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The *Lactococcus* genus as a potential emerging mastitis pathogen group: A report on an outbreak investigation

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ABSTRACT

The bacterium *Lactococcus lactis* is widely used in food production and in medical applications, and is considered safe for human and animal use. However, studies have also linked *Lactococcus* bacteria to infection. For example, certain variants of *Lactococcus* species have been associated with bovine mastitis (e.g., *Lactococcus lactis* and *Lactococcus garvieae*). In this study, we investigated an outbreak of bovine mastitis thought to be associated with *Lactococcus* bacteria by using microbiological and molecular techniques. We used bacterial isolation, next-generation sequencing, DNA fingerprinting, and other methods to test our hypothesis that *Lactococcus* microbes were the primary pathogen causing the mastitis outbreak. Twenty-eight *Lactococcus* isolates were obtained from mastitic milk of 28 dairy cows. The isolates were identified as *L. lactis* (27 isolates) and *L. garvieae* (1 isolate). Phylogenetic analysis based on 16S rDNA gene sequence comparison indicated similarity among the *L. lactis* isolates as well as between the isolates and reference sequences. The DNA fingerprinting analysis based on random amplified polymorphic DNA results of the 27 *L. lactis* isolates identified different random amplified polymorphic DNA profiles, which suggests they originated from multiple sources. Microbiome analysis determined *Lactococcus* to be the dominant genus in the majority of the mastitic milk samples, whereas it was found in low relative abundance in healthy milk samples. The *Lactococcus* genus was detected in all environmental samples tested, and sampling of bulk tank milk corroborated that *Lactococcus* was not abundant in healthy milk from the same dairy herd. In summary, our findings suggest that *Lactococcus* bacteria are a potential etiological agent in the mastitis outbreak studied. Further studies should be conducted to understand the importance of *Lactococcus*, especially *L. lactis*, as pathogenic microbes in veterinary medicine and food safety.
Key words: mastitis, *Lactococcus*, microbiome, next generation sequencing

INTRODUCTION

Mastitis is an important disease in dairy cows causing reproduction problems (Hertl et al., 2010), culling (Gröhn et al., 2005), and economic losses due to reduced milk production, treatment expense, and discarded milk (Bar et al., 2008). Clinical mastitis is also a painful disease and is associated with behavioral changes (Medrano-Galarza et al., 2012).

For treatment of mastitis, identifying the microorganisms responsible should be considered (Royster and Wagner, 2015). Many microbial species have been identified as etiological agents, typically through bacterial culture (Oikonomou et al., 2012). *Lactococcus* species have been isolated from bovine mastitis, and their association with the disease has been discussed (Werner et al., 2014; Plumed-Ferrer et al., 2015a). Lactococci are gram-positive, nonmotile cocci, homofermentative, poorly α-hemolytic, and exclusively produce L(+)-lactic acid (Casalta and Montel, 2008). They are members of the group lactic acid bacteria (**LAB**) and are routinely found on animal skin and plants (Casalta and Montel, 2008). Lactic acid bacteria are generally not considered harmful to humans (Mofredj et al., 2007) or animals (Klostermann et al., 2008; Espeche et al., 2012; Bouchard et al., 2015) and have been used for the prevention and treatment of human (Mofredj et al., 2007) and animal diseases (Klostermann et al., 2008; Espeche et al., 2012; Bouchard et al., 2015). Lactic acid bacteria have been reported to produce proteins, chemical mediators, and other molecules that stimulate local immune responses (Mofredj et al., 2007).

Interestingly, in dairy cows, the potential of LAB for treatment or prevention (or both) of mastitis has been considered (Klostermann et al., 2008; Espeche et al., 2012; Bouchard et al., 2015). Klostermann et al.

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(2008) evaluated the use of a live culture suspension of *Lactococcus lactis* DPC3147 to treat naturally infected mastitic animals. Trials were conducted for subclinical and acute clinical mastitis, which demonstrated that treatment with *L. lactis* DPC3147 culture potentially had a similar level of efficacy as common antibiotics (Klostermann et al., 2008).

Lactococcus lactis, in particular, is of considerable economic importance (Cavanagh et al., 2015) and is widely used as a starter culture in dairy fermentation (Casalta and Montel, 2008). *Lactococcus lactis* is known to produce bacteriocin (Klostermann et al., 2008) and is frequently used as a probiotic (Furtado et al., 2014). Moreover, *L. lactis* is included on the Qualified Presumption of Safety list of the European Food Safety Authority (Plumed-Ferrer et al., 2013) and is accepted as generally recognized as safe (Casalta and Montel, 2008). However, some species of *Lactococcus* have been reported to be the cause of human (Davies et al., 2009; Hadjisymeou et al., 2013; Inoue et al., 2014) and animal infections (Plumed-Ferrer et al., 2013, 2015a; Khoo et al., 2014). Nevertheless, it is unclear if these cases represent the emergence of novel pathogenic strains or were detected due to the availability of improved identification methods (Plumed-Ferrer et al., 2015b).

Werner et al. (2014) confirmed, by using a DNA sequencing approach, that the majority of isolates from bovine mastitis milk samples, which were phenotypically identified as *Streptococcus* spp., were in fact *L. lactis*. *Lactococcus* species are closely related to streptococci and *Streptococcus*-like genera such as *Enterococccus* and *Aerococcus*. Therefore, the role of *Lactococcus* spp. as an etiological agent of mastitis may have been underreported throughout the years (Werner et al., 2014). Considering this, *Lactococcus* species isolated from bovine intramammary infections are now being characterized both genotypically and phenotypically (Plumed-Ferrer et al., 2013, 2015a; Werner et al., 2014). However, the mechanism of pathogenicity is not yet fully understood (Plumed-Ferrer et al., 2015b), and few studies have been conducted on lactococci as potential bovine mastitis pathogens.

Therefore, the aim of the present study was to use current microbiological and molecular techniques to investigate an outbreak of mastitis that was thought to be associated with *Lactococcus* infection. Specifically, we hypothesized that a member(s) of the *Lactococcus* genus was the primary pathogen causing the mastitis outbreak. To explore our hypothesis, we used next-generation sequencing of the 16S rRNA gene, random amplified polymorphic DNA-PCR (**RAPD-PCR**), and phylogenetic techniques.

MATERIALS AND METHODS

Animal Care Statement

All experimental procedures in this study conformed to the recommendations of The Animal Welfare Act of 1966 (P.L. 89–544) and its amendments of 1970 (P.L. 91–579), 1976 (P.L. 94–279), and 1985 (P.L. 99–1998), which regulate the transportation, purchase, and treatment of animals used in research. The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University (protocol number: 2013–0056).

Farm and Management

The study was conducted on a single commercial dairy farm located in upstate New York. During the experimental period, from July until October 2015, the farm milked approximately 1,200 cows. Primiparous and multiparous cows were housed separately in freestall barns bedded with sand. Cows were fed a TMR to meet or exceed the nutrient requirements of a 650-kg lactating Holstein cow producing 45 kg/d of milk with 3.5% fat and 3.2% true protein when DMI is 25 kg/d (NRC, 2001). Cows were milked thrice daily in a double-20 milking parlor.

The target length of the dry period was 55 d. Cows were dried off by abrupt interruption of milking and dry cow therapy was equally performed for all quarters of all cows and consisted of intramammary infusion with ceftiofur hydrochloride (Spectramast DC, Zoetis, Madison, NJ) followed by the administration of an internal teat sealant (Orbeseal, Zoetis). Before the outbreak of *Lactococcus* spp., the common mastitis pathogens encountered based on aerobic mastitic milk culture were represented by approximately 33% gram-negative microbes, mainly *Escherichia coli* and *Klebsiella*; 33% gram-positive microbes, mainly *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus* spp., and *Staphylococcus* spp.; and 33% were culture negative from a total of approximately 500 mastitic milk samples evaluated during a period of 1 yr prior to the *Lactococcus* outbreak. The historical clinical cure rates following intramammary antibiotic therapy of clinical mastitis were approximately 70%. The bulk tank SCC ranged from 150,000 to 250,000. The herd is a closed herd with no other major concomitant disease problems. The incidence of displaced abomasum, ketosis, metritis, and retained placenta were 2.5, 5, 12, and 6%, respectively.