Exotoxin diversity of *Staphylococcus aureus* isolated from milk of cows with subclinical mastitis in Central Russia

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ABSTRACT

Mastitis, a major veterinary problem widespread in many regions, is caused mainly by *Staphylococcus* spp. However, there is no current reliable information about the role of *Staphylococcus aureus* and their toxins in the development of mastitis in cows in the territory of the Russian Federation. The aim of this investigation was to determine the profile of exotoxins of *S. aureus* from cow milk from farms of Central Russia. A total of 60 isolates of *S. aureus* were obtained from milk samples of cows with the subclinical form of mastitis. The exotoxin genes were identified using 2 types of PCR assays. The diversity of enterotoxin genes was studied by multiplex PCR. The percentage occurrence of enterotoxin genes was as follows: *sea*, 53.3%; *seb*, 3.3%; *sec*, 50%; *sed*, 4%; *see*, 46.6%; *seg*, 70%; *sei*, 10%; *seip*, 3.3%; and *tsst1*, 1.6%. The *seh* gene was not detected. The genes of pore-forming toxins and phenol-soluble modulins were identified by singleplex PCR and consisted of the following: *hla*, 70%; *lucS*, 46.6%; *psmA*, 81.6%; *psmB*, 95%; and *hld*, 78.3%. The most abundant genes were *psm* (*psmB*, 95%), which codes for pore-forming toxins, and *seg* (70%), which codes for enterotoxins. The production of some enterotoxins in bacterial culture medium was detected by ELISA. The level of toxin production was near 1 ng/mL for SEA, SEE, SEG, SEI, SELP, and TSST-1 and reached a maximal level of 18 ng/mL for SEE. In the present work, we show that subclinical mastitis in cows is associated with *S. aureus* in the central region of the Russian Federation. Most of the isolates containing enterotoxin genes also had cytotoxin genes.

Key words: *Staphylococcus aureus*, cow mastitis, enterotoxin, cytotoxin

INTRODUCTION

Bovine mastitis is a major and widespread veterinary problem (Zhang et al., 2016). This widespread disease inflicts considerable damage on agriculture and the economy as a whole. One of the consequences of this disease is reduced productivity of animals (Hertl et al., 2014). The main cause of mastitis is *Staphylococcus* spp. (Barkema et al., 2006; Hummerjohann et al., 2014). In the Russian Federation, the clinical form of mastitis is diagnosed in 20 to 25% of cows; subclinical mastitis is diagnosed in more than 50% (Artem'eva et al., 2015). Due to mastitis, annual milk production decreases every year. The specific agents frequently causing cow mastitis in the Russian Federation are *Staphylococcus aureus* and *Staphylococcus epidermidis* (Shurbuda et al., 2014; Artem'eva et al., 2015). Along with the *Staphylococcus* genus, a broad range of other microorganisms and viruses are associated with mastitis. Some of these microorganisms can induce the disease, whereas others can protect the host organism by inhibiting the pathogenic microflora (Falentin et al., 2016; Browne et al., 2017).

Staphylococcus spp. produce a set of exotoxins or factors of pathogenicity that include enterotoxins and cytolytic toxins. Enterotoxins represent the most common family of staphylococcal exotoxins; their direct participation in the development of subclinical and clinical forms of mastitis is well known. However, the subclinical form of mastitis is often associated with coagulase-negative staphylococci, which are not characterized by the production of enterotoxins. *Staphylococcus aureus*, on the other hand, is characterized by the production of enterotoxins.

Cytolytic toxins, which include hemolysins, leukocidins, and phenol-soluble modulins, play an important role in the development of mastitis. All of these toxins participate in the development of mastitis associated with *Staphylococcus* spp. The most studied are the hemolysins, which can lyse different types of cells, including epithelial cells, and are the main pathogenesis factor in mastitis.

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Panton–Valentine leukocidin is a 2-component toxin consisting of leukocidin S and leukocidin F. Panton–Valentine leukocidin is a cytotoxin that causes leukocyte destruction and tissue necrosis and has been detected in staphylococcal isolates associated with mastitis (Unal and Cinar, 2012).

The phenol-soluble modulins (**PSM**) constitute a relatively new family of cytotoxins, which are secreted, amphipathic, α -helical peptides with various biological functions. Phenol-soluble modulins possess powerful cytolytic activity against many types of cells, including neutrophils, monocytes, erythrocytes, keratinocytes, and osteoblasts. Last, it has been shown that the isolates associated with mastitis have a high level of expression of δ -toxin (Deplanche et al., 2016). The aim of this work was to study the composition of enterotoxin genes of *S. aureus* associated with subclinical mastitis from cows in Central Russia and to investigate the production of enterotoxins.

MATERIALS AND METHODS

Antibodies and Toxins

We used recombinant staphylococcal toxins (SEA, SEB, SEC, SED, SEE, SEG, SEI, SELP, and TSST-1) and their genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *sei*, *selp*, *tsst1*, *seh*), which were kindly provided by Y. V. Vertiev (N. F. Gamaleya Scientific Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences, Moscow, Russia).

Murine monoclonal antibodies to these staphylococcal toxins, as well as their conjugates with EZ-Link-Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA), were produced earlier, and their specificity was analyzed in our previous work (Rubina et al., 2010). The monoclonal antibodies were produced in ascites fluid and purified by a combination of ammonium sulfate fractionation and ion-exchange chromatography on a mono Q column (FPLC System, Pharmacia Biotech, Uppsala, Sweden). The detailed information about immunoreagents for enterotoxin detection was published recently by Rubina et al. (2010).

S. aureus Isolation

Milk samples from Holstein cows with subclinical mastitis were collected from farms of Central Russia (Kaluzhskaya oblast) in April 2015. The samples were collected in sterile tubes after disinfecting the ostium with iodized alcohol (2.5%), and they were transported to the laboratory under refrigeration (4–8°C) in cool boxes with ice packs. All of the handlers used sterilized latex gloves.

All collected samples were analyzed on traditional microbiological media as follows: coagulase-positive staphylococci were enumerated from colonies displaying an opaque zone on Baird-Parker agar with egg yolk tellurite emulsion (HiMedia Laboratories Pvt. Ltd., Telangana, India), and hemolytic staphylococci were enumerated from colonies displaying hemolysis zones on the Azide Blood Agar Pronadisa medium (Conda, Madrid, Spain) supplemented with 5% defibrinated sheep blood. In addition, we tested for the presence of positive plasma coagulation with rabbit dry citrate plasma (CJSC Ekolab, Moscow, Russia), and we performed biochemical identification using the API 20 Staph panel (BioMerieux, Marcy-l'Étoile, France). *Staphylococcus aureus* ATCC 25923 and *S. aureus* ATCC 6538 were used as reference strains (state collection of pathogenic microorganisms and cell cultures, Obolensk, Moscow region, Russia).

DNA Isolation

Individual isolates of *S. aureus* were cultivated in a tryptic soy broth (HiMedia Laboratories Pvt. Ltd.) at 37°C using an orbital shaker for 14 to 16 h. The cell culture was centrifuged at $4,000 \times g$ for 5 min at 4°C, and the cell pellet was used for DNA isolation. The culture medium was used to test SE expression by ELISA.

The individual bacterial pellet was resuspended in 0.5 mL of 0.01 M Tris-HCl (pH 8.0) containing lysostaphin (5 μ g/mL) and RNase A (30 μ g/mL) and incubated for 0.5 h at 37°C. Proteinase K (200 μ g/mL) and SDS (1%) were added, and the mixture was incubated for 20 min at 50°C. Further deproteinization of DNA was carried out by phenol-chloroform extraction and centrifugation at $12,000 \times g$ for 10 min. Purified DNA was precipitated from the aqueous phase by adding isopropanol to 40% (vol/vol) and collected by centrifugation at $12,000 \times g$ for 10 min. The DNA pellet was washed with 70% (vol/vol) ethanol, dried at room temperature for 5 min, and dissolved in 50 μ L of deionized water (MilliQ, Millipore Corp., Billerica, MA). The DNA extracts were analyzed by electrophoresis in a 1% agarose gel stained by ethidium bromide (5 μ g/mL).

Detection of Enterotoxin Genes

The multiplex PCR assay was used for the detection of staphylococcal enterotoxin genes (*sea*, *seb*, *sed*, *see*, *seg*, *seh*, *sei*) and *tsst1*. Singleplex PCR for *sec* was carried out to avoid the overlapping between *seg* and *sec*. In addition, *selp* was amplified separately. Primers and the sizes of the amplicons are listed in Table 1. The template DNA was amplified by thermal cycling at 94°C for 2 min, followed by 30 cycles of 94°C for 25

s, 50°C for 20 s, and 72°C for 40 s, and a final extension for 6 min at 72°C. The reaction mixtures used 0.5 μ M of each primer, 250 μ M of deoxyribonucleotide triphosphate, and 5 units of Taq DNA polymerase (Evrogen JSC, Moscow, Russia) in a reaction volume of 50 μ L. Positive controls were carried out in parallel and contained template DNA with the gene of an individual toxin. Negative controls used ultrapure water instead of template DNA during PCR. The reaction mixture without the DNA matrix was used as a negative control. The PCR product was analyzed by electrophoresis in a 1.5% agarose gel stained by ethidium bromide (5 μ g/mL). The gels were photographed under UV light using the Kodak Gel Logic 100 Imaging System (Kodak, Rochester, NY).

Sequencing of PCR products of these genes was carried out by Evrogen JSC. The DNA sequences were analyzed using the BLAST search tool (<https://blast.ncbi.nlm.nih.gov/>).

Detection of Cytotoxin Genes

The detection of genes of PSM (*psmA*, *psmB*, *hld*), α -hemolysin (*hla*), and Panton–Valentine leukocidin (*lucS*) was performed by real-time PCR. The primers

for the toxin genes and the sizes of the amplification products are given in Table 1. Due to the small sizes of the sequences for PSM (*psmA1*, *psmA2*, *psmA3*, *psmA4*, *psmB1*, and *psmB2*), which are organized into 2 separate operons (*psmA1*–*psmA4* and *psmB1*–*psmB2*) and coded for short peptides, each operon was totally amplified. The PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 58°C for 20 s, and 72°C for 20 s; and 72°C for 5 min. The PCR Master Mix and SYBRGreen dye (Syntol JSC, Moscow, Russia) were used in the reactions. Melting curves analysis and random electrophoresis in a 2% agarose gel were carried out. Each sample was amplified in triplicate. The relative values of fluorescence for each sample were calculated according to the manufacturer's recommendations. A reaction mixture without a DNA template was used as a negative control.

Solid-Phase Sandwich ELISA

The production of SEA, SEB, SEC, SED, SEE, SEG, SEI, SELP, and TSST-1 in staphylococcal culture medium was determined by ELISA. The antibody pairs for sandwich assay were selected for each toxin and used for detection. Corresponding recombinant enterotoxins

Table 1. The PCR primers and the results of assay for the presence of toxin genes in the investigated *Staphylococcus aureus*

Target	Primer sequence (5'–3')	Amplicon size (bp)	Reference
<i>sea</i>	GGTTATCAATGTGCGGGTGG CGGCACTTTTTCTCTTCGG	102	Chapaval et al. (2006)
<i>seb</i>	GTATGGTGGTGTAAGTGAAGC CCAAATAGTGACGAGTTAGG	164	Chapaval et al. (2006)
<i>sec</i>	AGATGAAGTAGTTGATGTGTATGG CACACTTTTGAATCAACCG	451	Chapaval et al. (2006)
<i>sed</i>	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTT	278	Chapaval et al. (2006)
<i>see</i>	AGGTTTTTTTACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209	Chapaval et al. (2006)
<i>seg</i>	CCACCTGTTGAAGGAAGAGG TGCAGAACCATCAAACCTCGT	432	Cremonesi et al. (2005)
<i>sei</i>	CTCAAGGTGATATTGGTGTAGG CAGGCAGTCCATCTCCTGTA	529	Cremonesi et al. (2005)
<i>seh</i>	CACATCATATGCGAAAGCAGA CCTTTTAAATCATAAATGTCGAATGA	617	Xie et al. (2011)
<i>seip</i>	AAGACACCGCCAATATATAC ATCACCAGAAGAAGGGTG	356	This study
<i>tsst1</i>	TTTCCATGGATAATTCCTTAGGATCTATGCGT TTTGCGGCCGCTTTATTTTCATCAATATTTATAGGTG	500	This study
<i>psmA1</i> – <i>psmA4</i>	CTTTCACATGGGTATCATTGCAGG CAATAGCCATCGTTTTGTCTCCT	266	This study
<i>psmB1</i> – <i>psmB2</i>	TAATAATGACGGCGCAAATTAGG GCAACGATGTCTACGATACTTGTGC	238	This study
<i>lucS</i>	AAAGCAATGAGGTGGCCTTTCC CATTACCTCCTGTTGATGGACCAC	172	This study
<i>hla</i>	CTTCAGGGTTTTACACGAGACTTCG TTTCCAATTTGTTGAAGTCCAATGC	137	This study
<i>hld</i> (δ -toxin)	GAATTTGTTCACTGTGTCGATAATCC AATTAAGGAAGGAGTGATTTCATATG	91	Dastgheyb et al. (2015)

were used as positive controls. The culture medium of an *S. aureus* isolate without the enterotoxin genes was used as a negative control. The standard curves for quantitative analyses were constructed using recombinant enterotoxins. Culture supernatants of individual *S. aureus* isolates were preincubated with 20% (vol/vol) normal rabbit serum at 4°C overnight to avoid any nonspecific reaction caused by protein A (Omoe et al., 2002). The ELISA were performed in 96-well microplates (Greiner Bio-One GmbH, Kremsmünster, Austria) using the biotin-streptavidin system for detection. We used a streptavidin conjugated with peroxidase for signal visualization (Thermo Fisher Scientific). Orthophenylenediamine hydrochloride (0.5 mg/mL) in 0.1 M citrate buffer (pH 5.0) was used as a substrate solution, and the absorbance at 490 nm was read with an iMar Microplate Absorbance Reader (Bio-Rad, Hercules, CA). Data were collected from triplicate measurements of each culture. The concentration of each toxin in the culture supernatants was determined by converting the absorbance values to the corresponding concentrations by the use of a standard curve. The method was developed as described by Pujol et al. (1993) with a slight modification.

RESULTS

The multiplex PCR assay for the detection of staphylococcal enterotoxin genes was carried out for 60 isolates. Fifty-three (88%) isolates had toxicogenic properties with the following genes identified in the corresponding number of isolates: *sea* in 32 (53.3%), *seb* in 2 (3.3%), *sec* in 30 (50%), *sed* in 3 (4%), *see* in 28 (46.6%), *seg* in 42 (70%), *sei* in 6 (10%), *selp* in 2 (3.3%), and *tsst1* in 1 (1.6%) isolate (Table 1). The *seh* gene was not identified. Simultaneous detection of *seg* and *sei* was observed in 5 isolates. The genes encoding SEG, SEC, and SEA toxins were the most abundant (Figure 1).

Figure 1. Polymerase chain reaction amplification of staphylococcal enterotoxin genes in *Staphylococcus aureus* isolates. Products of multiplex PCR in 1.5% agarose gel. L = GeneRuler DNA Ladder Mix (Fermentas, Waltham, MA); *se* = marker of toxin genes; lanes 1 to 8 = PCR products of *S. aureus* isolates; lane 9 = *sec* gene (control marker).

The sequences of *se* and *tsst1* genes were determined. The AA sequences were confirmed to correspond to GenBank accession numbers: SEA, AAA26681.1; SEB, EXP38233.1; SEC, BAF78878.1; SED, ANJ16443.1; SEE, AAA26617.1; SEI, EYO43459.1; SEG, EHT83812.1; SELP, EWO51483.1; and TSST-1, AAL71885.1.

The results of PCR for detecting the presence of genes encoding PSM and pore-forming toxins were as follows: *hla*, 70%; *lucS*, 46.6%; *psmA*, 81.6%; *psmB*, 95%; and *hld*, 78.3%. Considering the fact that the presence of genes does not guarantee their expression, we analyzed the ability of isolates to produce enterotoxins and TSST-1. All isolates containing the *seb*, *sed*, *see*, *sei*, *selp*, and *tsst1* genes were analyzed. For isolates that contained the *sea*, *sec*, *see*, and *seg* genes, 10 isolates were selected for the expression analysis of each gene. Each isolate that contained the *sea*, *see*, *seg*, *sei*, *selp*, and *tsst1* genes was capable of expressing the corresponding enterotoxin. Production of SEG in the culture medium did not exceed 1 ng/mL, SELP did not exceed 0.5 ng/mL, and SEA did not exceed 0.5 to 1 ng/mL. Concentrations of SEI in the culture medium ranged from 1 up to 4 ng/mL, and SEE concentrations ranged from 1 up to 10 ng/mL. The concentration of TSST-1 was 0.3 ng/mL, and TSST-1 was detected only once. Two isolates were characterized by simultaneous coexpression of 2 enterotoxins: SEE and SEI for isolate number 13, and SEE and SEG for isolate number 66. Another 2 isolates were able to simultaneously express 3 enterotoxins: TSST-1, SEG, and SEI for isolate number 17 and SEI, SEG, and SEE for isolate number 9 (Figure 2). The highest level of expression was detected for isolate number 66, which produced SEE at 18 ng/mL. The production of SEB, SEC, and SED was not detected in any isolate, although the corresponding genes were identified.

DISCUSSION

Cow mastitis is a common infectious disease both in the territory of the Russian Federation and around the world. In Russia, more than 20% of cows are discarded after the disease due to atrophy of one or several quarters of the udder. Because of mastitis, the annual herd yield is reduced by 8 to 12% (Artem'eva et al., 2015).

Studying the problem of mastitis and its associated pathogens is a current focus of research. *Staphylococcus aureus* plays an important role in the development of this disease. The presence of *S. aureus* in samples isolated from cows with subclinical mastitis varies from 4.8% in Brazil to 28.6% in Turkey (Karahana et al., 2009; Rall et al., 2014). It is considered usual to find enterotoxin genes in *S. aureus* isolated from affected

Figure 2. Detection of enterotoxins in the culture medium of *Staphylococcus aureus* isolates by sandwich ELISA. Recombinant enterotoxins were used as positive controls. Culture media of isolates without enterotoxin genes were used as negative controls. The *S. aureus* isolates were cultivated in vitro at 37°C with aeration for 14 to 16 h. Culture supernatants were preincubated with 20% (vol/vol) normal rabbit serum before analysis by sandwich ELISA. Data are from triplicate measurements of each culture and presented as the mean $\pm$ SD.

animals in various regions (Mello et al., 2016; Rola et al., 2016; Wang et al., 2016). However, data on the prevalence of staphylococcal exotoxins in the Russian Federation are very limited. In the present work, we investigated the presence of exotoxin genes in *S. aureus* isolates from cows with subclinical mastitis and analyzed the ability to express some of these genes.

One of the main enterotoxin genes of *S. aureus* isolated from cows with mastitis is *sea*. We detected *sea* in 32 (53.3%) isolates; *sea* was not the dominant enterotoxin gene among the isolates that we analyzed, but it was present at a high level. In previously published studies, this gene occurred at rates ranging from 23.6 to 50% (Rall et al., 2014).

The major enterotoxin gene in our investigation turned out to be *seg*. It was detected in 42 (70%) isolates. In previous works of other authors, this gene was detected at rates ranging from 30 to 40% of isolates (Wang et al., 2009; Rall et al., 2014). Despite the fact that the *seg* and *sei* genes are localized together in the *egc* cluster, we detected the *sei* gene in only 10% of our isolates.

High levels of detection were observed for the *sec* and *see* genes, which were detected at rates of 50 and 46.6% of our isolates, respectively. Based on previous work, these genes are rarely present in cow mastitis. The presence of the *sec* gene varied from almost 10% of coagulase-negative staphylococci to 9 to 20% of *S.*

aureus (Rola et al., 2015). The *see* enterotoxin gene has not been found in previous related works (Wang et al., 2009; Rola et al., 2015). Despite the fact that pathogenicity islands can carry the *sec-sell-tst* genes, in our case, *tsst1* was detected in 1.6% of the isolates.

All other enterotoxin genes were rarely present: *seb* in 2 (3.3%), *sed* in 3 (4%), and *selp* in 2 (3.3%) of the isolates. In addition, despite the previously demonstrated important role of SEH, we did not detect its gene; SEH is the inducer of mammary epithelial cell apoptosis (Liu et al., 2014), and thus this toxin plays an important role in the mastitis process.

Staphylococcal exotoxins can act in a cooperative manner by influencing both the mammary tissues and the immune system of the animal. Thus, the next step of our work was the detection of cytotoxin genes. A significant amount of effort has been devoted to studying the role of α -hemolysin in mastitis development (Vandenesch et al., 2012). We were able to identify the *hla* gene in 70% of the isolates. In a previous work, Wang et al. (2016) showed that 94% of staphylococcal isolates associated with mastitis contain the *hla* gene. The occurrence of the *hla* gene exceeded one-third of the investigated samples in an Italian region (Artursson et al., 2016). In addition, the high lethality associated with hemolysin production was shown on a murine model of mastitis caused by *S. aureus* (Bramley et al., 1989). The specific feature of the α -toxin is its ability to kill neutrophils, which are important constituents of natural immunity (Abtin et al., 2014). This effect of hemolysin production has been described for skin infections and presumably should occur at their site of action on mammary tissues.

Previous work of Unal and Cinar (2012) reported that the *pvl* gene was detected in 5% of coagulase-negative staphylococcal isolates. However, earlier works found a spread of 0 to 56% (Zecconi et al., 2006; Aires-de-Sousa et al., 2007). Here, we detected the *lukS* gene in 46.6% of our isolates.

The *psm* genes were the most commonly detected among our isolates. The gene *psmA* was identified in 81.6%, *psmB* in 95%, and the gene of the δ -toxin (*hld*) in 78.3% of the isolates. These peptide toxins play an important role in mastitis. According to Deplanche et al. (2016), PSM activate host immune system cells by regulating the spectrum and level of production of lymphokines. However, PSM have a wider range of functional activities, including their roles in quorum sensing, regulating the activity of the genes of staphylococcal virulence factors, and suppressing competitive microorganisms (Peschel and Otto, 2013; Joo et al., 2016).

Of great interest is the ability of isolates to produce enterotoxins. Considering that not all toxins are se-

creted into the culture medium (Oliveira et al., 2011), we tested for the actual capability of our isolates to produce enterotoxins. We analyzed the enterotoxin production levels in some isolates that contained enterotoxins genes. We found that all investigated isolates had a very low level of SE production. We also detected a minimal level of expression of TSST-1 (0.3 ng/mL), which was identified for 1 isolate. The maximal level of production was detected for SEE, which reached 18 ng/mL. The production of SEB, SEC, and SED in the culture medium was not confirmed. However, we found that 3.3, 50, and 4% of our isolates contained the *seb*, *sec*, and *sed* genes, respectively. The loss of toxin production, despite the presence of the enterotoxin gene, was described earlier by other authors (Rall et al., 2014). However, such a characteristic is inherent to coagulase-negative staphylococci. The combination of exotoxin genes determined in our isolates suggests a high potential for mastitis induction and necessitates further investigation into how they may cooperate to induce and develop mastitis in cows.

CONCLUSIONS

In the present work, we showed that subclinical mastitis in cows is associated with *S. aureus* in the central region of the Russian Federation. Enterotoxin genes were identified, and we detected the potential ability to express several of these genes. In addition, most of the isolates containing enterotoxin genes also had cytotoxin genes. We assume that such co-occurrence represents a cooperative process that plays an important role in the development of mastitis due to *S. aureus* invasion of the tissue of the mammary glands.

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