus*) are a principal cause of gastroenteritis resulting from the consumption of contaminated food (Le Loir et al., 2003). In this case, meat and meat products have been one of the most frequently reported types of food involved in such outbreaks (Hennekinne et al., 2012; van Loo et al., 2007). Currently, a great popularity of ready-to-eat meat products that are obtained and cut into appropriate portions or slices at the store upon request can be observed. Such products can be purchased in small quantities and eaten relatively quickly, which may reduce the risk of pathogenic microorganisms growing in the food. On the other hand, such food products are touched repeatedly by shop staff and also come into frequent contact with various equipment and store surfaces, which may be conducive to their contamination.

Therefore, the aim of this study was to analyze the staphylococci isolated from ready-to-eat meat products, including pork ham, chicken cold cuts, pork sausage, salami and pork luncheon meat, sliced in the store upon request, along with species identification and determination of antibiotic resistance. Genes encoding staphylococcal enterotoxins, staphylococcal enterotoxin-like proteins, exfoliative toxins, and toxic shock syndrome toxin 1 were also investigated.

2. Material and methods

2.1. Bacterial isolates

Bacteria were isolated from 45 ready-to-eat meat products, including pork ham, chicken cold cuts, pork sausage, salami, pork luncheon meat, sliced upon request in 5 randomly selected butcher shops in Szczecin, Poland. A 10 g portion of each food sample was homogenized in 90 ml buffered peptone water (1%, w/v Graso, Poland), incubated overnight at 37 °C and 0.1 ml was plated on Mannitol Salt Agar medium (MSA, Graso, Poland), which allowed to culture all currently described species of staphylococci. MSA plates were incubated at 37 °C for 48 h. All isolates representing morphologically different colonies and/or similar morphotypes were picked and purified for further analysis. After the initial basic phenotypic analysis (microscopic examination, Gram staining, catalase production, coagulase tube test using rabbit plasma) the isolates were identified at the species level by the methods described below.

2.2. DNA extraction

All bacterial isolates were plated onto Columbia Agar Base with 5% sheep blood (Grasso, Poland) and cultivated for 24 h at 37 °C. After incubation, one colony-forming unit of each isolate was transferred into Luria-Bertani broth (Oxoid, UK) and incubated for 24 h at 37 °C. After cultivation, the optical density (at 600 nm) of bacterial cultures was adjusted to 1.0. The total DNA was extracted from bacterial cultures using the Genomic Mini Kit (A&A Biotechnology, Poland), according to the manufacturer's instructions.

2.3. PCR and multiplex PCR

The presence of the *gap* and *mecA* genes was detected using primers previously described by Yugueros et al. (2000) and Oliveira and de Lencastre (2002), respectively. The PCR reaction mixture (25 μl) consisted of 0.7 U AmpliTaq Gold DNA Polymerase (5 U/μl) with 1× PCR buffer (Applied Biosystems Inc., USA), 2 mM of $MgCl_2$, 0.2 mM dNTP mix (Applied Biosystems Inc.), 0.8 μM of each primer for the *gap* gene and 0.5 μM of each primer for the *mecA* gene, and approximately 20–50 ng of DNA.

The presence of genes encoding SEs, SEl, ETA, ETD and TSST-1 in the investigated isolates was determined by multiplex PCR with 5 different sets of primers as described by Fijałkowski et al. (2014), Holtfreter et al. (2007), Jarraud et al. (2002) and Zhang et al. (1998). Each multiplex PCR reaction mixture (25 μl) consisted of 1 U AmpliTaq Gold DNA Polymerase (5 U/μl) with 1× PCR buffer (Applied Biosystems Inc.), 5 mM of $MgCl_2$, 100 nM of dNTP mix (Applied Biosystems Inc.), 0.15 to 0.4 μM of each primer and 20–50 ng of DNA. The PCR and multiplex PCR conditions were as follows: initial denaturation of DNA at 95 °C for 10 min, 40 cycles for the *gap* gene, 30 cycles for the *mecA* gene, 35 cycles for superantigenic toxin genes (95 °C for 30 s, 50 °C (*gap* gene), 55 °C (*mecA* gene) for 30 s, 55 °C for 45 s (superantigenic toxin genes), 72 °C for 60 s), final extension at 72 °C for 7 min.

PCR and multiplex PCR products were characterized by 1.5% agarose gel (peqGOLD, Peqlab, Germany) electrophoresis in 1× Tris-borate-EDTA (TBE) buffer (Bio-Rad, USA), followed by staining with ethidium bromide (Merck, Germany), visualization under UV light and analysis using GeneTools software (Syngene, UK).

S. aureus control strains included A920210 (*eta*) (Wu et al., 2011), Col (*seb*, *selk*, *selq*) (Wu et al., 2011), FRI1151m (*sed*, *selj*, *ser*) (Holtfreter et al., 2007), FRI137 (*sec*, *seh*, *sell*, *selu*) (Wu et al., 2011), FRI913 (*sea*, *sec*, *see*, *selk*, *sell*, *selq*, *tst-1*) (Wu et al., 2011), N315 (*sec*, *seg*, *sei*, *sell*, *selm*, *seln*, *selo*, *selp*, *tst-1*) (Kuroda et al., 2001), TY114 (*etd*) (Wu et al., 2011), 8325-4 (no SAgs genes) (Holtfreter et al., 2007), ATCC 43300 (*gap*, *mec*A) and ATCC 25923 (no *mec*A) (EUCAST, Oxoid). Amplification of non-template controls was also included in each analysis to determine if DNA contamination had occurred.

2.4. Species identification by PCR-RFLP

The *gap* gene amplification products were digested with *Alu*I (Fermentas, Waltham, Massachusetts, USA) and *Hpy*CH4V (New England Biolabs, Hitchin, UK) restriction enzymes, according to the manufacturer's recommendations. PCR-RFLP products were separated on 2% agarose gel in 1× TBE buffer, visualized by staining with ethidium bromide and analyzed using GeneTools software. The RFLP profiles of the *gap* gene of staphylococci after digestion with *Alu*I and *Hpy*CH4V enzymes, published previously (Karakulska et al., 2012; Karakulska and Fijałkowski, 2014) were used as positive controls for species identification.

2.5. Phenotypic antibiotic resistance

Antibiotic susceptibility was examined according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing