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Prevalence of enterotoxin genes and antimicrobial resistance of coagulase-positive staphylococci recovered from raw cow milk

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ABSTRACT

Raw milk may be contaminated by enterotoxigenic coagulase-positive staphylococci (CPS). Several of these microorganisms show antimicrobial resistance, which poses a potential risk for consumers. The aim of this study was to determine the occurrence of enterotoxin genes and antimicrobial resistance of CPS isolated from cow milk. A total of 115 samples were analyzed for the presence of CPS according to the International Organization for Standardization standard (ISO 6888–2). The genes were identified using 2 multiplex PCR assays. Resistance of the isolates to 10 antimicrobials was determined using the minimum inhibitory concentration method. Overall, 71 samples (62%) were contaminated with CPS and 69 isolates were further analyzed. Among them, 20 (29%) strains harbored the enterotoxin genes. The most commonly detected staphylococcal enterotoxin markers were *sed*, *sej*, and *ser*, whereas none of the analyzed isolates possessed the *seb* and *see* genes. Almost one-half of the tested strains (43%) were resistant to one or more antimicrobial agents. Resistance to penicillin was the most common, followed by sulfamethoxazole and chloramphenicol. On the other hand, all strains were susceptible to ciprofloxacin, erythromycin, gentamicin, cefoxitin, and streptomycin. None of the strains was positive for the *mecA* and *mecC* (methicillin-resistant *Staphylococcus aureus*) genes. These results indicate that enterotoxigenic and antimicrobial resistant CPS strains are present in raw milk, which may be a potential risk for public health.

Key words: milk, staphylococci, antimicrobial resistance, enterotoxin genes

INTRODUCTION

Staphylococcus aureus is a common microorganism present on the skin and mucosal surfaces of humans

and animals as well as in the environment. Contamination of raw milk with *S. aureus* may occur from infected dairy animals, but human handling, water, and milking equipment may also be important sources of these bacteria (Bergonier et al., 2003; Jørgensen et al., 2005a,b). *Staphylococcus aureus* produces a variety of extracellular proteins, including staphylococcal enterotoxins (STE), which cause staphylococcal food poisoning (SFP; Yesim Can and Haluk Celik, 2012). The symptoms of this illness include nausea, vomiting, abdominal cramps, and diarrhea occurring 1 to 8 h after consumption of contaminated food. Five main STE types (A, B, C, D, and E) responsible for the symptoms of SFP have been identified. In addition, several other variants of STE or staphylococcal-like toxins have been described (Lina et al., 2004; Ono et al., 2008).

The emergence of antibiotic-resistant *S. aureus* in farm environment is a potential risk for public health. Antibiotics on dairy farms are used to treat infections such as mastitis and as a preventive measure during dry cow therapy (Haran et al., 2012). The major groups of antimicrobial agents introduced for therapeutic use in food-producing animals are β -lactams, tetracyclines, aminoglycosides, macrolides, and sulfamethoxazole. The discovery of the third generation of fluoroquinolones with a broader spectrum activity has led to interest in their use in animals (Brown, 1996). In the last decade, bacterial isolates from food have shown a considerable increase in resistance against most antibiotics (Yesim Can and Haluk Celik, 2012). Since 1960, methicillin and oxacillin have been used for the effective control of staphylococcal infections; however, methicillin-resistant *S. aureus* (MRSA) strains are now found with increasing frequency in many countries (Lowy, 2003). Such microorganisms are also often resistant to other antimicrobial agents, including aminoglycosides, macrolides, chloramphenicol, tetracyclines, and fluoroquinolones (Türkyilmaz et al., 2010). Methicillin resistance in MRSA is determined by the chromosomally located *mecA* gene, which encodes a penicillin binding protein (PBP2') with a low affinity for β -lactams; therefore, such strains are resistant to all β -lactam antibiotics (Bystron et al., 2009). The MRSA genotypically classified under clonal complex 398 has been detected among

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pig farmers in the Netherlands and other countries (Khanna et al., 2008; Denis et al., 2009) and is known to cause infections in humans and animals (Witte et al., 2007). Such strains were also identified in cattle, and their presence in milk possess a potential risk to people working with cattle such as farm workers and veterinarians as well as milk consumers (Vanderhaeghen et al., 2010).

The objective of this study was to determine the prevalence of enterotoxin genes and antimicrobial resistance in coagulase-positive staphylococci isolated from raw cow milk collected in Poland.

MATERIALS AND METHODS

Milk Samples

A total of 115 samples of raw milk collected between 2009 and 2013 from 15 dairy farms and 15 dairies located in the eastern part of Poland were used in this study. After sampling, milk was transported to the laboratory under refrigeration within 24 h for further analyses.

Isolation and identification of CPS

Isolation of CPS was performed using Baird-Parker agar with rabbit plasma fibrinogen (bioMérieux, Marcy l'Etoile, France) at $37 \pm 1^\circ\text{C}$ for 48 h (ISO 6888-2, 1999). One suspected colony from each sample was cultured in brain heart infusion broth (Oxoid, Basingstoke, UK) at 37°C for 24 h for further analysis, including catalase reaction, hemolytic properties, and ability to coagulate rabbit plasma (coagulase tube test).

DNA Isolation

Staphylococcus aureus isolates were grown in brain heart infusion broth at 37°C for 24 h, and 1 mL of the culture was transferred to Eppendorf tubes and centrifuged at $13,000 \times g$ for 1 min at room temperature. Then, the bacterial cells were treated with 10 μL of lysostaphin (1 mg/mL; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C and DNA was extracted using the Genomic-Mini kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions.

Detection of Enterotoxin Genes

Two multiplex PCR (mPCR) assays were used to detect STE-encoding genes. The first (mPCR1), performed with 6 pairs of primers, allowed detection of the following genes: *sea*, *seb*, *sec*, *sed*, *see*, and *ser* (De Buyser et al., 2009a). The second reaction (mPCR2)

enabled us to identify the *seg*, *seh*, *sei*, *sej*, and *sep* genes (De Buyser et al., 2009b). Both mPCR were carried out in a TProfessional Standard Thermocycler (Biometra, Jena, Germany) with the conditions as follows: 94°C for 3 min, then 35 cycles at 94°C for 30 s, 55°C for 40 s (mPCR1), and 52°C for 30 s (mPCR2), 72°C for 90 s with final extension at 72°C for 7 min. The amplified PCR products were visualized by standard gel electrophoresis in a 2% agarose gel stained by ethidium bromide (5 $\mu\text{g}/\text{mL}$). The gels were photographed under UV light using the Gel-Doc 2000 system (Bio-Rad, Hercules, CA).

Identification of the MRSA

The MRSA was identified by PCR detection of the *mecA*, *nuc*, and *16S rRNA* genes as described previously (National Food Institute, 2008). The presence of the *mecC* gene was also analyzed according to the PCR protocol recommended by the European Union Reference Laboratory for Antimicrobial Resistance (National Food Institute, 2012). Briefly, in both PCR DNA was amplified by 30 cycles of denaturation (94°C for 30 s), annealing (55°C and 59°C for 30 s, respectively), and elongation (72°C for 1 min).

Determination of Phenotypic Antimicrobial Resistance

Staphylococcus aureus isolates were cultured on Columbia agar supplemented with 5% sheep blood (bioMérieux) at $37^\circ\text{C} \pm 1^\circ\text{C}$ for $24 \text{ h} \pm 2 \text{ h}$. After incubation, a suspension of 0.5 McFarland density was prepared and 50 μL was transferred to 11 mL of Mueller-Hinton broth (Trek Diagnostic Systems, East Grinstead, UK). Afterward, 50 μL of bacterial suspension in Mueller-Hinton broth was used to inoculate microplates (DKVP, Trek Diagnostic Systems) with the following antimicrobials ($\mu\text{g}/\text{mL}$): chloramphenicol (2–64), ciprofloxacin (0.12–8), erythromycin (0.25–16), gentamicin (0.25–16), penicillin (0.06–16), streptomycin (4–64), sulfamethoxazole (32–512), tetracycline (0.5–32), trimethoprim (0.5–32), and cefoxitin (0.5–32). The reference strain of *S. aureus* ATTC 25923 was used as a control for each microplate. The plates were incubated for 18 to 24 h at $36^\circ\text{C} \pm 1^\circ\text{C}$ and MIC, defined as the lowest concentration of antibiotics in which the bacterial growth was totally inhibited, were read using the Vision system (Trek). The cutoff values for the interpretation of the MIC results were in accordance with the European Committee on Antimicrobial Susceptibility Testing and the European Union Reference Laboratory for Antimicrobial Resistance.

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