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Genomic Typing of Enterococci Isolated from Bovine Mammary Glands and Environmental Sources[1]

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ABSTRACT

Enterococcal isolates (n = 102) from various sources of bovine origin on 1 farm were characterized using pulsed field gel electrophoresis analysis of *Sma*I restriction patterns. Isolates originated from feed samples (n = 6), bedding samples (n = 15), and bovine quarter-milk samples (n = 81). Isolates collected from milk samples included those from high-somatic cell count cows (n = 42), postpartum milk samples (n = 16), and clinical mastitis samples (n = 23). Species evaluated included *Enterococcus faecium* (n = 68), *Enterococcus casseliflavus* (n = 29), and *Enterococcus faecalis* (n = 5). A total of 20 clusters representing 44 isolates were detected when a similarity cut-off level of 75% was applied to interpret the pulsed field gel electrophoresis results. Fifteen of the clusters contained only isolates from milk samples. Four clusters contained isolates from bedding and milk samples. One cluster contained only isolates from feed samples. Clusters comprised of a single species represented 17 of the 20 total clusters. These results suggest enterococci from bovine origin were genetically diverse, whereas a limited number of isolates from various sources appeared to cluster together.
Key words: *Enterococcus* species, mammary, epidemiology

INTRODUCTION

Enterococcus spp. have commonly been included in the heterogeneous grouping of environmental streptococci or non-*agalactiae* streptococci when rate or prevalence of naturally occurring IMI have been reported for individual herds or survey results (Hogan et al., 1989; Gonzalez et al., 1990). Although enterococcal mastitis is relatively uncommon compared with subclinical and clinical mastitis caused by *Streptococcus* spp., milk quality and udder health problems within herds caused by enterococcal IMI have been documented (Todhunter et al., 1995). A concern about enterococci in the environment of the cow relates to the emerging prominence of nosocomial infections caused by enterococci, prevalence of resistance among these bacteria to commonly used antibiotics, and the shared virulence traits expressed by isolates from hospital patients and isolates from food animals (Mannu et al., 2003).

The epidemiology of IMI caused by *Enterococcus* spp. is relatively undefined with regards to common farm management practices that may lead to the control of mastitis caused by these organisms. The use of pulsed field gel electrophoresis (**PFGE**) to compare restriction fragments of DNA among bovine isolates has been valuable in determining the epidemiological traits of *Staphylococcus aureus* (Zadoks et al., 2000; Middleton et al., 2002) and *Streptococcus uberis* (Douglas et al., 2000; McDougall et al., 2004) isolated from bovine mastitis. Similarly, comparison of PFGE patterns of isolates from nosocomial infections are common to determine if hospital outbreaks with enterococci are from a single clone or multiple strains (Baldassarri et al., 2005). Comparison of enterococci isolated from milk with isolates in the environment may provide insight into the epidemiology of enterococcal mastitis on a dairy farm. The purpose of the current study was to use PFGE to compare genetic similarity among restriction endonuclease patterns for enterococci isolated from quarter-milk samples, forage, and bedding of a commercial dairy.

MATERIALS AND METHODS

Origin of Isolates

The survey herd consisted of approximately 1,200 lactating cows managed in dry lot corrals and fed a green chop alfalfa-based diet. The herd was selected based on reports by extension personnel of milk quality problems due to enterococci. A total of 102 enterococcal isolates were collected from the dairy herd during April 2004 to October 2004 for PFGE analysis. The distribution of the 102 isolates tested by bacterial species and source are in Table 1.

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Table 1. Distribution of enterococci isolated from mammary secretions and the environment of a commercial dairy farm

	Enterococcus species		
Origin of isolates	*Enterococcus faecium* (n = 68)	*Enterococcus casseliflavus* (n = 29)	*Enterococcus faecalis* (n = 5)
Mammary[1] (n = 81)			
High SCC	28	14	0
Postpartum	9	5	2
Clinical mastitis	21	1	1
Environment (n = 21)			
Bedding	6	9	0
Feed	4	0	2

[1]Isolates of mammary origin were from quarter-milk samples collected using aseptic technique from high-SCC milk within 2 wk after DHI testing (high SCC), mammary quarters with clinical mastitis before antibiotic therapy (clinical mastitis), and cows within 3 d after parturition (postpartum).

Milk Samples

Isolates of mammary origin were from quarter-milk samples collected using aseptic technique from high-SCC milk within 2 wk after DHI testing (n = 1,368), mammary quarters with clinical mastitis before antibiotic therapy (n = 453), and cows within 3 d after parturition (n = 3,162). All milk samples were frozen at –20°C after collection and thawed at room temperature before primary isolation of bacteria. Milk was plated on tryptic soy agar (Becton, Dickinson and Company, Sparks, MD) containing 5% bovine blood and 0.1% esculin (MP Biomedicals LLC, Aurora, OH). Primary isolation plates were incubated aerobically at 37°C for 48 h. Gram-positive, catalase-negative cocci that hydrolyzed esculin and grew in the presence of 6.5% NaCl were presumptively identified as enterococci (NMC, 1999). The number of isolates presumptively identified as enterococci were 42 from SCC milk, 23 from clinical mastitis, and 16 from postpartum milk. Isolates presumptively identified as enterococci and subsequently identified as an *Enterococcus* spp. by the API 20 STREP system (bioMérieux Industry, Hazelwood, MO) with greater than 95% confidence were included for testing. If enterococci were isolated from a mammary quarter multiple times during the survey, only the isolate from the initial sample was tested by PFGE.

Bedding and Feed

Twenty-five grams of composite-dried manure bedding sample was collected monthly from locations in lactating cow corrals (n = 12) during the survey period. Twenty-five grams of green chop alfalfa, sampled after harvest and before mixing into the diet of lactating cows, was collected twice (n = 4) during the last month of the survey. Bacteria were isolated in bedding and feed by adding 10 g of sample to 90 mL of sterile PBS and mixing the solution for 40 s in a stomacher (Stomacher Lab-Blender 400, Tekmar Co., Cincinnati, OH). Serial dilutions of the liquid phase in sterile PBS were plated on the surface of kanamycin (20 mg/L) esculin azide agar (Oxoid Ltd., Basingstoke, Hampshire, UK) for selection and differentiation of enterococci. Serial dilutions plated were $1:10^2$ to $1:10^6$. Inoculated plates were incubated 24 h at 37°C. Random colonies with black pigment and halos of esculin hydrolysis were selected and plated on trypticase soy agar containing 5% bovine blood and 0.1% esculin. Presumptive identification and testing with the API 20 STREP system were the same as isolates from milk samples. To reduce possible repetition of clones, a single isolate of each species identified from randomly selected colonies of each bedding sample was included in PFGE testing. Each of the isolates collected from forage and subsequently identified as *Enterococcus* spp. were included in PFGE analysis.

PFGE

Genomic DNA was prepared using a previously described method (Saeedi et al., 2002). Restriction digestion of bacteria DNA was carried out using 40 units of *Sma*I (New England Biolabs, Ipswich, MA) in individual digestion tubes (25°C, 4 h). The PFGE was performed on a Chef Mapper (BioRad, Hercules, CA) in 0.5× Tris-borate EDTA (TBE) running buffer (0.9 *M* Tris base, 0.9 *M* boric acid, 0.02 *M* EDTA, pH 8.0) with recirculation at 14°C. The DNA restriction fragments were resolved in 1.0% SeaKem Gold agarose (Cambrex, East Rutherford, NJ) in 0.5× TBE buffer. Lambda ladder (BioRad) was used as a size marker on each gel. Electrophoresis was carried out at 6 V/cm for 14 h with pulse timed of 2.16 s to 35.07 s (linear ramping) with a 120° angle. Gels were stained with ethidium bromide and viewed with UV illumination. Gel images were saved in the TIFF format and exported to the Fingerprinted Informatix II software (BioRad). Data were analyzed using the Dice coefficient-unweighted pair-group method with arithmetic averages with 1.0% band tolerance and 1.0% optimization setting for the whole profile. A dendrogram was generated to examine relatedness of PFGE profiles for all study isolates. Similarity cut-off value of 75% was applied (Seong et al., 2004).

RESULTS

Pulsed field gel electrophoresis analysis of *Sma*I restriction patterns determined banding patterns ranged among isolates from 9 to 20 fragments with a range in size from 20 to 250 kb. Using a similarity cut-off value