Polish Journal of Veterinary Sciences Vol. 17, No. 3 (2014), 407–411

DOI 10.2478/pjvs-2014-0058

Original article

Distribution of classical enterotoxin genes in staphylococci from milk of cows with- and without mastitis and the cowshed environment

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Abstract

The aim of this study was to analyze by PCR 185 isolates of *Staphylococcus* from milk of cows with- and without mastitis and from the cowsheds environment for their potential ability to produce five classical staphylococcal enterotoxins. Among *S. aureus* isolates 8 (32%) carried enterotoxin genes and only 2 of them had more than one gene. The enterotoxin genes were detected in 22 (13.7%) coagulase-negative staphylococci (CNS) isolates, among them in 9 (11.4%) isolates of *S. xylosus*, 5 (16.7%) *S. sciuri*, 3 (10.3%) *S. epidermidis* and in 5 (22.7%) *Staphylococcus* spp. In some CNS 2 or 3 genes were detected simultaneously. Among the investigated enterotoxin genes, *sec* was the most prevalent (70%). The genes encoding enterotoxin B and D were detected in 5 (16.7%) and 6 (20%) isolates, respectively. The lowest number of isolates had *sea* and *see* genes.

The genes encoding enterotoxins were often identified in staphylococci from milk of cows with mastitis (73.4% of detected genes), while only 6 (20%) isolates from milk of cows without mastitis and 2 (6.6%) isolates from cowshed environment were positive for enterotoxin genes.

The results showed that CNS from bovine milk, like *S. aureus*, carried enterotoxin genes and may pose a risk for public health.

Key words: coagulase-negative staphylococci, *S. aureus,* staphylococcal enterotoxin genes, PCR

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Introduction

Historically, *S. aureus* have been considered the most important pathogen among staphylococci causing mastitis, whereas coagulase-negative staphylococci (CNS) were considered minor pathogens (Njage et al. 2013). However, recently CNS have become a significantly predominant pathogen in bovine mastitis (Sampimon et al. 2009, Kot et al. 2012a). Similarly as *S. aureus*, CNS produce a number of virulence factors that determine the ability of adhesion, avoidance of host immune mechanisms, production of toxins and enzymes (Dinges et al. 2000). The main toxins produced by staphylococci are classified as having the characteristics of staphylococcal enterotoxins (SEs). SEs have the properties of superantigens (SAgs) and induce uncontrolled proliferation of T lymphocytes. Superantigen production leads to excessive secretion of cytokines and lymphokines, and consequently damage to the blood vessels, lowering blood pressure and inducing fever (Baker and Acharya 2004). The International Nomenclature Committee for Staphylococcal Superantigens developed standard nomenclature of enterotoxins according to which staphylococcal enterotoxins (SEA-SEI) show emetic activity which was confirmed in tests on primates. Other enterotoxins, marked J-V, are called the putative SEls (Staphylococcal Enterotoxin-like superantigens) because they showed no emetic properties in animal studies (Lina et al. 2004, Collery et al. 2008, Podkowik et al. 2013). Another classification of enterotoxins involves their division into classical enterotoxins (five antigenically different types of SEA, SEB, SEC, SED, SEE) and new enterotoxins (SEG-SElV) (Bania et al. 2006, Park et al. 2011). Genes encoding staphylococcal enterotoxins may be located on plasmids, bacteriophages, transposons, or pathogenicity islands (PIs) and are transferred by horizontal gene transfer. Staphylococci present in food, showing the potential to produce enterotoxins, are serious threat to public health (Blaiotta et al. 2006). Enterotoxins SEA, SEB, SEC, SED, called classic, cause more than 90% of cases of staphylococcal food poisoning (SFP). Milk and dairy products can be a source of enterotoxic staphylococci (Orwin et al. 2001, Asao et al. 2003, Boynukara et al. 2008). Due to the growing importance of CNS as etiological agents of mastitis it is important to study the potential ability of production of enterotoxins by these bacteria.

The aim of this study was the analysis of the occurrence of classical enterotoxin genes by PCR in CNS and *S. aureus* isolated from milk of cows with – and without mastitis symptoms and from cowshed environmental samples.

Materials and Methods

Staphylococcal isolates

A total of 125 staphylococcal isolates from milk of cows with subclinical and clinical mastitis and 29 isolates from milk samples from cows without mastitis symptoms, as well as 31 isolates from cowshed environment, were used in this study. The diagnosis of mastitis was made on the basis of clinical examination of the udder by the veterinarian. Moreover, a quarter was identified as infected when somatic cell counts (SCC) were above 200 000/ml (Kot et al. 2012b). The quarter milk samples and the samples from the cowshed environment (the swabs from milker's hands, teat skin, teat cups milking machine, samples of cow's beddings, fodder and water) were collected in the eastern part of the Mazovia and the north Lublin region of Poland in years 2009-2010. The sampling methods, isolation and identification of staphylococcal isolates were described previously (Kot et al. 2012a).

DNA isolation

Genomic DNA was isolated from bacterial cells by using the IT 1-2-3^{TM} DNA Sample Purification Kit (Idaho Technology Inc. Salt Lake City, Utah, USA) according to the manufacturer's protocol.

Detection of enterotoxin genes

The specific primers used in the present study are shown in Table 1. The primers were synthesized by DNA-Gdańsk (Poland). Amplification of DNA was performed using the Multi Gene II thermal cycler (Labnet International, Inc., USA). PCR for enterotoxin genes was carried out under the following conditions: an initial denaturation of DNA at 95°C for 10 min was followed by 35 cycles of amplification (95°C for 30 s, 53°C for 45s, 72°C for 90 s), ending with a final extension at 72°C for 10 min. Duplex PCR was performed with two different sets of primer mixtures: set 1: *sea* and *seb*, set 2: *sec* and *sed*. Monoplex PCR was used to detect the presence of the *see* gene. The volume of each PCR mixture was 25 µL and contained 2.5 µL of DNA template, 1 × PCR buffer, 0.2 mM of dNTP mix (Fermentas, Lithuania), 200 nM each primer, 1 U of RedTag Genomic DNA polymerase (Sigma-Aldrich, Germany) and sterile deionized water. Positive controls with the genomic DNA from *S. aureus* for the *sea*, *seb*, *sec, sed* and *see* genes were included in each test run. All PCR

Table 1. Nucleotide sequences of primers and expected size of PCR products (bp) of staphylococcal superantigenic toxins used in this study.

Gene	Primer	Oligonucleotides sequence (5'-3')	PCR product (bp)	References
sea	SEA-F	CAGCATACTATATTGTTTAAAGGC	400	Park et al. 2011
	SEA-R	CCTCTGAACCTTCCCATC		
seb	SEB-F	GTATGGTGGTGTAACTGAGCA	351	Park et al. 2011
	SEB-R	TCAATCTTCACATCTTTAGAATCA		
sec	SEC-F	CTCAAGAACTAGACATAAAAGCTAGG	271	Becker et al. 1998
	SEC-R	TCAAAATCGGATTAACATTATCC		
sed	SED-F	CTAGTTTGGTAATATCTCCTTTAAACG	319	Becker et al. 1998
	SED-R	TTAATGCTATATCTTATAGGGTAAACATC		
see	SEE-F	CAGTACCTATAGATAAAGTTAAAACAAG	178	Becker et al. 1998
	SEE-R	CTAACTTACCGTGGACCCTTC		

(F) – forward primers, (R) – reverse primers.

Table 2. Distribution of enterotoxin genes among staphylococcal isolates.

Species (n)	Results (no. of isolates) of PCR for:				
	sea	*seb*	*sec*	*sed*	*see*
S. aureus (n=25)	+(2)	+(1)	+(6)	+(2)	–
S. xylosus (n=79)	–	–	+(6)	+(2)	+(2)
S. sciuri (n=30)	–	–	+(5)	–	–
S. epidermidis (n=29)	+(1)	+(1)	+(2)	+(1)	+(1)
Staphylococcus spp. (n=22)	–	+(3)	+(2)	+(1)	–
Total (185)	3	5	21	6	3

products were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide in 2 × Tris-borate-EDTA buffer. Molecular size marker (GenoPlast Biochemicals, Poland) was also run for product size verification.

Results

The presence of enetrotoxin genes *sea*, *seb*, *sec*, *sed*, *see* was investigated in 185 isolates including 25 *S. aureus* and 160 CNS originating from bovine milk and cowshed environmental samples. The presence of enterotoxin genes was detected in 8 (32%) *S. aureus* isolates in which two carried more than one gene encoding enterotoxins. Among CNS strains 22 (13.7%) had enterotoxin genes. *S. xylosus* was the most prevalent CNS species and 9 (11.4%) of the isolates were positive for enterotoxin genes. Six *S. xylosus* isolates from milk of cows with mastitis symptoms, two isolates from milk of cows without mastitis and one isolate originating from skin of milker's hands were positive for enterotoxin genes. In the case of *S. sciuri* and *S. epidermidis* isolates the enterotoxin genes were detected in 5 (16.7%) and 3 (10.3%), respectively. In *S. sciuri* isolates only the *sec* gene was detected, present in 3 isolates from milk of cows with mastitis, one isolate originated from milk of cow without mastitis and one isolate from sample of cow's bedding. The isolates of *S. epidermidis* positive for enterotoxin genes originated from milk of cows with- and without mastitis. Among *Staphylococcus* spp. isolates 5 (22.7%) were positive for enterotoxin genes. Most of isolates with genes encoding enterotoxins detected in staphylococci originated from milk of cows with mastitis symptoms 22 (73.4%), while only 6 (20%) isolates from milk of cows without mastitis symptoms, and one (3.3%) isolate from the milker's hands and one (3.3%) isolate from samples of cow's beddings were positive for enterotoxin genes. In some *S. aureus*, *S. xylosus*, and *Staphylococcus* spp. isolates *sec*+*sed* genes were detected simultaneously. The *sea*+*seb* genes were detected in one *S. epidermidis* isolate. Three genes *sea*+*seb*+*sec* and *sec*+*sed*+*see* were detected simultaneously in one *S. aureus* and *S. epidermidis*, respectively. The gene coding enterotoxin C was present

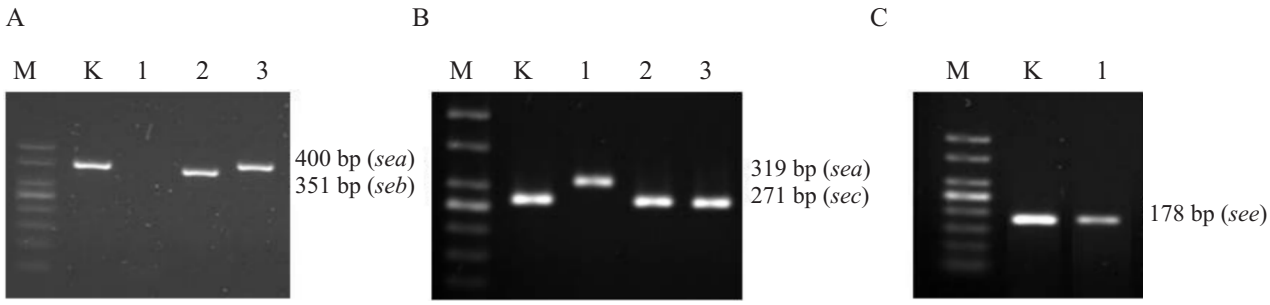

Fig. 1. Electrophoresis in 1.5% agarose gel of PCR products obtained by using specific primers for *sea* and *seb* genes (A), *sec* and *sed* genes (B), *see* gene (C). A, B, C – Lanes M – molecular weight markers (500, 400, 300, 250, 200, 150, 100, 50 bp – GenoPlast Biochemicals). A – Lanes: K – 400 bp product obtained by using genomic DNA of *S. aureus* (positive control); 2 – product obtained by using genomic DNA of *S. epidermidis* isolate; 1 – no products by using genomic DNA of *S. xylosus* isolate; 2,3 – products obtained by using genomic DNA of *S. epidermidis* isolate. B – Lanes: K – 271 bp product obtained by using genomic DNA of *S. aureus* (positive control); 1 – product obtained by using genomic DNA of *S. sciuri* isolate; 2, 3 – products obtained by using genomic DNA of *S. xylosus* isolates. C – Lanes: K – 178 bp product obtained by using genomic DNA of *S. aureus* isolate; 1 -product by using genomic DNA of *S. epidermidis* isolate.

the most, 21 (11.3%), of staphylococcal isolates (Table 2). The PCR product of 271 bp, corresponding to the fragment of the gene *sec* (Fig. 1B) was detected in 21 (70%) of the isolates positive for enterotoxin genes. Among staphylococcal isolates having enterotoxin genes, the gene encoding enterotoxin D (amplification product of 319 bp) (Fig. 1B) was detected in 6 (20%) isolates and gene encoding the enterotoxin B was present in genome of 5 isolates (16.7%).

The lowest number of the tested isolates of staphylococci had genes *sea* and *see* because the products of 400 bp specific for *sea* gene (Fig. 1A) were detected only in 3 (10%) isolates, and fragments of 178 bp corresponding to the *see* gene (Fig. 1C) were detected in other 3 (10%) isolates (Table 2).

Discussion

The family of toxins belonging to superantigens (SAgs) harbours staphylococcal exoprotein enterotoxins. Superantigenic toxins can cause lesions through the overproduction of cytotoxins by T-cells and by monocytes (Herman et al. 1991). Production of enterotoxins by staphylococci is an important virulence factor responsible for staphylococcal food poisoning (SFP) in humans. Major symptoms of staphylococcal intoxication such as vomiting, nausea, abdominal pain, diarrhea and headache occur after the ingestion of food contaminated with *Staphylococcus* produced enterotoxin. Clinical symptoms may be mild but sometimes they are serious and require hospitalization. Severity of intoxication depends on the amount of toxin ingested and individual susceptibility (Cunha and Calsolari 2008). Farm animals, particularly their milk, are considered a reservoir of enterotoxigenic staphylococcal species (Weese 2012). *S. aureus* is the most common pathogen of mastitis and milk is a good substrate for their growth. Several studies have shown that *S. aureus* isolates frequently contain SE genes and produce one or more enterotoxins that cause SFP in humans (Scherrer et al. 2004, Podkowik et al. 2013).

In our research 32% of *S. aureus* isolates were positive for enterotoxin genes which is in agreement with the results obtained by de Freitas Guimarãeses et al. (2013) who observed that 35% of coagulase-positive staphylococci from bovine milk had genes related to production of enterotoxins. Jørgensen et al. (2005) reported that *sec* gene was most commonly detected in *S. aureus* from bovine bulk milk in Norway which is consistent with our results. Boynukara et al. (2008), who examined 106 *S. aureus* strains, found that 25.5% of them had enterotoxin genes, among which 23.5% were positive for *sea* and 1.9% were positive for *seb* genes. In our study, we also detected *S. aureus* isolates with *sea* and *seb* genes. Moreover, we detected isolates with *sed* gene and isolates with more than one enterotoxin gene, contrary to the results obtained by Boynukara et al. (2008), who did not obtain isolates positive for *sec* or *sed* genes or isolates with more than one type of enterotoxin gene.

Currently, the CNS are the most prevalent microorganisms isolated from milk samples of cows with mastitis symptoms and there are very little data on eterotoxigenic properties of CNS from bovine milk (Park et al. 2011, de Freitas Guimarães et al. 2013). In our study, 15.4% of CNS from bovine milk and 6.5% from cowshed environment were positive for enterotoxin genes. The highest percentage of CNS isolates with genes related to production of enterotoxins (66%) was found by de Freitas Guimarãeses et al. (2013) who investigated CNS from bovine milk in Brazilian dairy herds. The differences in obtained

results can by caused by spread of certain CNS types within specific geographical areas.

In this study, CNS isolates from cowshed environment carried only the *sec* gene and this gene also was detected most commonly in CNS from bovine milk, which is different from results obtained by Park et al. (2011), who showed that among the classical enterotoxins the *seb* gene was the most common in CNS isolates from bovine intramammary infections. Our results revealed that *sec* gene was detected in 15 (68.2%) CNS isolates among 22 positive for enterotoxin genes. Lower percentage of CNS isolates with genes encoding the enterotoxin A (4.5%), B and D (18.2%), E (13.6%) were detected.

The SEs genes are diversely distributed among different CNS species (Park et al. 2011, de Freitas Guimarãeses et al. 2013). In our study, the enterotoxin genes were detected in 9 isolates of *S. xylosus* (11.4%), 5 (16.7%) of *S. sciuri*, 3 (10.3%) of *S. epidermidis* and in 5 (22.7%) of *Staphylococcus* spp.

Our results revealed that not only *S. aureus* isolates carry enterotoxin genes, but CNS isolated from bovine milk may be a source of these genes. Therefore, these microorganisms require more attention in microbiological evaluation of food and should not be considered mere contaminants.

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