J. Dairy Sci. 96:1–11
http://dx.doi.org/10.3168/jds.2012-6091

Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk

Yvonne Frey, Joan Peña Rodriguez, Andreas Thomann, Sybille Schwendener, and Vincent Perreten[1]
Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, CH-3012 Bern, Switzerland

ABSTRACT

Coagulase-negative staphylococci (CNS; n = 417) were isolated from bovine milk and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Nineteen different species were identified, and *Staphylococcus xylosus*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, and *Staphylococcus sciuri* were the most prevalent species. Resistance to oxacillin (47.0% of the isolates), fusidic acid (33.8%), tiamulin (31.9%), penicillin (23.3%), tetracycline (15.8%), streptomycin (9.6%), erythromycin (7.0%), sulfonamides (5%), trimethoprim (4.3%), clindamycin (3.4%), kanamycin (2.4%), and gentamicin (2.4%) was detected. Resistance to oxacillin was attributed to the *mecA* gene in 9.7% of the oxacillin-resistant isolates. The remaining oxacillin-resistant CNS did not contain the *mecC* gene or *mecA1* promoter mutations. The *mecA* gene was detected in *Staphylococcus fleurettii*, *Staphylococcus epidermidis, Staph. haemolyticus*, and *Staph. xylosus*. Resistance to tetracycline was attributed to the presence of *tet*(K) and *tet*(L), penicillin resistance to *blaZ*, streptomycin resistance to *str* and *ant(6)-Ia*, and erythromycin resistance to *erm*(C), *erm*(B), and *msr*. Resistance to tiamulin and fusidic acid could not be attributed to an acquired resistance gene. In total, 15.1% of the CNS isolates were multidrug resistant (i.e., resistant to 2 or more antimicrobials). The remaining CNS isolates were susceptible to antimicrobials commonly used in mastitis treatment. Methicillin-resistant CNS isolates were diverse, as determined by *mecA* gene sequence analysis, staphylococcal cassette chromosome *mec* typing, and pulsed-field gel electrophoresis. Arginine catabolic mobile element types 1 and 3 were detected in both methicillin-resistant and methicillin-susceptible *Staph. epidermidis* and were associated with sequence types ST59 and ST111. Because this study revealed the presence of multidrug-resistant CNS in a heterogeneous CNS population, we recommend antibiogram analysis of CNS in persistent infections before treatment with antimicrobials.

Key words: methicillin-resistance, coagulase-negative staphylococci, genotyping, antibiotic resistance

INTRODUCTION

Coagulase-negative staphylococci are the microorganisms most commonly isolated from bovine milk in many countries, and they are an important cause of mastitis (Pyörälä and Taponen, 2009; Rajala-Schultz et al., 2009; Piessens et al., 2011; De Vliegher et al., 2012). The CNS are opportunistic pathogens that are usually diagnosed as a group without species identification. They cause subclinical IMI that result in an increase in SCC and reduced milk quality, leading to economic losses (Pyörälä and Taponen, 2009). Because simple subclinical CNS infections can be self-limiting, they are usually not treated with antibiotics. However, CNS often appear with other major pathogens such as *Staphylococcus aureus*, *Streptococcus* spp., or coliform bacteria. In these cases and in persistent CNS infections, the cows undergo antimicrobial treatment. Currently, β-lactam antimicrobials (including penicillin and cephalosporins), aminoglycosides (gentamicin and neomycin), and macrolides (spiramycin) are commonly used to treat mastitis in Switzerland (Büttner et al., 2011). Resistance to these antibiotics has been increasingly reported in CNS associated with bovine mastitis (Walther and Perreten, 2007; Sawant et al., 2009; Sampimon et al., 2011). The CNS may also harbor antimicrobial resistance elements and pathogenicity islands, such as the staphylococcal cassette chromosome (**SCC*mec***) element (Wielders et al., 2001; Barbier et al., 2010; Tsubakishita et al., 2010) and the arginine catabolic mobile element (**ACME**; Diep et al., 2006, 2008; Miragaia et al., 2009) that can be transferred to *Staph. aureus*. Arginine catabolic mobile elements are genomic islands in *Staph. epidermidis* that are associated with host colonization, fitness, and pathogenicity. Mobility of ACME is associated with recombinase genes present on the SCC*mec* elements (Goering et al., 2007; Diep et al., 2008). The SCC*mec* elements contain the *mec* genes—*mecA* or *mecC* ($mecA_{LGA251}$)—which

Received August 27, 2012.
Accepted November 11, 2012.
[1]Corresponding author: vincent.perreten@vetsuisse.unibe.ch

encode alternative penicillin-binding proteins (**PBP 2a**) and confer resistance to all β-lactam antimicrobials (García-Álvarez et al., 2011; Ito et al., 2012). In *Staph. sciuri*, the *mecA* gene homolog *mecA1* is a native gene that is not part of the *mec* gene complex (Couto et al., 1996, 2000; Wu et al., 1998, 2001; Tsubakishita et al., 2010). Most *Staph. sciuri* isolates are susceptible to β-lactam antimicrobials. However, alterations in the promoter regions of *mecA1* upregulate *mecA1* expression and confer methicillin resistance (Wu et al., 2001, 2005; Couto et al., 2003). Methicillin-resistant staphylococci are often also resistant to other classes of drugs such as aminoglycosides and macrolides (Woodford 2005). Nevertheless, little is known about the molecular mechanisms of antimicrobial resistance (Lüthje and Schwarz, 2006) or the genetic background of multidrug-resistant CNS strains in bovine milk.

We identified different CNS species in milk from cows with clinical and subclinical bovine mastitis, characterized their antimicrobial resistance mechanisms, and determined whether specific methicillin-resistant and multidrug-resistant CNS clones are common in dairy cows.

MATERIALS AND METHODS

Origin of Milk Samples

Coagulase-negative staphylococci (n = 417) were isolated from milk (n = 370) obtained from cows diagnosed with clinical (n = 115) and subclinical (n = 255) mastitis and control samples (n = 47) in Switzerland. Control samples were collected from cows that had suffered from mastitis previously and had been treated; the control milk samples contained <150,000 cells/mL. The 417 isolates came from 363 different cows and from 2 different mammary quarters of 7 cows. The 363 cows originated from 195 different farms ($\mathbf{n_f}$) in the cantons of Berne (n_f = 91), Jura (n_f = 56), Fribourg (n_f = 26), Vaud (n_f = 8), Lucerne (n_f = 5), Valais (n_f = 4), Solothurn (n_f = 3), Aargau (n_f = 1), and Thurgau (n_f = 1). In 47 cases, 2 different CNS strains were found in the same milk sample.

Isolation and Identification of CNS

Milk samples were centrifuged at 590 × *g* for 10 min at room temperature. The milk pellets were cultivated on tryptone soy agar containing 5% defibrinated sheep blood (Becton, Dickinson and Co., Franklin Lakes, NJ) and incubated at 37°C for 18 to 24 h. Staphylococci were selected based on colony morphology, gram-positive staining of cocci, and catalase production and were subcultured on tryptone soy agar containing 5% defibrinated sheep blood.

The isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-TOF MS**) analysis using the ethanol–formic acid extraction method for better resolution (Microflex LT, Bruker Daltonics GmbH, Bremen, Germany; Application Note MT-80, Bruker Daltonics GmbH). Species identification was considered valid when the matching score with reference spectra of the MALDI Biotyper v3.0 database (Bruker Daltonics GmbH) was ≥2, according to the criteria proposed by the manufacturer. Isolates whose measured spectra had score <2.0 were further identified by DNA sequencing of the 16S rDNA (Kuhnert et al., 1996). The CNS strains were stored at −80°C in trypticase soy medium containing 30% glycerin (Becton, Dickinson and Co.).

DNA Extraction and Amplification

To obtain total DNA, cells were incubated in 100 μL of Tris-EDTA buffer containing 0.1 mg/mL lysostaphin for 15 min at 37°C; then, 450 μL of lysis buffer (0.1 *M* Tris-HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/mL proteinase K) was added and incubated at 60°C for 45 min. The DNA was then denatured at 95°C for 15 min. The PCR was performed with HOT FIREPol DNA Polymerase (Solis BioDyne, Tartu, Estonia) using the primers and conditions listed in Table 1.

Antimicrobial Resistance Tests

The CNS isolates were tested for antimicrobial susceptibility with the broth microdilution technique (Clinical and Laboratory Standards Institute, 2009) using Sensititre susceptibility plates (NLEUST plates; Trek Diagnostics Systems, East Grinstead, UK) that contained the following 19 antimicrobials: chloramphenicol, ciprofloxacin, clindamycin, dalfopristin-quinupristin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, oxacillin, penicillin, rifampicin, streptomycin, sulfamethoxazole, tetracycline, tiamulin, trimethoprim, and vancomycin. The resistance breakpoints were those proposed for CNS in the guidelines of the European Committee on Antimicrobial Susceptibility Testing (**EUCAST**, www.eucast.org; Table 2), except for streptomycin and kanamycin, for which breakpoints came from the French Society for Microbiology (www.sfm-microbiologie.org). The production of β-lactamase was tested on nitrocefin dry slides (Becton, Dickinson and Co.) using colonies grown on Mueller Hinton agar for 18 h at 37°C with 0.05 μg/mL penicillin to induce β-lactamase production (Schnellmann et al., 2006). The