**Short communication: Genotypic and phenotypic identification of environmental streptococci and association of *Lactococcus lactis* ssp. *lactis* with intramammary infections among different dairy farms**


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**ABSTRACT**

*Lactococcus* species are counted among a large and closely related group of environmental streptococci and streptococci-like bacteria that include bovine mastitis pathogenic *Streptococcus*, *Enterococcus*, and *Aerococcus* species. Phenotypic and biochemical identification methods can be inaccurate and unreliable for species within this group, particularly for *Lactococcus* spp. As a result, the incidence of *Lactococcus* spp. on the farm may have been historically underreported and consequently little is known about the clinical importance of this genus as a mastitis pathogen. We used molecular genetic identification methods to accurately differentiate 60 environmental streptococci and streptococci-like bacteria isolated from cows with high somatic cell count and chronic intramammary infection (IMI; >2 somatic cell scores above 4) among 5 geographically distinct farms in New York and Minnesota that exhibited an observed increase in IMI. These isolates were phenotypically identified as *Streptococcus uberis* and *Streptococcus* spp. Genetic methods identified 42 isolates (70%) as *Lactococcus lactis* ssp. *lactis*, including all 10 isolates originally phenotypically identified as *S. uberis*. Antibiotic inhibition testing of all *Lc. lactis* ssp. *lactis* showed that 7 isolates were resistant to tetracycline. In the present study, a predominance of *Lc. lactis* ssp. *lactis* was identified in association with chronic, clinical bovine IMI among all 5 farms and characterized antimicrobial resistance for treatment therapies. Routine use by mastitis testing labs of molecular identification methods for environmental streptococci and streptococci-like bacteria can further define the role and prevalence of *Lc. lactis* ssp. *lactis* in association with bovine IMI and may lead to more targeted therapies.

**Key words:** streptococci-like bacteria, *Lactococcus lactis* ssp. *lactis* identification, *Streptococcus* spp., bovine intramammary infection

**Short Communication**

Environmental streptococci and streptococci-like bacteria are significant contributors to the incidence of clinical mastitis in the United States (Gröhn et al., 2004), accounting for up to 15% of cows diagnosed annually (NMC, 1990). Only a small percentage of these infections become chronic (18% of infections exceeding 100 d), and although the cure rate for these cases is low, targeted therapy can sometimes reduce disease severity (Jones and Swisher, 2009).

*Streptococcus uberis* has been implicated in most bovine IMI, with less than 18% of reported cases attributed to other environmental streptococci and streptococci-like bacteria (Jones and Swisher, 2009). Among this 18%, species of *Enterococcus* and *Aerococcus* have been well characterized in terms of incidence and role in bovine IMI; however, similar data for *Lactococcus* spp. are few (Malinowski et al., 2003; Kuang et al., 2009; Plumed-Ferrer et al., 2013). This lack of information could be attributed to limitations in routine procedures used to speciate environmental streptococci and streptococci-like bacteria. Phenotypic and biochemical tests recommended by the National Mastitis Council for identification of environmental streptococci and streptococci-like bovine milk isolates (NMC, 1999), as well as commercially available biochemical test kits for
these microorganisms, are not inclusive of Lactococcus spp. and can produce variable or erroneous results that cause misidentifications (Fortin et al., 2003; Odierno et al., 2006; Svec and Sedláček, 2008; Gordonillo et al., 2010). Thus, it is possible that the incidence of Lactococcus spp. associated with bovine IMI has been severely underreported.

In recent years, molecular genetic methods such as PCR and sequencing-based methods have proven a more reliable means of accurately differentiating environmental streptococci and streptococci-like bacteria isolated from bulk tank and composite milk samples (Holm et al., 2004; Reinoso et al., 2010; de Garnica et al., 2014; Kanyó and Nagy, 2014) and have, in some cases, enabled the unequivocal identification of pure cultures of Lactococcus lactis ssp. lactis and Lactococcus garvieae originating from bovine IMI cases (Devriese et al., 1999; Kuang et al., 2009).

The objective of this report was to use molecular genetic identification methods to accurately differentiate environmental streptococci and streptococci-like bacteria isolated from cows with high SCC and chronic IMI among 5 geographically distinct farms in New York and Minnesota that exhibited an observed increase of IMI due to these bacteria. Where Lc. lactis ssp. lactis isolates were identified, antimicrobial resistance profiles were determined to contribute to the understanding of possible therapies and cure rates.

Isolates were included from 2 New York State dairy farms (herds A, B, and C), where each herd exhibited a high prevalence of chronic infections as determined by 2 or more consecutive monthly test-day linear scores greater than 4.0 (SCC ≥200,000 cells/mL; 18, 21, and 23% of the total of lactating cows, respectively). Herd A, a 160-lactating cow Holstein-Friesian herd in freestall facilities bedded with sand, had an average daily milk production of 40.1 kg and a rolling bulk tank SCC of 286,000 cells/mL. Quarter samples (n = 42) from 12 cows with high SCC were submitted to the Quality Milk Production Services (QMPS; Ithaca, NY) for bacteriological culture. Ten of the 12 culture-positive samples were identified as Streptococcus spp. Isolates from herds B and C were submitted through a private veterinary clinic located in New York State and were initially identified as Streptococcus spp. or Streptococcus uberis. Herd B, an 895-lactating cow Holstein-Friesian herd in freestall facilities bedded with recycled sand, had an average daily milk production of 43 kg and a bulk tank SCC of 365,000 cells/mL. Ten isolates from 10 cows with high SCC were submitted to QMPS for sequence analysis. Herd C, a 914-lactating cow Holstein-Friesian herd in a freestall facility bedded with recycled sand and sand lane, had an average daily milk production of 39 kg and a bulk tank SCC of 365,000 cells/mL. Ten isolates from 10 cows with high SCC milk were submitted to QMPS for sequence analysis.

The initial phenotypic speciation by all laboratories was performed using National Mastitis Council laboratory identification procedure recommendations (NMC, 1999). These biochemical and serological tests, including Gram stain, β-hemolysis, catalase test, esculin hydrolysis, Christie, Atkins, Munch-Peterson (CAMP) test, growth in enterococcal medium, and growth in 6.5% NaCl, were used to identify isolates as Streptococcus spp. or Streptococcus uberis. For 2 isolates, the BBL Crystal Gram-Positive ID Kit (Becton Dickinson, Franklin Lakes, NJ) was used for further identification. Polymerase chain reaction and sequence analysis of 16S rDNA and rpoB were used for molecular identification of all isolates. For these analyses, a crude cell DNA lysate was first prepared for each isolate (Furrer et al., 1991). Briefly, pure culture streaks were made on a tryptic soy agar with 5% sheep blood plate (Becton Dickinson) and incubated for 18 to 24 h at 37°C. One isolated colony was transferred to a 1.5-mL tube of Todd Hewitt Broth (Becton Dickinson) and incubated 18 to 24 h at 37°C. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine DNA quantity. Two hundred fifty microliters of each broth culture was transferred to 1.5-mL microcentrifuge tubes and centrifuged to pellet for 10 min at 6,000 × g at room temperature (20°C ± 2°C). The supernatant was removed and discarded, and the pellet was resuspended in 95 μL of 1× PCR buffer (Roche Applied Science, Indianapolis, IN). A 4-μL aliquot of freshly prepared 50 mg/mL lysozyme (Promega, Madison, WI) solution was added to this suspension and mixed well by pipetting to result in a final concentration of 2 mg/mL lysozyme. After a 15-min incubation at room temperature, 1 μL of a 20 mg/mL proteinase K solution (Promega) was added and vortexed to mix, resulting in a final concentration of 200 μg/mL proteinase K. The tubes were incubated for up to 2 h in a heat block set
at 58°C until the lysates cleared. The enzymes were inactivated for 8 min at 95°C. After equilibrating to room temperature, tubes were centrifuged for 3 s at 6,000 × g to remove condensate from the sides and lids. Crude cell lysates were stored at −20°C until further analysis.

Two different PCR amplification steps were performed to amplify 2 different gene targets, rpoB (Drancourt et al. 2004) and 16S rDNA (Greisen et al., 1994). Primers rpoBF (5′-AARYTIGGMCCTGAAGAAAT-3′), rpoBR (5′-GCCTTTAACTTCACTATCA-3′), DG74 (5′-AGGAGGTGATCCAACCGCA-3′), and P5SH (5′-TGAAGAGTTTGATCMTGGCTCAG-3′) were purchased from Integrated DNA Technologies (IDT, Coralville, IA). A PCR amplification mix was prepared consisting of 0.25 μL of a 100 μM solution of each forward and reverse primer, 25.00 μL of GoTaqGreen (Promega), 27.50 μL of H2O, and 2.0 μL of DNA. The mix was amplified on a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA) with running conditions of an initial denaturation step of 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; and a final 7-min extension time at 72°C. The PCR amplicons were analyzed by gel electrophoresis to confirm a 740-bp rpoB amplicon and a 1,040-bp 16S rDNA amplicon.

In preparation for sequencing, excess primers and nucleotides were removed from each sample by treatment with ExoSAP-IT (USB Corporation, Cleveland, OH). Sequencing of each sample was performed in 2 directions using Big Dye Terminator chemistry on an ABI Prism 3700 DNA analyzer (Applied BioSystems, Foster City, CA). Sequences were proofread in SeqMan (version 5.08, Lasergene; DNASTar Inc., Madison, WI), compared with publicly available sequence data using the National Center for Biotechnology Information (NCBI) BLAST programs (Altschul et al., 1997), and identified using interpretive criteria provided by the Clinical and Laboratory Standards Institute (CLSI, 2008a). Briefly, the criteria for BLAST were as follows: maximum identity ≥99.0% to species level with greater than 0.8% separation between different species and an expected value (E-value) = 0.0.

Antimicrobial MIC for Lc. lactis ssp. lactis isolates were determined using the Sensititer Mastitis Plate Format veterinary panel and Sensititer ARIS system (Trek Diagnostics Systems, Cleveland, OH). This panel includes antibiotics and the respective MIC dilution ranges listed in Table 1. Results were interpreted according to the European Food Safety Authority (EFSA) technical guides (European Commission, 2005, 2008).

Genotypic and phenotypic identification results for all isolates analyzed are shown in Table 2. Sequence analysis of all 60 isolates among the 5 herds identified overall 42 (70%) as Lc. lactis ssp. lactis, 10 (17%) as

<table>
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<tr>
<th>Antimicrobial</th>
<th>Breakpoint (μg/mL)</th>
<th>No. of strains at MIC (μg/mL)</th>
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<th>0.25</th>
<th>0.50</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
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<td>22</td>
<td>29</td>
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<td>43</td>
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<td>31</td>
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1. Shaded areas indicate the concentration ranges of each of the antimicrobials tested. Resistance breakpoints are indicated by vertical lines when available and as NA when not.

2. Resistance breakpoints proposed by the EFSA (European Commission, 2008).

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Resistance breakpoints proposed by the EFSA (European Commission, 2008).

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For herd A, 9 of the 10 isolates phenotypically reported as *Streptococcus* spp. were identified by sequencing as *Lactococcus lactis* spp. *lactis*, whereas 1 was identified as *Enterococcus faecium*. For herd B, where 8 of the 18 isolates were identified as *Streptococcus* spp., 4 (7%) as *Lactococcus lactis* spp. *lactis*, whereas 1 was identified as *Enterococcus saccharolyticus*. For herd D, where 11 isolates were identified, 1 (1.5%) as *Streptococcus dysgalactiae* spp. *dysgalactiae*, 1 (1.5%) as *Lactococcus lactis* spp. *lactis*, and 1 (1.5%) as *Streptococcus uberis*. For herd E, 10 isolates were identified, 1 (1.5%) as *Enterococcus saccharolyticus*.
isolates were initially phenotypically identified as *Streptococcus* spp. and 10 of the isolates as *Streptococcus uberis*, 15 of these were identified by molecular methods as *Lc. lactis* ssp. *lactis*, 2 as *Ent. saccharolyticus*, and 1 as *Enterococcus* spp. All 10 of the isolates phenotypically identified as *Streptococcus uberis* were genetically identified as *Lc. lactis* ssp. *lactis*.

For herd C, 2 isolates were biochemically identified by the BBL Crystal Gram-Positive ID kit as *Leuconostoc* spp. and *Aerococcus viridans*. These isolates were identified by sequencing as *Lc. lactis* ssp. *lactis* and *Enterococcus saccharolyticus*, respectively. Among the remaining 9 isolates that were phenotypically identified as *Streptococcus* spp., 2 were identified by molecular methods as *Enterococcus saccharolyticus* and 7 as *Lc. lactis* ssp. *lactis*.

All isolates from herds D and E were initially phenotypically identified as *Streptococcus* spp. For herd D, DNA sequence analysis identified 5 as *Lc. lactis* ssp. *lactis*, 4 as *Enterococcus saccharolyticus*, 1 as *Streptococcus uberis*, and 1 as *Streptococcus dysgalactiae* ssp. *dysgalactiae*. For herd E, 4 of these isolates were identified by sequence analysis as *Lc. lactis* ssp. *lactis*, 1 as *Lc. lactis*, 3 as *Streptococcus uberis*, and 2 as *Enterococcus saccharolyticus*.

Antibiotic inhibition testing of all *Lc. lactis* ssp. *lactis* showed that all isolates were susceptible to ampicillin, erythromycin, and penicillin, and 7 isolates were resistant to tetracycline. These data are shown in Table 1. Results showed that, among these 5 farms, *Lc. lactis* ssp. *lactis* was the predominant streptococci-like bacterium associated with bovine IMI and identified potential antimicrobial agents for therapy. Overall, use of phenotypic identification methods resulted in overreporting the incidence of *Streptococcus uberis* and did not provide discriminatory information about the incidence of other genera and species. In this case, phenotypic and biochemical identification produced erroneous results and failed to identify *Lc. lactis* ssp. *lactis*. From a clinical perspective, it is important to understand the role played by *Lc. lactis* ssp. *lactis* within the farm and, furthermore, to study the epidemiology in order to define the behavior of this potential mastitis pathogen and management of therapy decisions. Routine use of molecular methods for speciation of environmental streptococci and streptococci-like bacteria in association with chronic, clinical IMI will contribute to this understanding. Antimicrobial susceptibility profiling along with accurate identification will enable targeted and early intervention for reduction of chronic IMI.

REFERENCES


NMC (National Mastitis Council). 1990. Procedures for the identification of specific groups or species of microorganisms that cause


