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Validation of a mycoplasma molecular diagnostic test and distribution of mycoplasma species in bovine milk among New York dairy farms

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ABSTRACT

Mycoplasma mastitis is a contagious and costly disease of dairy cattle that significantly affects animal health and milk productivity. *Mycoplasma bovis* is the most prevalent and invasive agent of mycoplasma mastitis in dairy cattle, and early detection is critical. Other mycoplasma have been isolated from milk; however, the role and prevalence of these species as mastitis pathogens are poorly understood. Routine screening of milk for mycoplasma by bacteriological culture is an important component of a farm control strategy to minimize a herd mycoplasma outbreak, but phenotypic methods have limited ability to speciate mycoplasma, affecting how farms and practitioners can understand the role and effect of species other than *M. bovis* in herd health. Fastidious mycoplasma culture can be lengthy and inconclusive, resulting in delayed or false negative reports. We developed and validated a multitarget PCR assay that can in the same day confirm or reject a presumptive positive mycoplasma culture found upon bacteriological testing of clinical specimens, further discriminate between *Acholeplasma* and *Mycoplasma*, and identify *M. bovis*. Coupled with sequence analysis isolates can be further identified as bovine mycoplasma *Mycoplasma arginini*, *Mycoplasma alkalescens*, *Mycoplasma canadense*, *Mycoplasma bovirhinis*, *Mycoplasma bovigenitalium*, *Mycoplasma californicum*, *Acholeplasma laidlawii*, and *Acholeplasma oculi*. Assay validation included analysis of 845 mycoplasma representing these species and 30 additional bacterial species obtained from routine milk submissions to the Quality Milk Production Services from New York farms and veterinary clinics between January 2012 and December 2015. Among 95 herds, we found 8 different *Mycoplasma* species and 3 different *Acholeplasma* species, with an overall prevalence of *M. bovirhinis* of 1%, *A. oculi* of 2%, *M. arginini* of 2%, *M. californicum* of 3%, *M. canadense* of 10%, *M. bovigenitalium* of 10%, *A. laidlawii* of 11%, *M. alkalescens* of 17%, and *M. bovis* of 78%. More than one mycoplasma was found in 14% of the herds tested, and both *M. bovis* and *Acholeplasma* were found in 6% of the farms. Incorporation of the validated molecular diagnostic assay into routine bacteriological screening as a supportive confirmation and identification tool will lead to an improved assessment of *Mycoplasma* and *Acholeplasma* prevalence data, which will facilitate increased knowledge about the role of these mycoplasma in mastitis.

Key words: *Mycoplasma*, *Acholeplasma*, bovine, mastitis, molecular diagnostic assay

INTRODUCTION

Mycoplasma IMI of dairy cattle is a serious, highly contagious disease that can have a significant financial effect on a farm through loss of animals and reduced milk quality and production (Wilson et al., 1997; Maunsell et al., 2011). Mycoplasma are resistant to antibiotic treatment, and carriage in the animal can be persistent and fluctuate broadly (Jasper, 1982; Biddle et al., 2003). Readily transmitted through blood and lymph, mycoplasma transmission between cows commonly occurs through direct contact or via equipment during milking (USDA APHIS, 2003, 2008; Fox, 2012). Because of these factors, control strategies to reduce spread and effect rely on regular bacteriological culture screening of bulk-tank milk or pooled animal milk. When positive cultures are identified, smaller pools or individual cows are tested to pinpoint infection, and positive animals are culled or segregated (USDA APHIS, 2008; Maunsell et al., 2011; Fox, 2012).

After initial isolation of mycoplasma from clinical samples by bacteriological culture, in some cases positive confirmation can be a lengthy and inconclusive process that can increase the response time of a farm to a mycoplasma outbreak. Fastidious mycoplasma require specialized media and incubation conditions for isolation and often grow slowly or exhibit atypical colony morphology that can confound a definitive culture report or lead to an erroneous identification (Freundt,

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1983; Gourlay and Howard, 1983; Nicholas et al., 2008). Atypical colonies on mycoplasma media can feasibly be due to growth of unstable L-form bacteria induced by penicillin included in the media, cellular material present in a clinical sample, or insoluble precipitates produced by older media (Razin, 1983); however, few bacteriological tools exist that can discriminate between these and mycoplasma. The National Mastitis Council recommends incubating cultures 7 to 10 d before a negative report (NMC, 1999), and subculture of atypical growth is commonly recommended (Nicholas et al., 2008). However, failure of a suspect mycoplasma to subculture may be due to the fastidious nature or uncultureability of a particular mycoplasma strain, resulting in a false negative report.

Several different mycoplasma species have been reported to be isolated from bovine milk including *Mycoplasma bovis*, *Mycoplasma arginini*, *Mycoplasma alkalescens*, *Mycoplasma canadense*, *Mycoplasma bovirhinis*, *Mycoplasma bovigenitalium*, *Mycoplasma californicum*, *Mycoplasma dispar*, *Acholeplasma laidlawii*, *Acholeplasma oculi*, and *Acholeplasma granularum* (USDA APHIS, 2003; Nicholas et al., 2008; Justice-Allen et al., 2011). These mycoplasma have been implicated in causing mastitis as well as other reproductive and respiratory diseases in dairy cattle (Jasper, 1994; Nicholas et al., 2008). The frequently researched *M. bovis* has been considered the most prevalent and clinically important mycoplasma species in dairy cattle, associated with acute mastitis, arthritis, otitis, and pneumonia (Gonzalez and Wilson, 2003; Maunsell et al., 2011; Fox, 2012). Not all species have been as comprehensively studied as *M. bovis*, and less genetic data exists to contribute to our understanding of the significance of each species as pathogens. Serological and biochemical tests have been standardized for further identification of mycoplasma; however, both approaches are time consuming and difficult to perform accurately, reliably, and unambiguously (Volokhov et al., 2012). Indeed, the scarcity of available diagnostic tools for routine, reliable identification of mycoplasma species has contributed to the historical lack of knowledge about individual species. In particular, very little has been reported about the distribution among herds and role of *Acholeplasma* species in mastitis, and it is unclear whether *Acholeplasma* are simply environmental contaminants (Jasper, 1982). In the future, new knowledge of the clinical relevance of different *Mycoplasma* and *Acholeplasma* along with information gained from routine differentiation of mycoplasma to genus or species level may be an important contribution to a farm developing a treatment and disease management response.

Alternative molecular methods that can, within the same day of detection, confirm a bacteriological diagnosis of mycoplasma as well as further speciate are of value to the mastitis laboratory. Several research groups have developed for culture DNA various PCR-based strategies with and without additional culture strategies to discriminate between *Mycoplasma* and *Acholeplasma* (Boonyayatra et al., 2012a), to speciate bovine mycoplasma (Eissa et al., 2013), or to rapidly identify a few species of concern, particularly *M. bovis* (Baird et al., 1999; Bashiruddin et al., 2005; Boonyayatra et al., 2012b). All of these approaches have distinct merit; however, we have found no single strategy that can within the same day of detection confirm a culture positive mycoplasma and identify as *M. bovis*, *Mycoplasma*, or *Acholeplasma* and that can allow for further speciation of the mycoplasma targeted in the current research work.

Our objective for the current study was to develop and validate a molecular diagnostic assay combining conventional PCR and sequencing to quickly confirm and identify mycoplasma isolated from bacteriological culture of milk routinely submitted by farms and practitioners to the Animal Health Diagnostic Center, Quality Milk Production Services laboratories (Cornell University, Ithaca, NY). Within the same day as detection in culture, the assay can confirm or reject a presumptive positive mycoplasma found upon bacteriological testing of clinical specimens, where a single colony or atypical growth prevents a conclusive identification as mycoplasma. At the same time, the assay can discriminate between *Mycoplasma* and *Acholeplasma*, and identify the most significant mycoplasma pathogen, *M. bovis*. Through subsequent sequence analysis, other bovine *Mycoplasma* and *Acholeplasma* can be identified to species level, adding to our knowledge about the prevalence of these species among dairy farms. This new knowledge will contribute to a better understanding of the role of these species in bovine mycoplasma mastitis.

MATERIALS AND METHODS

Method Description

The molecular diagnostic assay developed includes a multiplex conventional PCR followed by optional sequencing. Composing the assay are 3 separate PCR amplifications that use separate amplification mixes but run concurrently in the same thermocycler under the same running conditions. The first assay amplifies *M. bovis uvrC* (***uvrC***) and *Mycoplasma* and *Acholeplasma* 16S rDNA (**16S**) gene targets and incorporates 2 forward and reverse primer sets. A second assay amplifies the *Mycoplasma* 16S to 23S rDNA intergenic transcribed spacer region (**MITS**), and a third assay