



***Mycoplasma* species isolated from bovine milk collected from US dairy herds between 2016 and 2019**

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ABSTRACT

Determining the species of mycoplasma isolated from culture-positive milk samples is important for understanding the clinical significance of their detection. Between August 2016 and December 2019, 214,518 milk samples from 2,757 dairy herds were submitted to Quality Milk Production Services (QMPS) at Cornell University for mycoplasma culture. From these samples, 3,728 collected from 204 herds were culture positive. Based on the request of herd managers, owners, or veterinarians, 889 isolates from 98 herds were subjected to molecular identification by PCR and amplicon sequencing. The largest proportion of the identified isolates were from New York State (78.1%), while the others came from the eastern United States (17.8%), Texas (2.0%), and New Mexico (2.1%). As expected, *Mycoplasma* spp. were the most common (855 isolates, 96.2%) and *Acholeplasma* spp. accounted for the remainder (34 isolates, 3.8%). *Mycoplasma bovis* was the most prevalent *Mycoplasma* species (75.1%), followed by *M. bovis genitalium* (6.5%), *M. canadense* (5.9%), *M. alkalescens* (5%), *M. arginini* (1.7%), *M. californicum* (0.1%), and *M. primatum* (0.1%). A portion of the isolates were confirmed as *Mycoplasma* spp. other than *M. bovis* but were not identified at the species level (16 isolates, 1.8%) because further information was not requested by the manager, owner, or veterinarian. *Mycoplasma bovis* was the only species identified in 59 of the 98 herds. However, more than 1 *Mycoplasma* sp. was identified in 29 herds, suggesting that herd infection with 2 or more mycoplasmas is not uncommon. Moreover, a *Mycoplasma* sp. other than *M. bovis* was the only species identified in 8 herds. From the subset of 889 mycoplasma culture-positive isolates from 98 herds,

we determined that over a third of the herds had either more than 1 *Mycoplasma* sp. or a *Mycoplasma* sp. other than *M. bovis* detected in their milk samples. In conclusion, we observed that *M. bovis* is the most common pathogenic *Mycoplasma* species found in mastitic milk, but other *Mycoplasma* species are not uncommon. Our results suggest that it is critical to test milk samples for mycoplasmas using diagnostic tests able to identify both the genus and the species.

Key words: *Mycoplasma bovis*, *Mycoplasma* spp., *Acholeplasma* spp., mastitis, molecular identification

INTRODUCTION

Mastitis leads to substantial economic losses in the dairy industry, and the timely identification of etiological agents can significantly contribute to controlling the disease (Blosser, 1979). This identification is even more relevant for contagious pathogens such as *Mycoplasma* spp., which represent an important target for herd survey screening in the United States and other parts of the world (Fox et al., 2005; Barkema et al., 2009; Nicholas et al., 2016). *Mycoplasma* spp. are considered among the most important pathogens in cattle systems of industrialized and developing countries (Nicholas, 2011), causing clinical, subclinical, and chronic IMI that can persist through the current lactation and into subsequent lactations (Fox, 2012; Nicholas et al., 2016; Parker et al., 2018). Although the prevalence is not as high as for some other pathogens, *Mycoplasma* IMI can result in severe udder disease (Fox et al., 2003). *Mycoplasma* IMI are usually highly contagious; they can affect more than one quarter, cause significant decreases in milk yield, and do not respond to antimicrobial treatment (Jasper, 1981; Britten, 2003) or therapy (Bushnell, 1984). Culling remains the most common recommendation for controlling *Mycoplasma* infections (Britten, 2003). In countries with large dairy herds, *Mycoplasma* IMIs are a significant concern, and more than 21% of large dairies with over 500 cows may be affected in the United States (APHIS, 2003).

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Historically, the most relevant *Mycoplasma* species reported to cause IMI in cattle is *M. bovis*, but other species have also been isolated from cow milk (Boonyayatra et al., 2011; Fox, 2012), and mixed *Mycoplasma* infections may occur in a herd and be associated with disease (González and Wilson, 2003; Pinho et al., 2013). Therefore, knowledge of the circulating *Mycoplasma* species, their co-occurrence with *M. bovis*, and their involvement in mastitis cases could result in the use of appropriate molecular diagnostic tests for their detection, enabling timely management decisions to control outbreaks and prevent new cases.

According to the National Mastitis Council (NMC) guidelines, the gold standard method to detect mycoplasmas in milk samples is based on bacteriological culture on selective solid media and the growth of colonies with the typical “fried-egg” appearance in positive samples (NMC 2017; Parker et al., 2018). Standard bacteriological culture does not differentiate *Mycoplasma* spp. from *Acholeplasma* spp. due to their similar colony morphology. Knowing the species of *Mycoplasma* isolated from the milk is useful information, but the distinction between bacterial genera is also critical because it is not entirely clear if *Acholeplasma* spp. can act as a primary pathogen. Deciphering *Mycoplasma* spp. from *Acholeplasma* spp. is important because historically *Acholeplasma* spp. have often been considered as nonpathogenic saprophytic milk contaminants originating from the soil, compost, and wastewater (Jasper, 1982). An alternative culture-based test for discriminating *Mycoplasma* spp. from *Acholeplasma* spp. relies on antimicrobial discs with digitonin or nisin producing inhibition zones with different sizes for the 2 genera (Boonyayatra et al., 2012), but the results are often difficult to interpret (Parker et al., 2018). To overcome these limitations, the Quality Milk Production Services (QMPS) laboratories at the Animal Health Diagnostic Center, Cornell University (Ithaca, NY) have developed a molecular speciation test that is offered after the detection of suspected *Mycoplasma* colonies from a milk sample (Gioia et al., 2016).

This study presents the results obtained from milk samples submitted over a 40-mo period to QMPS for mycoplasma detection from dairy herds located in New York and in other eastern US states, Texas, and New Mexico. We determined the species of a subselection of mycoplasma culture-positive isolates and describe the geographical distribution of the identified species isolated by culture according to the location of the dairy herds from which the milk samples were obtained. From the outcome of our data, we evaluated the occurrence and the distribution patterns of the different *Mycoplasma* species, and we investigated the occurrence of mixed infections within dairy herds.

MATERIALS AND METHODS

Milk Samples

Milk samples (214,518) from 2,757 US dairy herds were received for mycoplasma testing at QMPS laboratories between August 2016 and December 2019. Milk was collected or received from individual quarters (clinical quarter milk samples), combined from multiple quarters of a cow into one sample tube (composite milk samples), or from bulk tanks. Composite samples were tested as a herd survey or for the screening of freshly calved cows, cows with high SCC, or cows in the hospital pen. Pooled samples were quarter or composite samples from 3 to 5 cows combined.

Bacteriology

Milk samples were subjected to standard mycoplasma culture methods described in the NMC guidelines (NMC, 2017) in the context of the diagnostic routine at QMPS. Modified Hayflick medium (Northeast Laboratory) was used for mycoplasma isolation. Milk samples were mixed by inversion and a sterile cotton swab applicator was used to spread milk samples onto one-half of a standard Petri dish. After 7 d of incubation at 37°C in an atmosphere of >95% humidity and 5% carbon dioxide, all plates were examined under an illuminated stage stereomicroscope (Olympus SZH-ILLB) for visualizing colonies with morphology consistent with *Mycoplasma* spp.

Molecular Identification

Based on voluntary requests by herd owners, managers, or veterinarians, a subset of the mycoplasma-positive samples were subjected to further molecular testing. This testing used a multitarget PCR assay that enabled identifying *M. bovis* at the species level and discriminating *Mycoplasma* from *Acholeplasma* at the genus level (Gioia et al., 2016). Molecular tests were performed directly on colonies grown on the mycoplasma agar plates that exhibited suspicious or typical fried-egg morphology. Agar plates submitted for molecular testing were processed immediately or after a maximum storage of 15 d at 4°C. In the diagnostic routine, the assay can confirm or reject a presumptive positive mycoplasma culture result for which a single colony or atypical growth prevents a conclusive identification as *Mycoplasma* spp. Isolates confirmed as *Mycoplasma* spp. but not identified as *M. bovis* can be identified at the species level through an optional further step that includes Sanger sequencing of the PCR amplicons and subsequent sequence analysis. The same process is

Table 1. Distribution of the 3,728 samples testing positive for *Mycoplasma* by bacterial culture from 2016 to 2019 at the Quality Milk Production Services laboratories (Ithaca, NY)

Geographical origin	Sample type			
	Tank ¹	Pooled ²	Composite ³	Quarter ⁴
New York State	458	740	1,324	480
Non-New York State	164	142	314	106
Total	622	882	1,638	586

¹Milk collected from the bulk tank.²Combined milk from 3 to 5 cows.³Combined milk from quarters of the same cow.⁴Quarter milk from 1 cow.

also done in the case of *Acholeplasma*-positive samples in the PCR. All sequence analyses were offered as an optional step performed only if the client's consent was received. A small portion of veterinarians or farmers declined to pursue the availability of further speciation when samples resulted in *Mycoplasma* species other than *M. bovis*. For those clients, a *Mycoplasma* confirmation test with only the identification or exclusion of *M. bovis* presence was considered enough to make management decisions. Most veterinarians or farmers who sent samples that were identified as containing *Acholeplasma* spp. declined the option to proceed for further speciation because they perceived no additional value in knowing which species of *Acholeplasma* was present.

Data Analysis

The data were extracted from the Veterinary Hospital & Laboratory Management System (VetView), which is used to enter all client and testing information submitted to the Animal Health Diagnostic Center. Using Tableau (Tableau Software), an intelligence software used for data analysis and data visualization, we ran queries to obtain relevant information. The data were exported using .csv format and imported into Microsoft Excel (2016 version, Microsoft Corp.) for further analyses. The data related to the speciation results were taken from Excel worksheet records maintained in the QMPS Molecular Laboratory. All the data used for the present study were summarized and descriptive statistics are presented.

RESULTS

Herd Locations, Milk Sample Types, and Proportion of Samples Positive for *Mycoplasma* Culture

Out of 214,518 milk samples cultured for the presence of *Mycoplasma*, 199,885 (93.2%) were from New York State and 14,633 (6.8%) were from other states

(eastern states, Texas, and New Mexico). The distribution based on sample types included 12,231 bulk tank (5.7%), 84,098 pooled (39.2%), 88,407 composite (41.2%), and 29,782 quarter (13.9%). Of all the milk samples tested, 3,728 (1.7%) grew *Mycoplasma* colonies on culture. The state distribution (NY and non-NY) of culture-positive milk samples according to the sample type is summarized in Table 1.

Mycoplasma and *Acholeplasma* Species Identified in Culture-Positive Milk Samples

Of the 3,728 *Mycoplasma* culture-positive samples, 889 (23.8%) were subjected to molecular identification of the isolate; 694 (78.1%) were from New York and 195 (22.8%) were from other states. Figure 1 illustrates their geographical distribution. Details on samples and herds are provided in Table 2.

At the genus level, 96.2% of all 889 speciated isolates were *Mycoplasma* spp., while 3.8% were *Acholeplasma* spp. *Mycoplasma bovis* was the most frequently identified species (75.1%), followed by *Mycoplasma bovigenitalium* (6.5%) and *Mycoplasma canadense* (5.8%). All identified species are presented in Table 3.

The 889 identified isolates originated from the following sample types: 190 bulk tank, 196 pooled, 354 composite, and 149 quarter milk. The distribution of the *Mycoplasma* spp. varied in the different sample types. Figure 2 illustrates the percentage of *Mycoplasma* and *Acholeplasma* species according to the sample type (bulk tank, pooled, composite, and quarter).

Mycoplasma bovis was the species identified most frequently in all sample types, accounting for 82.2%, 79.7%, and 65.8% of isolates identified from composite, bulk tank, and quarter samples, respectively. Among *Mycoplasma* spp. other than *M. bovis*, *M. bovigenitalium* was identified most often in quarter samples (13.4%), *M. canadense* in pooled samples (14.3%), and *Mycoplasma alkalescens* and *Mycoplasma arginini* in bulk tank samples (10.0 and 3.7%, respectively). *Mycoplasma californicum* was identified in only 1 pooled

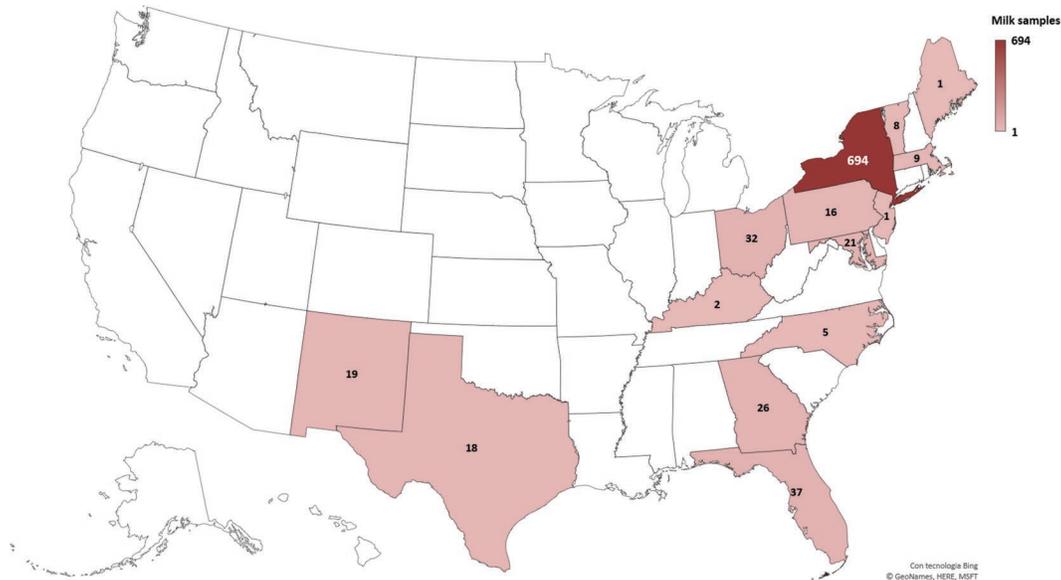


Figure 1. Geographical distribution of 889 milk samples submitted from 2016 to 2019 to Quality Milk Production Services laboratories (Ithaca, NY) producing the *Mycoplasma* and *Acholeplasma* isolates that were subjected to molecular identification.

sample, and *Mycoplasma primatum* in only 1 quarter sample. Some isolates that were not *M. bovis* (*Mycoplasma* spp.) were not identified further because the request was not made. These isolates originated most frequently from quarter milk samples (4.0%). Finally, *Acholeplasma* spp. were isolated most often in composite samples (6.2%).

Isolation of Single or Multiple *Mycoplasma* and *Acholeplasma* Species in the Tested Herds

The presence of single or multiple species of *Mycoplasma* and of *Acholeplasma* in the tested herds is re-

Table 2. Geographical distribution of 889 *Mycoplasma* or *Acholeplasma* isolates from milk samples submitted for bacterial culture from 2016 to 2019 to Quality Milk Production Services laboratories (Ithaca, NY), and of the 98 herds from which they originated

State	No. of milk samples (%)	No. of herds (%)
New York	694 (78.1)	66 (67.4)
Florida	37 (4.1)	4 (4.1)
Georgia	26 (3.1)	1 (1.0)
Kentucky	2 (0.2)	1 (1.0)
Massachusetts	9 (1.0)	2 (2.0)
Maryland	21 (2.4)	4 (4.1)
Maine	1 (0.1)	1 (1.0)
North Carolina	5 (0.6)	1 (1.0)
New Jersey	1 (0.1)	1 (1.0)
New Mexico	19 (2.1)	2 (2.0)
Ohio	32 (3.5)	5 (5.2)
Pennsylvania	16 (1.8)	4 (4.1)
Texas	18 (2.0)	2 (2.0)
Vermont	8 (0.9)	4 (4.1)

ported in Table 4. In most herds, *M. bovis* was the only species isolated from milk (60.2%). A smaller percentage of herds had positive results due to *Mycoplasma* spp. other than *M. bovis* (8.2%), and 2 herds showed only *Acholeplasma* spp. (2.0%). In several herds, however, we detected the presence of *M. bovis* together with other *Mycoplasma* spp. (20.4%), sometimes also together with *Acholeplasma* spp. (9.2%). The species found most often in these co-infections with *M. bovis* were *M. bovisgenitalium*, *M. alkalescens*, *M. canadense*, and *M. arginini*, respectively. Most of the herds (65%) with more than 1 species detected showed the presence of only 1 other *Mycoplasma* sp. in addition to *M. bovis*, while the remaining herds had 2 or more other *Mycoplasma* species detected. *Mycoplasma arginini* was detected only in herds with a large variety of other species found.

Table 3. Molecular identification of 889 *Mycoplasma* or *Acholeplasma* isolates from milk samples submitted for bacterial culture from 2016 to 2019 to Quality Milk Production Services laboratories (Ithaca, NY)

Species	New York State	Non-New York State	Total, no. (%)
<i>M. bovis</i>	511	157	668 (75.1)
<i>M. bovisgenitalium</i>	53	5	58 (6.5)
<i>M. canadense</i>	51	1	52 (5.9)
<i>M. alkalescens</i>	24	20	44 (5.0)
<i>M. arginini</i>	12	3	15 (1.7)
<i>M. californicum</i>	1	0	1 (0.1)
<i>M. primatum</i>	1	0	1 (0.1)
<i>Mycoplasma</i> spp. ¹	8	8	16 (1.8)
<i>Acholeplasma</i> spp. ¹	33	1	34 (3.8)

¹The species was not identified with the PCR method.

Table 4. Occurrence of *Mycoplasma bovis*, other *Mycoplasma* spp., and *Acholeplasma* spp., in 98 herds sending their samples from 2016 to 2019 to Quality Milk Production Services laboratories (Ithaca, NY) for which molecular identification of *Mycoplasma* or *Acholeplasma* isolates was performed

Bacterial species present at the farm	No. of herds (%)
<i>M. bovis</i> only	59 (60.2)
<i>M. bovis</i> and other <i>Mycoplasma</i> spp.	20 (20.4)
<i>M. bovis</i> , other <i>Mycoplasma</i> spp., and <i>Acholeplasma</i> spp.	9 (9.2)
<i>Mycoplasma</i> spp. different than <i>M. bovis</i> only	8 (8.2)
<i>Acholeplasma</i> spp. only	2 (2.0)

DISCUSSION

We present the results from routine mycoplasma cultures of bulk tank, pooled, composite, and quarter milk samples submitted to QMPS laboratories between 2016 and 2019. For a portion of the isolates testing positive for *Mycoplasma* in culture, further molecular tests were performed to identify the species. This study demonstrated the presence of *M. bovis* across several states. Additionally, other *Mycoplasma* species with pathogenic potential were detected in notable numbers from several herds. This information is important for herd owners, managers, and veterinarians to know when selecting appropriate species-specific PCR tests for diagnosis of mycoplasmal mastitis. Diagnoses could be missed if only a *M. bovis* PCR is carried out in herds in which mastitis is caused by other *Mycoplasma* species. Co-infections with *M. bovis* were also detected in some herds. This study also highlighted the presence of *Acholeplasma* spp., which could be mistaken for *Myco-*

plasma spp. if only standard bacteriological cultures are used for diagnostic testing.

The detection of mycoplasmas in the different sample types has various interpretations: (1) the detection in bulk tank milk shows that *Mycoplasma* is present within a herd, (2) the detection within composite samples without clinical signs of mastitis is indicative of subclinical infections, and (3) the detection within clinical quarter milk samples suggests that *Mycoplasma* is possibly causing the mastitis. The value of knowing the genus and species of *Mycoplasma* is different for each sample type and is highly dependent on the individual farm situation.

Bulk Tank Milk Samples

Testing bulk tank milk samples is a good way to survey herds for infectious agents capable of causing mastitis, including *Mycoplasma* species. In our survey, *M. bovis* was the most common mycoplasma species detected for 190 mycoplasma culture-positive bulk tank milk samples from 98 herds across 14 states. *Mycoplasma alkalescens*, *M. arginini*, *M. bovigenitalium*, and *M. canadense* were also detected, in order of abundance. Of these, *M. bovigenitalium* and *M. canadense* have been reported to cause mastitis (Jackson and Boughton, 1991; Mackie et al., 2000; Fox, 2012). *Mycoplasma alkalescens* and *M. arginini* have also been previously isolated from bulk milk, but less evidence exists to suggest they cause mastitis (Kokotovic et al., 2007; Justice-Allen et al., 2011). In the present study, *M. arginini* was isolated most frequently from bulk tank samples from farms where we also detected other *Mycoplasma*

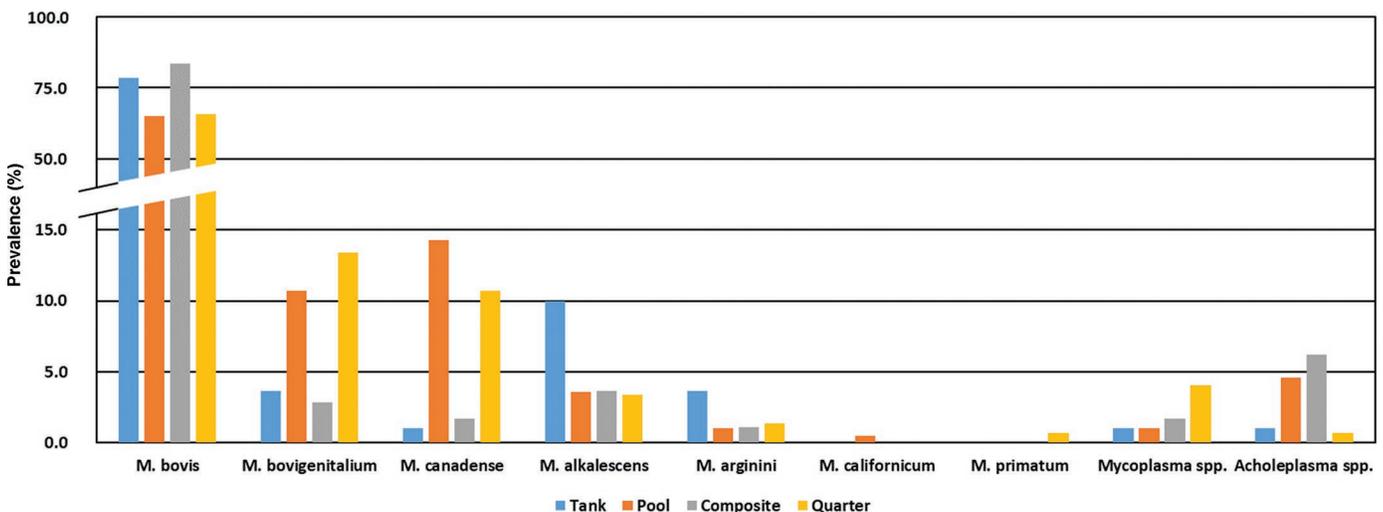


Figure 2. Percentages of *Mycoplasma bovis*, other *Mycoplasma* spp., and *Acholeplasma* spp., in bulk tank, pool, composite, and quarter milk. The class *Mycoplasma* spp. includes the isolates that were not *M. bovis* but were not processed for further speciation. The y-axis is broken for a better visualization of lower-abundance species.

species. We found no reports of mastitis caused by *M. arginini*, although this species is frequently detected in bulk milk originating from herds affected by mastitis of different etiologies (Fox et al., 2005; Higuchi et al., 2011; Justice-Allen et al., 2011).

The observation of *M. bovis* as the main species identified in bulk tank milk is consistent with the data presented by Britten et al. (2020) at the 59th National Mastitis Council Annual Meeting. *Mycoplasma bovis* and *M. alcalescens* were the second and third most commonly identified species, respectively. However, *M. californicum* was more common than *M. canadense*.

In our experience, the vast majority of herd managers, owners, or veterinarians decide to pursue the genus and species identification for bulk tank samples once they are given the option. According to guidelines of QMPS's veterinarians, if a bulk tank sample is positive for *M. bovis*, then action is needed to determine which individual cows are infected, so that they can be removed. If a bulk tank sample comes back as positive for *Acholeplasma*, then the advice is to resample the bulk tank after a period of time. If a bulk tank sample is positive for *Mycoplasma* species other than *M. bovis*, including *M. alcalescens*, *M. bovis*, *M. californicum*, or *M. canadense* (González and Wilson, 2003; Mackie et al., 1982, 1986), then the herd manager, owners, or veterinarian may decide if it is worth the effort and expense to find the individual cows. If it is any of the *Mycoplasma* spp. other than *M. bovis*, then resampling the bulk tank after a period of time would be advised.

Composite Milk Samples

Composite milk samples submitted to our laboratories were from herds surveying groups of cows that had freshly calved, had high SCC, or were contained within the hospital pen. We observed high numbers of *Mycoplasma*-infected cows based on composite milk samples (41.2%). Most of the composite samples found to be positive were collected from cows without signs of clinical mastitis in the context of herd screening programs or from cows belonging to herds in which clinical *Mycoplasma* cases had been diagnosed. These subclinical infections could be self-limiting or the species identified may vary in pathogenicity (González and Wilson, 2003; Nicholas et al., 2016). Intermittent shedding is an important feature of subclinical *Mycoplasma* infections (Biddle et al., 2003; Caswell and Archambault, 2007; Hazelton et al., 2018) and therefore needs to be considered a limiting factor if attempting to detect subclinical infections. Interestingly, Hazelton et al. (2020) do not advise testing whole herds for the detection of *M. bovis*

subclinical infections after all clinical mastitis cases have been culled following an outbreak. To overcome this issue, it is important that farms are periodically screened (Rosenbusch, 2003).

In our mycoplasma culture-positive composite samples, most contained *M. bovis* (82.2%), followed by *Acholeplasma* spp. (6.2%), *M. alcalescens* (3.7%), or *M. bovis* (2.8%). For composite samples, the value of pursuing genus or species testing was highly dependent on the individual herd situation. Very little information on this sample type for *Mycoplasma* spp. other than *M. bovis* or for *Acholeplasma* has been published in the literature, making a definitive decision more difficult.

For some herd situations in which the tolerance for *Mycoplasma* is very low, the veterinarian or manager stated that pursuing genus or species identification has no value because the cow will be culled anyway to reduce the risk to the rest of the herd. In other herd situations, however, the veterinarian or manager elected to pursue genus or species identification to make sure that cows were not inappropriately culled for being mycoplasma culture positive with *Acholeplasma* or with a *Mycoplasma* sp. other than *M. bovis* that has questionable mammary gland pathogenicity. When these latter situations are found for composite samples, a review of the sample collection procedure is initiated and the individual cows are segregated, monitored by SCC, and retested.

Quarter Milk Samples

Quarter milk samples submitted to our laboratories were from clinical mastitis cases. In quarter samples, *M. bovis* (65.8%) was followed by *M. bovis* (13.4%), *M. canadense* (10.7%), *M. alcalescens* (3.4%), and *M. arginini* (1.3%); other *Mycoplasma* spp. (4.7%) and *Acholeplasma* spp. (0.7%) were also found. For quarter samples that were assumed to be from cows with clinical mastitis, some herd managers, owners, and veterinarians stated that knowing the species of *Mycoplasma* had no value because the cow would be a cull candidate in any case for the benefit of the herd (Pfütznner and Sachse, 1996). Others perceived value in case of sample contamination at the time of collection and wanted to differentiate *Mycoplasma* from *Acholeplasma* (Jasper et al., 1979, 1981; Bushnell, 1984) or to identify *Mycoplasma* spp., such as *M. arginini*, that have not been shown to cause mastitis (Stipkovits et al., 2013). Previous studies have suggested that *M. arginini* does not cause mastitis but significantly predisposes the animal to infection with *Streptococcus dysgalactiae* leading to severe mastitis (Stipkovits et al., 2013). *Mycoplasma arginini* is frequently isolated from various animal spe-

cies with different pathological conditions, such as from cattle with respiratory disease (Thomas et al., 2002; Ayling et al., 2004). It has been demonstrated that *M. arginini* can persist for a long time under different environmental conditions (Nagatomo et al., 2001). Only a mild decrease in milk production and a slight increase in SCC were noticed after challenging cows with *M. arginini* into the udder (Stipkovits et al., 2013).

When *Acholeplasma* or *Mycoplasma* spp. other than *M. bovis* with questionable pathogenicity are found, the veterinarian and managers have typically advised segregating the cow, so that she is not a risk to the herd, and monitoring her over time to determine if she clears the infection and returns to low SCC and mycoplasma culture-negative status.

Concerning *Mycoplasma* species other than *M. bovis*, we found *M. bovisgenitalium* and *M. canadense* mostly in quarter and pooled samples. Also, we often identified *M. bovisgenitalium* in farms showing the concurrent presence of multiple species. Their mammary gland pathogenicity and virulence factors are poorly known, and our prominent identification of these non-*M. bovis* species in quarter milk, typically collected from clinical mastitis cases, suggests their possible role in mammary gland health and deserves further investigation.

Transmission of *Mycoplasma* can occur internally, and a single clone of bovine *Mycoplasma* could affect and cause disease in multiple body sites over several months in cattle of all ages (Djordjevic et al., 2001; Punyapornwithaya et al., 2010). This is the case for *M. bovisgenitalium*, which is frequently isolated from milk samples but is also reported to be associated with diseases of the reproductive tract (Catania et al., 2014; Hata et al., 2017; Parkinson, 2019).

In addition to the species typically found in cow milk, we isolated *Mycoplasma primatum* from a clinical mastitis quarter milk sample. This species has not been isolated from milk samples or the dairy environment previously, but it is commonly found in the oral and urogenital tracts of primates (DelGiudice et al., 1971).

Independent of sample type considerations, another interesting observation emerging from our study was the presence of co-infections by *M. bovis* and other *Mycoplasma* spp., sometimes also together with *Acholeplasma* spp., in the same herd. The species most commonly found in these coinfections were *M. bovisgenitalium*, *M. alkalescens*, *M. canadense*, and *M. arginini*, respectively. *Mycoplasma arginini* was detected only in herds with a large variety of other species found. This finding also calls for more investigations to determine if other species have a direct influence on mammary gland health or if they have only a nonclinical or production-related effect. Also, other diagnostic approaches may

be useful in these cases, such as evaluating the possible co-occurrence of other mastitis pathogens detectable by aerobic cultures.

Mycoplasma species identification using molecular methods may help to clarify the role played by different *Mycoplasma* spp. in udder health, and it also differentiates *Mycoplasma* from *Acholeplasma*. The consequences of *Acholeplasma* isolation from milk have not been investigated in detail, and it remains unknown whether these bacteria are present in milk as IMI agents or as simple contaminants (Quinn et al., 2013; Parker et al., 2018), resulting from incorrect sampling procedures or from transient presence in the udder. In this study, *Acholeplasma* was only identified in 3.8% of culture-positive samples and, of these, in only 0.7% of all quarter milk samples (most likely with clinical mastitis), demonstrating no significant association with mastitis. Bulk tank, pooled, and composite milk samples were more likely to be contaminated with *Acholeplasma*. With regard to the sampling practices, it is more difficult to collect composite samples than quarter samples. Composite samples have a higher chance of contamination because the vials are kept open for a long time and undergo repositioning under the cow udder. This could explain why a greater proportion of the composite samples were *Acholeplasma* positive.

In the future, implementing direct quantitative PCR in milk may significantly reduce time to results, but assay conditions will have to be carefully defined to avoid specificity issues, and cost-benefit considerations for the farmers will have to be made (Parker et al., 2018). Also, as mentioned above, most of the quantitative PCR assays available only target *M. bovis*, and the current study further confirms that other species are routinely isolated from milk. A PCR for *M. bovis* alone would not be suitable in 8.2% of the herds in which *M. bovis* was not the species cultured, and this fact will have to be accounted for when implementing PCR as the only testing method.

In conclusion, *M. bovis* was the most common mycoplasma species identified in the milk samples tested in our laboratories, but *Mycoplasma* spp. other than *M. bovis* as well as *Acholeplasma* spp. were routinely found. In addition, isolation of multiple species from the same herd was not uncommon. Therefore, determining the species of any mycoplasma culture-positive sample is important to inform the herd owner, manager, or veterinarian on the clinical significance of the isolate and guide possible PCR tests available to those herds for future mastitis cases due to *Mycoplasma*. In the future, a better knowledge of the distribution of non-*M. bovis* species and of their relationships with clinical signs in the herd will improve our understanding of the epide-

miology and the relevance of other *Mycoplasma* species in milk production losses, enabling more meaningful testing and management decisions.

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