Differentiation of Toxigenic *Staphylococcus aureus* Strains Isolated from Retail Meat Products

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Abstract

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Staphylococcus aureus is a saprophyte and commensal of the skin and mucous membranes in both animals and humans. As a pathogen, it can cause a number of diseases ranging from minor skin infections to fatal sepsis. Toxigenic strains of *S. aureus* are currently among the leading causes of food-borne intoxication (staphylococcal enterotoxicosis). Food contamination sources can be humans, raw materials, environment, technological equipment, etc. The identification of the origin of *S. aureus* would be helpful in the detection of the sources and routes of contamination. The aim of our study was to determine the probable origin of the selected *S. aureus* isolates coming from retail meat products intended for direct consumption with the use of phenotypic and genotypic methods. A set of 45 *S. aureus* isolates producing staphylococcal enterotoxins (SEs) with the potential to cause food-borne intoxication were selected for the study. These isolates were producers of the following enterotoxins: SEA (n = 10), SEB (n = 8), SEC (n = 10), SED (n = 7), SEH (n = 9), and SEB along with SED (n = 1). The phenotypic method used was based on the assessment of the growth on crystal violet agar (CV agar). A PCR-based genotypic method enabled the screening of the isolates for the *sak* gene encoding the enzyme staphylokinase typically found in human *S. aureus* isolates. As can be inferred from the type of growth on CV agar and the presence of the *sak* gene, all the isolates analysed were probably of human origin. These results confirm that humans are a major source of the bacteria *S. aureus* in both the food industry and retail sale of food products.

Keywords: SA 442; origin; staphylococcal enterotoxins; staphylokinase; crystal violet agar; phenotype; food

The bacterial species *Staphylococcus aureus* (*S. aureus*) is considered to be one of the most successful human pathogens of the present time. It lives on the skin or mucous membranes in about one third of the population in a commensal-like relationship and does not cause any problems. Nevertheless, when the host's natural defence is compromised, the pathogen is able to enter the tissues and to cause a variety of diseases ranging from minor skin infections to severe, possibly fatal infections of internal organs (Lowy 1998).

From the perspective of food microbiology, the most relevant characteristic of *S. aureus* is the production of heat-stable enterotoxins implicated in food-borne intoxications. Currently, 20 staphylococcal enterotoxins (SEs) are known: 5 classical and 15 newly described (Ono *et al*. 2008). The potential to cause food-borne intoxications has

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been reported in all classical SEs (SEA–SEE) and a single new SE, SEH (Omoe *et al*. 2002).

The presence of *S. aureus* in food products poses a consumer safety risk. SEs are formed as metabolic products in foods where these bacteria multiply. The *S. aureus* count of 10^5 CFU/g (ml) in food is considered as the critical dose for the possible accumulation of enterotoxin with the potential to cause disease. The food products often associated with staphylococcal enterotoxicosis are meat and meat products such as ham or ready-to-eat cooked sausage (Lancette & Tatini 1992). The bacteria *S. aureus* that contaminate food can originate from raw materials and the food industry staff or environment (Varnam & Evans 1991). Another possible source is an improper handling under retail sale conditions (slicing, packaging, etc.) (Karpíšková & Gelbíčová 2009).

Staphylococcal enterotoxicosis has a rapid onset and course. The first symptoms of intoxication, i.e. vomiting, headache, abdominal pain, and possibly diarrhoea, develop as early as within 2–6 h after the ingestion of food containing SEs (Zhang *et al*. 1998; Atanassova *et al*. 2001; Loir *et al*. 2003). The symptoms resolve spontaneously within 24 to 48 h while a more severe course has been observed particularly in high-risk population groups (Loir *et al*. 2003).

Given the wide spread of this pathogen, it is difficult to determine and eliminate the source of infection. Genotyping (Karpíšková & Gelbíčová 2009) would make it possible to identify the probable origins of the isolates and would be thus greatly helpful in epidemiological investigations.

The aim of our study was to determine the origins of the selected toxigenic *S. aureus* isolates coming from retail meat products intended for direct consumption, using phenotypic and genotypic methods. The phenotypic method used was the assessment of the growth on crystal violet agar (CV agar) (Skalka & Smola 1981). A PCR-based genotypic method served for screening the isolates for the *sak* gene encoding the enzyme staphylokinase typically found in human *S. aureus* isolates (Sung *et al*. 2008).

MATERIAL AND METHODS

Characteristics and confirmation of isolates. A set of 45 *S. aureus* isolates producing staphylococcal enterotoxins (SEs) with the potential to cause food-borne intoxication were selected for the study. They were producers of the following enterotoxins: SEA (n = 10), SEB (n = 8), SEC (n = 10), SED (n = 7), SEH (n = 9), and SEB along with SED (n = 1). These

Table 1. Overwiev of primers used for confirmation *S. aureus* isolates and for the detection of genes encoding SEs and staphylokinase

Primer	Sequence (5'–3')	Product size (bp)	Gene	Reference
SA 422-1 SA 422-2	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	108	fragment DNA 442 bp	Martineau *et al.* (1998)
InKo1 InKo2	GGA GGA AGG TGG GGA TGA CG ATG GTG TGA CGG GCG GTG TG	241	16S rRNA	Martineau *et al.* (1996)
sea 1 sea 2	GCA GGG AAC AGC TTT AGG C GTT CTG TAG AAG TAT GAA ACA CG	520	*sea* gen	Monday and Bohach (1999)
seb-sec 1 seb-sec 2	ACA TGT AAT TTT GAT ATT CGC ACT G TGC AGG CAT CAT GTC ATA CCA	667	*seb* and *sec* gen	Løvseth *et al.* (2004)
sec 1 sec 2	CTT GTA TGT ATG GAG GAA TAA CAA TGC AGG CAT CAT ATC ATA CCA	283	*sec* gen	Monday and Bohach (1999)
sed 1 sed 2	GTG GTG AAA TAG ATA GGA CTG C ATA TGA AGG TGC TCT GTG G	384	*sed* gen	Monday and Bohach (1999)
seh 1 seh 2	CAA CTG CTG ATT TAG CTC AG GTC GAA TGA GTA ATC TCT AGG	360	*seh* gen	Monday and Bohach (1999)
sak 1 sak 2	TGAGGTAAGTGCATCAAGTTCA CCTTTGTAATTAAGTTGAATCCAGG	403	*sak* gen	Sung *et al.* (2008)

isolates were obtained from retail meat products in the Czech Republic in 2005–2009. All isolates were confirmed by the PCR method for the detection of the specific specific SA 442 fragment, the gene for 16S rRNA having been used as an internal control with the primers and conditions designed by Martineau *et al*. (1998). The PCR products were amplified using PPP Master mix polymerase (Top-Bio, Prague, Czech Republic). The presence of the genes encoding SEs (*ses*) was confirmed by the multiplex PCR assay according to the conditions described by Monday and Bohach (1999) and Løvseth *et al*. (2004). For the amplification, HotStart Taq polymerase (Qiagen Inc., Turnberry Lane Valencia, USA) was used. The primers used for PCR assays are listed in Table 1.

Phenotypic determination of the origin. Crystal violet agar was used for phenotypic differentiation of *S. aureus* isolates based on the type of growth as a possible indicator of the strains origin (Table 2). The type of growth (colour of colonies) was maeked with the following letters: A (yellow), C (blue-violet), and E (white) (Skalka & Smola 1981).

Genotypic determination of the origin. A PCR-based assay was used for the determination of the strain origin, targeting the *sak* gene encoding staphylococcal staphylokinase (Sung *et al*. 2008). The reaction was designed as a multiplex PCR assay for the detection of the *sak* gene while the gene for 16S rRNA was used as an internal control (Martineau *et al*. 1996). The primers used are shown in Table 1. The conditions of the PCR reaction were taken from the study by Sung *et al*. (2008). PCR products were amplified using PPP Master mix polymerase (Top-Bio, Prague, Czech Republic).

RESULTS AND DISCUSSION

Determination of the origin of isolates

The phenotypic test revealed two types of growth, A and C. These are typically associated with human *S. aureus* isolates. The genotypic, phenotypic, and other characteristics of the *S. aureus* isolates under study are presented in Table 3. The *sak* gene was detected in all (100%) of them.

Table 2. Origin of isolates according to the type of growth on crystal violet agar (Skalka & Smola 1981)

Colour of colonies	Symbol	Probable origin
Yellow	A	fowl, pig, cattle, humans
Blue-violet	C	humans, cattle, sheep, hare
White	E	dog, horse, pigeon

The distribution of the phenotypic and genotypic characteristics among the *S. aureus* isolates analysed was as follows: all isolates carrying the *sea* gene showed type C growth, the *seb* gene was associated with both type C growth (in 5 strains, i.e. 62.5%) and type A growth (in three strains, i.e. 37.5%), the isolate with genes *seb+sec* showed type C growth. Nine (90%) of 10 isolates possessing the *sec* gene showed type C growth and one of these isolates (10%) type A growth, the *sed*-positive strains varied in the type of growth depending on the presence/absence of other *ses* genes, the isolates carrying the *sed+sej* genes (n = 3) showed correspondingly type C growth while the *sed+seg+sei+sej* positivity (n = 4) was associated with type A growth, and the isolates carrying the *seh* gene also displayed both types of growth, more precisely five isolates (55.6%) formed type A colonies and four isolates (44.4%) type C colonies.

Based on our results, it can be concluded that all the isolates tested showed both phenotypic and genotypic characteristics suggestive of human origin. Forty-three of 45 meat products from which the *S. aureus* isolates had been recovered were heat-processed meat products. *S. aureus* contained in the raw material is likely to be inactivated when heat processed. The presence of this bacterial species in the final retail products (not packaged directly by the producer) is a result of contamination during the distribution, storage, slicing, packaging, and retail sale of the products. The sources of contamination are primarily the shop staff and environment.

The study of Sung *et al*. (2008) focused on the differentiation between human and animal *Staphylococcus aureus* strains suggests that the human isolates of *S. aureus* belong to specific clonal lineages that differ from the animal lineages of *S. aureus*. Each lineage carries a unique combination of core variable (CV) genes encoding mainly the surface proteins or structures and their regulators. Surprisingly, the animal lineages of *S. aureus* carried a variety of CV genes previously identified as belonging to human *S. aureus*. The *S. aureus* lineages have evolved by horizontal recombination between all lineages. The animal lineages carry a variety of CV markers detected in sequenced human lineages. This implies that they may have evolved along with the human lineages and are not located on a separate branch of the phylogenetic tree.

Table 3. Characteristics of *S. aureus* isolates from retail meat products

Isolate No.	Year of isolation	Type of meat product	Detection of		Growth on CV agar
			ses[1]	*sak*[2]	
SA 748 A	2005	pork ham	*a*	+	C
SA 986	2006	sausage, preserved, heat processed	*a*	+	C
SA 1142	2007	pork ham	*a*	+	C
SA 1143	2007	Czech liver sausage	*a*	+	C
SA 1163	2007	raw sausage, soft	*a*	+	C
SA 1420	2008	raw sausage, soft	*a*	+	C
SA 1421	2008	frankfurters	*a*	+	C
SA 1423	2008	pork ham	*a*	+	C
SA 1424	2008	pork pudding	*a*	+	C
SA 1610	2009	bacon	*a*	+	C
SA 760 C	2005	sausage, preserved, heat processed	*b*	+	A
SA 762 C	2005	raw sausage, soft	*b*	+	A
SA 978	2006	sausage, preserved, heat processed	*b*	+	A
SA 979	2007	pork ham	*b*	+	C
SA 992	2006	pork ham	*b*	+	C
SA 1003	2006	sausage, preserved, heat processed	*b*	+	C
SA 1004	2006	raw sausage, soft	*b, d*	+	C
SA 1533	2009	Czech liver sausage	*b*	+	C
SA 1655	2009	poultry ham	*b*	+	C
SA 1141	2007	sausage	*c*	+	C
SA 1144	2007	raw sausage, soft	*c*	+	C
SA 1145	2007	pork ham	*c*	+	C
SA 1164	2007	raw sausage, soft	*c*	+	A
SA 1372	2008	sausage, preserved, heat processed	*c*	+	C
SA 1375	2008	raw sausage, soft	*c*	+	C
SA 1377	2008	blood sausage	*c*	+	C
SA 1428	2008	sausage, preserved, heat processed	*c*	+	C
SA 1498	2008	poultry ham	*c*	+	C
SA 1539	2009	sausage	*c*	+	C
SA 1425	2008	sausage, preserved, heat processed	*d*	+	C
SA 1426	2008	frankfurters	*d*	+	C
SA 1524	2009	liver sausage	*d*	+	C
SA 1553	2009	pork ham	*d*	+	A
SA 1559	2009	raw sausage, soft	*d*	+	A
SA 1560	2009	pork ham	*d*	+	A
SA 1652	2009	poultry ham	*d*	+	A
SA 991	2006	sausage, preserved, heat processed	*h*	+	C
SA 998	2006	sausage, preserved, heat processed	*h*	+	C
SA 1127	2007	sausage, preserved, heat processed	*h*	+	C
SA 1128	2007	sausage, fermented, preserved	*h*	+	A
SA 1129	2007	sausage, fermented, preserved	*h*	+	A
SA 1130	2007	raw sausage, soft	*h*	+	C
SA 1131	2007	frankfurters	*h*	+	A
SA 1133	2007	pork pudding	*h*	+	A
SA 1371	2008	Czech liver sausage	*h*	+	A

[1]genes encoding staphylococcal enterotoxins; [2]gene encoding staphylokinase

The study of Sung *et al.* (2008) attempted at identifing the host-specific genes. They found most differences to be lineage specific rather than host specific. Nevertheless, some genes are more often detected in human isolates than in animal. Ones, the *sak* gene was detected sporadically in animal isolates with Sung *et al.* (2008). In the study by Callon *et al.* (2008), staphylokinase gene analysis was always positive in *S. aureus* strains of human origin (noses and hands), thus confirming the presence of this gene in the human biotype in accordance with the previous study by Hajek and Marsalek (1991). The *sak* gene is associated with the defence of *S. aureus* against human immune response and is likely, along with a variety of proteins such as chemotaxis inhibiting protein (CHIPS), staphylococcal complement inhibitor (SCIN), and staphylococcal enterotoxin A (SEA), to interfere specifically with the human and not with the animal immune system. These genes are located on the IEC (immune evasion cluster) encoded on β-haemolysin converting bacteriophage (Rooijakkers *et al.* 2005a,b; van Wamel *et al.* 2006; Sung *et al.* 2008). Notably, animal isolates are significantly less likely to carry some of these IEC genes, including the genes for CHIPS, SCIN and *sak* gene (van Wamel *et al.* 2006). Monecke *et al.* (2007) and Kumagai *et al.* (2007) have already reported a low incidence of these genes in bovine isolates of *S. aureus*.

The SEA production is often associated with *S. aureus* strains of human origin and the SEA producers along with the SED producers are the leading cause of staphylococcal enterotoxicosis. On the other hand, the SEC producers are often linked to dairy product-borne intoxications (Wilson *et al.* 1991; Jablonski & Bohach 1997; Balaban & Rasooly 2000). Nevertheless, based on our results, even the strains carrying the *sec* gene were phenotypically and genotypically (based on the presence of the *sak* gene) classified as coming from *S. aureus* of human origin.

The above-mentioned study of Sung *et al.* (2008) has detected the *sak* gene in 83% of human isolates and in 20% of animal isolates, and the *sea* gene in 25% and 14% of isolates, respectively. Therefore, the *sea* gene is not an unambiguous marker for the differentiation of *S. aureus* strains by origin.

The differentiation between *S. aureus* strains, including that between the human and animal origins, can also be performed using the phenotypic methods such as the growth on CV agar. The phenotypic methods provide less accurate results (also due to the subjective assessment) but are complementary to the genotypic ones.

For a more objective assessment, it would be necessary to test more markers, to analyse a wider range of strains, and to perform statistical analysis of the results. Moreover, the *spa* and MLST typing might also be helpful, taking into account the correlation between the clonal complexes and origin of *S. aureus* strains as reported Juhasz-Kaszanyitzky *et al.* (2007) and Sung *et al.* (2008). Further data would cast more light on *S. aureus* in general and on the routes of its spreading in the food chain in particular, and thus would contribute to a better understanding of the sources of *S. aureus* strains in foods.

CONCLUSION

The presence of *S. aureus* in the final retail products can be very often a result of contamination during the retail sale of the products. Because the sources of contamination are primarily the shop staff and environment, it is necessary to emphasise the importance of respecting the principles of good manufacturing and hygienic practices not only in the manufacture but also in the retail sale. New knowledge of the differences between the human and animal strains of *S. aureus* would facilitate tracing the sources of staphylococcal intoxication, i.e. epidemiological investigations of the sources and routes of contamination of foods and the environments in the food industry plants and retail shops as well as in staphylococcal enterotoxicosis outbreaks.

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