J. Dairy Sci. 101:1–7
https://doi.org/10.3168/jds.2017-14074
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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%; *selp*, 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.

Received October 31, 2017.
Accepted January 16, 2018.
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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 × *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 μg/mL) and RNase A (30 μg/mL) and incubated for 0.5 h at 37°C. Proteinase K (200 μg/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 × *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 × *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 μL 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 μg/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

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