Characterization of erythromycin-resistant methylase genes from multiple antibiotic resistant Staphylococcus spp isolated from milk samples of lactating cows

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Objective—To isolate and characterize erythromycin-resistant methylase genes in multiple-antibiotic resistant staphylococci isolated from milk samples.

Animals—300 lactating cows.

Procedure—23 erythromycin-resistant staphylococci were isolated from milk samples of 300 lactating cows. The prevalence of erythromycin-resistant methylase (erm) genes, ermC and ermA genes, and the multicomponent macrolide efflux pump in staphylococci msrA genes were identified and characterized by use of multiplex polymerase chain reaction (PCR), Southern hybridization, restricted fragment length polymorphism (RFLP) analysis, and dot-blot hybridization.

Results—Biochemical characterization indicated that 3 of 23 (13%) isolates were coagulase-positive Staphylococcus aureus, and the rest were coagulase-negative. Multiplex PCR resulted in amplification of a 520-base pair (bp) region of the ermC gene from the cell lysates of a strain of S simulans M-21 and S sciuri M-28. The ermC gene in both isolates was found on a 3-kilobase plasmid. The ermA gene was found on the chromosome of 21 isolates, and 6 RFLP patterns were observed. None of the isolates harbored the msrA gene.

Conclusions—Erythromycin-resistant Staphylococcus spp isolated from milk samples of lactating cows may serve as reservoirs of erm genes homologous to those described in human isolates. However, the chromosomal insert patterns and prevalence of these genes, the sizes of plasmids harboring the genes, and the number of inserts of the genes (copy number) may differ from that of human isolates. (Am J Vet Res 2000;61:1128–1132)

Masititis, as a disease of dairy cattle, may be caused by Staphylococcus spp, Streptococcus spp, Micrococcus spp, Mycoplasma spp, Corynebacterium spp, Mycobacterium spp, and Klebsiella spp. Economic losses resulting from mastitis have been estimated to be more than $3 billion annually in the United States. Antibiotic treatment of dairy cows during nonlactation and lactation periods reduces infection by 40 to 75%. However, suboptimal treatment regimens with antibiotics may favor antibiotic-resistant bacteria. The development and dissemination of such antimicrobial resistant strains may have considerable impact on the efficacy of future treatment, the control of disease, and the health and productivity of dairy cows.

Erythromycin or its derivatives are commonly used for the control or prevention of mastitis in nonlactating and lactating cows. Extensive and indiscriminate use of erythromycin has led to the spread of resistant bacteria. This suggests that these bacteria may be coding for erythromycin-resistant methylase (erm) genes. In 1994, Roberts and Brown isolated several strains of erythromycin-resistant Streptococcus spp from milk from cows with mastitis and demonstrated that ermB, ermF, ermG, and ermQ genes were coding for resistance to erythromycin in these bacteria. Although Staphylococcus spp account for more than 60% of all mastitis infections, little information is available on the molecular nature of their erm genes. Although Staphylococcus spp may act as a reservoir of erm genes. The purposes of the study presented here were to determine the prevalence of erythromycin-resistant Staphylococcus spp in milk samples from lactating dairy cows, to characterize biochemical and drug resistance profiles of these isolates, and to characterize erm and msrA genes in these bacteria.

Materials and Methods

Bacterial isolation—Milk samples were collected from lactating cows and shipped on ice by the Arkansas Dairy Corporation. Milk samples (1 ml) were enriched in 10 ml of Luria Bertani broth overnight at 37 C. The bacterial suspension was plated on phenylethyl alcohol agar, which is specific for the isolation of gram-positive bacteria. Pure colonies were further characterized and identified by use of a commercially available system. All isolates were stored in Luria Bertani broth containing 20% glycerol at –70 C for preservation. Organisms were grown overnight at 37 C in Luria Bertani broth or on trypticase soy agar plates supplemented with 5% sheep blood for plasmid or chromosome isolation.

Susceptibility testing—Antibiotic susceptibilities of each strain of Staphylococcus spp were determined by use of a disk diffusion test with 150-mm-diameter culture plates that contained Mueller-Hinton agar medium. Disks of ampicillin (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg),...
penicillin (10 U), nalidixic acid (30 µg), streptomycin sulfate (10 µg), and tetracycline (30 µg) were used.

Multiplex polymerase chain reaction—Cells from the overnight cultures (1.5 ml) were centrifuged at 10,000 X g for 1 minute at 5 C and washed with 500 µl Tris-EDTA buffer (10 mM, pH 8). The resultant pellets were then suspended in 1 ml Tris-EDTA buffer containing 0.25% Triton X-100. Bacterial lysates were then prepared by placing the cell suspension for 15 minutes in a boiling water bath followed by quick cooling and centrifugation at 10,000 X g for 5 minutes at 4 C. A 10-fold dilution of the resulting supernatant was used as a source of template DNA for ermC and ermA gene detection. The gene-specific multiplex polymerase chain reaction (PCR) primers (Table 1) were used for simultaneous amplification of the ermC and ermA genes. The reaction was performed in a total volume of 50 µl. Each reaction tube contained 5 µl of the bacterial lysate (5 to 10 ng of DNA), 5 µl of 10X-PCR buffer (500 mM KCl, 20 mM MgCl2, 100 mM Tris-HCl, pH 8.3, 0.01% gelatin [wt/vol]), 12.5 µl of a 4 µM mixture of the ermC and ermA gene-specific primers (1 µM each), 8 µl of a 1.25 mM mixture of deoxynucleoside triphosphate (dNTP), 19.25 µl of water, and 0.25 µl of (5 U/µl) Taq DNA polymerase. The reaction mixture was layered with 50 µl of sterile mineral oil. Thirty-five cycles of amplification were performed. Each cycle consisted of a 60-second denaturation step at 95 C, a 55-second annealing step at 53 C, and a 60-second amplification step at 72 C. The first cycle of denaturation and the last cycle of amplification were extended by 3 minutes. The amplified products were separated on a 1.2% agarose gel at 40 mA for 4 hours, followed by ethidium bromide staining and photography. The authenticity of the *ermC* and *ermA* gene PCR products was confirmed by use of restriction digestion with *FokI*. Restriction digestion of the PCR products with *FokI* resulted in 477- and 132-base pair (bp) *ermC* gene digestion products and 333- and 187-bp *ermC* gene digestion products.

Southern-blot hybridization—Plasmid DNA was separated by use of electrophoresis in agarose gels (1.2%), deparaffinized, denatured, neutralized, vacuum blotted, and fixed onto a nylon membrane, using exposure to ultraviolet light. The *ermC* gene oligonucleotide probe (Table 1), labeled at the 3’-terminus by use of a terminal transferase, was used in the hybridization reaction. The nylon membrane was prehybridized for 2 hours and then hybridized at 42 C for 16 hours with the *ermC* gene-labeled oligonucleotide probe. Washing, blocking, development, and detection of DNA by use of Southern-blot analysis were performed according to the instructions in the DNA labeling and detection kit.

Isolation of chromosomal DNA—Total DNA was prepared as described by Thakker-Varia et al.12 For restriction analysis, 2 µg of DNA was digested with *EcoRI*. This enzyme does not cut the *ermA* gene internal probe (Table 1), so each chromosomal band that hybridizes with this probe represents 1 *ermA* gene insert. The use of agarose gel electrophoresis of restriction endonuclease-digested DNA was followed by Southern-blot analysis, prehybridization for 2 hours, and hybridization with the *ermA* gene probe at 58 C overnight. Washing, blocking, development, and detection of DNA by use of Southern-blot analysis were performed according to the instructions in the DNA labeling and detection kit. Color development was stopped after 2 hours of incubation.

Dot-blot hybridization—Chromosomal DNA suspensions (3 µg) were directly blotted on nylon membrane filters and allowed to air dry for 10 minutes. The blots were denatured and neutralized. The DNA was fixed by use of ultra violet light exposure, followed by prehybridization and hybridization with the *msrA* gene probe at 40 C. Purified plasmid *pUL5050*, which encoded for the *msrA* gene, was used as a probe.22 Washing, blocking, development, and detection of DNA by use of Southern-blot analysis were performed according to the instructions in the DNA labeling and detection kit. Color development was stopped after 2 hours of incubation.

Control plasmids—Positive controls for the *ermA* gene were plasmid *pEM9698* and *pEM9592*. The positive controls for *ermC* and *msrA* genes were plasmid *pE194* and *pUL5030*, respectively.

Results

Erythromycin-resistant bacteria—Twenty-three erythromycin-resistant *staphylococci* were selected from 360 gram-positive bacteria that had the typical *staphylococcal* morphologic characteristics. Of the 23 isolates, 3 were coagulase-positive and were identified as *S. aureus*. The remaining 20 coagulase-negative isolates were identified as *S. epidermidis* (4 isolates), *S. sciuri* (4), *S. auricularis* (3), *S. simulans* (3), *S. saprophyticus* (2), *S. equorum* (1), *S. hominis* (1), *S. lentus* (1), and *S. xylosus* (1). These isolates were also resistant to penicillin, ampicillin, tetracycline, and streptomycin (data not shown).

Detection of *ermC* and *ermA* genes—The PCR analysis was performed with template DNA from the 23 erythromycin-resistant *staphylococcal* isolates (Fig 1). Template DNA from *S. simulans* M-21 and *S. sciuri* M-28 was amplified with the *ermC* gene-specific

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**Figure 1**—Simultaneous polymerase chain reaction (PCR) amplification of *ermC* and *ermA* genes from erythromycin-resistant *staphylococcal* strains isolated from milk of lactating cows. Lanes 1 and 13, 100-base pair (bp) ladder; lane 2, 520-bp amplified DNA from *S. simulans* (strain M-21); lane 3, 610-bp amplified DNA from *S. sciuri* (strain M-28); lanes 4 and 5, 520-bp amplified PCR product from *S. simulans* (strain M-21) and *S. sciuri* (strain M-28); lanes 6 to 12, 610-bp amplified DNA from *S. aureus*, *S. auricularis*, *S. epidermidis*, *S. equorum*, *S. hominis*, *S. simulans*, and *S. sciuri* after PCR.
primers and subsequently produced a 520-bp amplified DNA fragment, indicative of the \textit{ermC} gene. The \textit{ermC} gene-specific primers failed to produce the 520-bp amplified DNA from the other 21 staphylococcal isolates. This indicated that these isolates do not harbor the \textit{ermC} gene. Purification and restriction digestion of the amplified DNA fragment with 
\textit{Fok}I yielded the predicted 333- and 187-bp \textit{ermC} gene digestion product. Template DNA from the other 21 of the 23 bacterial isolates was amplified with \textit{ermA} gene-specific primers and produced a 610-bp amplified DNA fragment, indicative of the \textit{ermA} gene. Restriction digestion of the 610-bp DNA fragment yielded the predicted 477- and 132-bp \textit{ermA} gene digestion products.

**Plasmid characterization**—Plasmid profiles of strains of \textit{Staphylococcus} spp isolated from contaminated milk were determined (Fig 2A). Fifteen of the 23 strains of \textit{Staphylococcus} spp did not harbor any plasmids. These strains were \textit{S hominis} M-1 (lane 3), \textit{S epidermidis} (strain M-2); \textit{S aeurulas} (strain M-7); lane 6, \textit{S aureus} (strain M-8); lane 7, \textit{S scuri} (strain M-9); lane 8, \textit{S aureus} (strain M-10); lane 9, \textit{S epidermidis} (strain M-11); lane 10, \textit{S saprophyticus} (strain M-12); lane 11, \textit{S lentus}; lane 12, \textit{S equorum}; lane 13, \textit{S epidermidis} (strain M-18); lane 14, \textit{S aureus} (strain M-20); lane 15, \textit{S simulans} (strain M-21); lane 16, \textit{S scuri} (strain M-21); lane 16, \textit{S scuri} (strain M-24); lane 17, \textit{S xylosus}; lane 18, \textit{S simulans} (strain M-26); lane 19, \textit{S simulans} (strain M-27); lane 20, \textit{S scuri} (strain M-26). Part B: plasmid profile of \textit{S simulans} strain M-21 (lane 3) and \textit{S scuri} strain M-28 (lane 4) that contain the \textit{ermC} gene. Lane 1 is the molecular weight marker, and Lane 2 is the 3.7-kb plasmid (positive control). Part C: Southern hybridization analysis of the 3-kb plasmid that encodes the \textit{ermC} gene of \textit{S simulans} strain M-28 (lane 3) and \textit{S scuri} strain M-28 (lane 4). Lane 2 is plasmid pE194 (positive control).
containing strains. For example, the plasmid profile of 1 strain of *S. aureus* M-10 (lane 8) was different from the other strain of *S. aureus* M-20 (lane 14). The *S. aureus* M-10 (lane 8) contained 3 plasmids measuring from 2 to 3 kb, and 10 kilobases (kb) each, whereas *S. aureus* M-20 (lane 14) had numerous plasmids measuring from 3 to 10 kb. Differences in plasmid profiles were also observed in plasmid containing strains of *S. epidermidis*, *S. simulans*, *S. sciuri*, and *S. lentus*. Plasmid profiles of *S. simulans* M-21 (Fig 2B, lane 3) and *S. sciuri* M-28 (lane 4) revealed that these staphylococcal isolates harbored the *ermC* gene. The 3-kb plasmids from these 2 strains hybridized with the *ermC* gene probe (Fig 2C, lanes 3 and 4).

Detection of *ermA* and *msrA* genes—Isolates with plasmids hybridized with a biotinylated *ermA* gene probe (Table 1). None of the plasmids hybridized with the *ermA* gene-specific probe. The chromosomal DNA from all the isolates was extracted and digested with *Eco* RI, separated by use of electrophoresis, and hybridized with *ermA* gene probe. Chromosomal DNA from 21 of the 23 staphylococcal isolates hybridized with the *ermA* gene probe. Six different *Eco* RI restriction fragment length polymorphism (RFLP) patterns were found in these isolates (Fig 3). The gene was found as a single chromosomal insert on 1 and 13 kb *Eco* RI fragments in 2 strains of *S. simulans* (lane 2) and 3 strains of *S. auricularis* (lane 3). Similarly, the *ermA* gene was found as a single chromosomal insert on 6 kb *Eco* RI fragments in 3 strains of *S. aureus* (lane 5). The gene was found as a single chromosomal insert on a 3 kb *Eco* RI fragment in 4 strains of *S. sciuri* (lane 6), 2 strains of *S. saprophyticus*, and in strains of *S. equorum*, *S. hominis*, *S. lentus*, and *S. xylosus*. Two different *Eco* RI RFLP patterns of the *ermA* gene were found in *S. epidermidis*. The *ermA* gene was found as a double chromosomal insert on a 2.4- and 1-kb *Eco* RI fragments in 1 strain of *S. epidermidis* (lane 4). In 2 other strains of *S. epidermidis*, the *ermA* gene was found as a triple chromosomal insert on 2.4-, 7-, and 9.4-kb *Eco* RI fragments (lane 7). None of the 23 isolates harbored the *msrA* gene.

**Discussion**

Fourteen species of staphylococci have been associated with bovine mastitis, and most are coagulase-negative. 2,4,6-17 In our study, we isolated 10 *Staphylococcus* spp from cows with mastitis. Three strains of *S. aureus* were coagulase-positive, and 21 strains of other species were coagulase-negative. Six percent of staphylococci in milk samples collected from mastitic cows were erythromycin resistant. The prevalence of erythromycin resistant staphylococci in our study is similar to an earlier report that indicated 7% of all streptococci isolated from dairy cows with mastitis were erythromycin-resistant. 7 The isolation, identification, and characterization of multiple-antibiotic resistant *Staphylococcus* spp from cows with mastitis indicate that these microbes harbor erythromycin-resistance as well as resistance to penicillin, ampicillin, tetracycline, and streptomycin, which are used to control or prevent mastitis.

The genetics of erythromycin resistance is well documented. 2,4,7-10,40 Several genes (*ermA*, *ermC*, and *msrA* genes) confer resistance to erythromycin in *Staphylococcus* spp; however, *ermA* or *ermC* genes are found in 98% of erythromycin-resistant strains isolated from human specimens. The *msrA* gene is known to confer resistance to erythromycin in a few *Staphylococcus* spp from animal origin. 2,4,7-10 The *ermC* gene is the most dominant *erm* gene determinant and is found in more than 84% of the erythromycin-resistant strains of *Staphylococcus* spp isolated from human specimens, and the gene is usually located on a small plasmid measuring approximately 2.5 kb. 2,4,10,22,23 In contrast, data from our study indicate that the gene is found in less than 10% of the isolates and was on a slightly larger plasmid of 3 kb. *Staphylococcus* spp that are isolated from human specimens contain multiple plasmids ranging from size 16 to 2 kb, whereas most of the *Staphylococcus* strains in our study lacked plasmids. The lack of plasmids in most of our isolates may have contributed to the low prevalence of the *ermC* gene in the *Staphylococcus* spp isolated from milk samples of lactating cows.

The *ermA* gene is located on an unusual noncomposite transposon, Tn534. 24 Westh et al17 found the *ermA* gene in less than 20% of erythromycin-resistant *S. aureus* isolated from human blood. Although these investigators observed several chromosomal insertion patterns of the *ermA* gene (*Eco* RI RFLP patterns), more than 60% of the isolates had the *ermA* gene as a single insert on a chromosomal digest (6-kb *Eco* RI fragment), and 37% of the isolates had the gene on 2 chromosomal inserts (6- and 10.5-kb *Eco* RI fragments). Results from another study with human isolates of *S. aureus* and coagulase-negative staphylococci indicate that the
ermA gene is almost always found as a single chromosomal insert on a 6 kb EcoRI fragment.12

Results of our studies indicate that the ermA gene is a dominant gene, found in 91% of the Staphylococcus spp isolated from milk samples of lactating cows, and we observed 6 chromosomal insert patterns of the ermA gene (by EcoRI RFLP pattern). The RFLP pattern of the ermA gene (a 6-kb EcoRI fragment) in bovine isolates of S aureus was similar to the 6-kb EcoRI RFLP pattern of the ermA gene observed in human isolates of S aureus. The RFLP pattern of the ermA gene (a 3-kb EcoRI fragment) in strains of S sciuri, S saprophyticus, S hominis, S lentus, and S xylosus were different from the RFLP pattern found in human strains of Staphylococcus spp. In addition, the sizes of the double and triple chromosomal inserts of the ermA gene in some bovine isolates of S epidermidis were also different from the sizes of the inserts observed in human isolates of staphylococci. The differing ermA gene RFLP patterns observed indicate that either Tn554 harboring the ermA gene has several attachment sites in these organisms or that the transposon may have lost its specificity so that it is attached randomly, thereby giving rise to various RFLP patterns.

The prevalence of erm genes from Staphylococcus spp from human specimens has been documented. However, little information is available on the characteristics of erm genes in Staphylococcus spp isolated from farm animals. Results of our experiments indicate that Staphylococcus spp from milk of lactating cows harbor erm genes homologous to those described in human isolates of Staphylococcus spp. However, the patterns and prevalence, sizes of plasmids, and sizes and numbers of inserts (copy number) of the erm genes differ from the erm genes found in erythromycin-resistant staphylococci isolated from human specimens.

References