Effect of atmospheric carbon dioxide concentration on the cultivation of bovine *Mycoplasma* species

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ABSTRACT

Recommendations for bovine mycoplasma culture CO₂ concentrations are varied and were not empirically derived. The objective of this study was to determine whether the growth measures of bovine mycoplasma isolates differed when incubated in CO₂ concentrations of 10 or 5% or in candle jars (2.7 ± 0.2% CO₂). Growth of *Mycoplasma bovis* (n = 22), *Mycoplasma californicum* (n = 18), and other *Mycoplasma* spp. (n = 10) laboratory isolates was evaluated. Isolate suspensions were standardized to approximately 10⁸ cfu/mL and serially diluted in pasteurized whole milk to achieve test suspensions of 10² and 10⁶ cfu/mL. One hundred microliters of each test dilution was spread in duplicate onto the surface of a modified Hayflick's agar plate. Colony growth was enumerated on d 3, 5, and 7 of incubation. A mixed linear model included the fixed effects of CO₂ treatment (2.7, 5, or 10%), species, day (3, 5, or 7), and their interactions, with total colony counts as the dependent variable. Carbon dioxide concentration did not significantly affect overall mycoplasma growth differences, but differences between species and day were present. Colony counts (log₁₀ cfu/mL) of *M. bovis* were 2.6- and 1.6-fold greater than *M. californicum* and other *Mycoplasma* spp., respectively. Growth at 7 d of incubation was greater than d 3 and 5 for all species. These findings were confirmed using field isolates (n = 98) from a commercial veterinary diagnostic laboratory. Binary growth responses (yes/no) of the field isolates were not different between CO₂ treatments but did differ between species and day of incubation. On average, 57% of all field isolates were detected by 3 d of incubation compared with 93% on d 7. These results suggest that the range of suitable CO₂ culture conditions and incubation times for the common mastitis-causing *Mycoplasma* spp. may be broader than currently recommended.

Key words: mastitis, *Mycoplasma* spp., culture, carbon dioxide

INTRODUCTION

Mycoplasma mastitis, first described by Hale et al. (1962), is an important disease of the dairy industry worldwide. Due to decreased milk production and quality, mycoplasma mastitis causes an approximately \$108 million annual loss in the US industry (Rosenbusch as cited by Rosengarten and Citti, 1999). The predominant *Mycoplasma* spp. causing mastitis are *Mycoplasma bovis* and *Mycoplasma californicum*; *Mycoplasma bovis*, *Mycoplasma genitalium*, *Mycoplasma alkalescens*, and *Mycoplasma canadense* are less frequently isolated from mastitic cows and bulk tank milk samples (González and Wilson, 2003). As contagious organisms, these pathogens can affect a cow in any stage of lactation, including the dry period (Jasper, 1982). As with other contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*, transmission of mycoplasma mastitis is believed to occur during milking (González and Wilson, 2003), but the ability of mycoplasmas to colonize other body sites that do not directly contact other animals suggests that other modes of transmission via blood, lymph, or aerosols may occur (Fox, 2012). Because of these factors, control strategies rely heavily on identification and culling of infected animals (Fox et al., 2005).

The National Mastitis Council (Middleton et al., 2017) currently recommends isolating mycoplasma by spreading 100 µL of milk onto a modified Hayflick's agar plate and incubating at 37°C under microaerophilic conditions (10% CO₂) for 7 to 10 d. This recommendation is derived from the initial report of *M. bovis* mastitis by Hale et al. (1962) in which incubation for 5 d in 10% CO₂ yielded isolates. However, other studies have reported successfully cultivating mycoplasma from avian, bovine, and cell culture sources when using either a candle jar to limit oxygen tension or 5% CO₂ (Fabricant et al., 1962; Stipkovits et al., 1975; Amin and Jordan, 1978; Polak-Vogelzang et al., 1983; Kotani et al., 1990; Nicholas and Baker, 1998; Parker et al.,

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2017). With such atmosphere variability in mycoplasma culture conditions reported in the literature and with *Mycoplasma* spp. becoming more prevalent, empirical evaluation of isolation protocols may lead to improved mycoplasma mastitis diagnosis. The objective was to determine whether bovine mycoplasma isolates differ in growth measures when incubated in CO₂ concentrations of 10 or 5% or in candle jars ($2.7 \pm 0.2\%$ CO₂). Using these findings, veterinary diagnosticians may be able to select a more optimal atmosphere for culturing mycoplasma pathogens from milk.

MATERIALS AND METHODS

Laboratory Isolates

Mycoplasma bovis (n = 22), *M. californicum* (n = 18), *M. bovis genitalium* (n = 5), and *Mycoplasma arginini* (n = 5) laboratory isolates were selected from the Washington State University Field Disease Investigative Unit culture collection and were filtration-cloned before use for the first study (Boonyayatra et al., 2012). Herein, these *M. bovis genitalium* and *M. arginini* isolates are collectively referred to as *Mycoplasma* spp. (n = 10). Species verification was performed by PCR-RFLP analysis (Tang et al., 2000; Boonyayatra et al., 2012). Bulk tank milk (3 L) was collected from the Washington State University herd, and 250-mL portions were split into sterilized 500-mL beakers for pasteurization by microwave. Milk was microwaved with a household microwave (model no. EM-1100S, Sanyo Electronics, Little Ferry, NJ) at the highest setting for 3.5 min; the microwaving was periodically interrupted to swirl the milk in the beaker. Aliquots (100 μ L) were removed from the beaker and cultured on Columbia blood agar (Hardy Diagnostic), and plates were incubated aerobically for 48 h at 37°C. Only microwaved milk free of bacterial growth as tested was used and was stored frozen (-15°C) until needed. Isolates to be tested were defrosted at ambient temperature, and 400 μ L of culture stock was transferred to 40 mL of sterile pleuropneumonia-like organisms (PPLO) broth. Inoculants were incubated at 37°C in 10% CO₂ for 5 d. Broth cultures of the mycoplasma strains were then centrifuged at $5,000 \times g$ for 30 min. The cell pellet was suspended in 20 mL of PBS solution and centrifuged for a total of 3 resuspensions or washings. Washed cells were suspended in 3 mL of PBS solution. The mycoplasma suspension was diluted to achieve an optical density of 0.2 at 540 nm (Punyapornwithaya et al., 2010). Mycoplasma suspensions were subsequently serially diluted with pasteurized bulk tank milk collected to achieve target concentrations that would produce several colonies in a visually countable range. The milk samples were incubated for

24 h at 37°C in 10% CO₂. To enumerate colony growth, a 100- μ L portion of inoculated samples was serially diluted and 100- μ L portions were spread onto the entire surface of a modified Hayflick's agar plate (BD BBL, Thermo Fisher Scientific Inc., Waltham, MA). Petri dishes were incubated at 37°C under various CO₂ concentrations: incubators set at 10 and 5% CO₂ and in 1-gallon jars with a metal screw cap containing a lit unscented votive candle (Mainstays, Walmart Inc., Bentonville, AR) that produced an average CO₂ atmosphere of $2.7 \pm 0.2\%$. To verify CO₂ concentrations, a 0 to 30% CO₂, temperature, and relative humidity data logger (CM-0017, CO2Meter.com, Ormond Beach, FL) was used. Candle jars were opened only on the day of enumeration, and all plates were discarded after counting. Colonies on each plate with the characteristic "fried egg" appearance as examined using a dissecting microscope (15 $\times$ magnification) were first enumerated on d 3, 5, and 7 of incubation.

Field Isolates

Clinical mycoplasma mastitis field samples (n = 304) were used in a second experiment to verify the findings from the laboratory isolates. These were collected over a 19-mo period by a commercial veterinary diagnostic laboratory (Dairy Vet Management, Sunnyside, WA) from sample submissions confirmed to contain mycoplasma-like species. Samples were kept frozen (-15°C) and were defrosted at ambient temperature immediately before use, and 100 μ L was spread onto the entire surface of a modified Hayflick's agar plate. Petri dishes were incubated and examined as above after 3 and 7 d of incubation.

A representative colony was extracted from plates with growth (n = 98) after 7 d of incubation, inoculated into 10 mL of sterile PPLO broth, and incubated for 5 d in 10% CO₂. Genomic DNA was extracted with the Pure-Link Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) according to the user manual provided with the kit. Species identification was performed using a nested PCR-RFLP analysis (Tang et al., 2000; Boonyayatra et al., 2012). Polymerase chain reaction was performed in a total volume of 50 μ L containing 1 $\times$ PCR buffer (20 mM Tris-HCl, 2 mM MgCl₂, and 50 mM KCl; pH 8.4) and 50 μ M each of deoxynucleoside triphosphates, 20 pmol of each primer, and 1 U of Taq DNA polymerase (Invitrogen). A set of primers including F2, R2, and R34 (Tang et al., 2000) was used to amplify the 16S and 23S rRNA intergenic spacer regions of *Mycoplasma* and *Acholeplasma*. Five microliters of DNA extract was used as a template by adding it into 45 μ L of reaction mixture. The thermal cycling protocol was performed on an iCycler (Bio-Rad, Hercules, CA) with the follow-

ing conditions: 1 cycle of 94°C for 30 s; 30 cycles for the first-stage PCR (or 35 cycles for the second-stage PCR) of 94°C for 30 s; 1 cycle of 55°C for 2 min; 1 cycle of 72°C for 2 min; and 1 cycle of 72°C for 5 min. Products were digested with *AseI* for RFLP analysis. Products were electrophoresed on a 2% agarose gel, and patterns of DNA fragments were visualized by UV fluorescence (Boonyayatra et al., 2012).

Statistical Analysis

Laboratory Isolates. The UNIVARIATE procedure of SAS 9.3 (SAS Institute Inc., Cary, NC) was used to confirm that the data were normally distributed. The MIXED procedure of SAS 9.3 was used to evaluate the fixed effects of treatment (2.7, 5, or 10% CO₂), species (*M. bovis*, *M. californicum*, or *Mycoplasma* spp.), and day (3, 5, or 7) and their interactions on enumerations (log₁₀ cfu/mL). All main effects were retained, and stepwise backward elimination was used to remove nonsignificant interactions ($P \geq 0.05$). Three individual species models were used to evaluate the fixed effects of treatment (2.7, 5, or 10% CO₂) and day (3, 5, or 7) and their interactions on enumerations (log₁₀ cfu/mL). In the instance of the *M. californicum* and *Mycoplasma* spp. models, a compound symmetry covariance structure was specified and used to account for unequal variances. Means for the respective models were compared using Fisher's least significant differences test.

Field Isolates. The GLIMMIX procedure in SAS 9.3 with a logit link and binomial error distribution was used to model the probability of growth (yes/no) for field isolates. The variables of interest were CO₂ concentration (2.7, 5, or 10%) and incubation length (3 or 7 d), and differences among species recovered (*M. bovis*, *M. californicum*, *Acholeplasma axanthum*, and *Mycoplasma* spp.) were compared.

RESULTS

Laboratory Isolates

All plates yielded growth, with a minimum organism recovery of 3.98 cfu/mL of sample. Overall, mean isolate log₁₀ colony-forming unit enumerations were not significantly influenced by the 3 tested CO₂ concentrations ($P = 0.65$); the average enumeration ($\pm$ SEM) of all mycoplasma isolates incubated within a candle jar, 5% CO₂, and 10% CO₂ were 4.72 ± 0.22 , 4.74 ± 0.22 , and 4.68 ± 0.22 log₁₀ cfu/mL, respectively. Day of incubation was significantly different across all species, with enumerations increasing from 4.43 ± 0.22 log₁₀ cfu/mL after 3 d of incubation to 4.91 ± 0.22 log₁₀ cfu/mL ($P = 0.0013$) at 7 d of incubation. Additionally,

differences in enumerated growth among species were identified ($P < 0.0001$). Across all CO₂ concentrations, *M. bovis* isolates had the greatest enumerated growth at 7 d of incubation (8.14 ± 0.04 log₁₀ cfu/mL), followed by *Mycoplasma* spp. (4.56 ± 0.07 log₁₀ cfu/mL) and *M. californicum* (4.46 ± 0.12 log₁₀ cfu/mL). Within bacterial species, significant differences were detected between strains of bacteria ($P < 0.05$).

The average *M. bovis* isolate enumeration ($\pm$ SEM) was 8.02 ± 0.04 , 8.09 ± 0.04 , and 8.08 ± 0.04 log₁₀ cfu/mL when incubated for 7 d within a candle jar, 5% CO₂, or 10% CO₂, respectively, and differences were not significant ($P = 0.14$; Figure 1). All isolates were detected by 3 d of incubation regardless of CO₂ concentration, though log₁₀ enumerations were significantly greater after 7 d of incubation ($P = 0.01$; Figure 2). Across all CO₂ concentrations, enumerations ($\pm$ SEM) increased from 8.04 ± 0.04 log₁₀ cfu/mL after 3 d of incubation to 8.21 ± 0.04 log₁₀ cfu/mL after 7 d of incubation.

For the *Mycoplasma* spp. isolates, the average enumerated isolate growth ($\pm$ SEM) was 4.62 ± 0.07 , 4.55 ± 0.07 , and 4.51 ± 0.07 log₁₀ cfu/mL when incubated for 7 d within a candle jar, 5% CO₂, or 10% CO₂, respectively, and differences were not significant ($P = 0.53$; Figure 1). Enumerated growth was significantly greater with increasing incubation length ($P < 0.0001$; Figure 2). Across all CO₂ concentrations, enumerations ($\pm$ SEM) increased from 4.24 ± 0.07 log₁₀ cfu/mL after 3 d of incubation to 4.89 ± 0.07 log₁₀ cfu/mL after 7 d of incubation.

Figure 1. Growth (log₁₀ cfu/mL) $\pm$ SEM of each *Mycoplasma* species when laboratory isolates were incubated within a candle jar with $2.7 \pm 0.2\%$ CO₂ (black bars) or in incubators of 5% (hatched bars) or 10% (gray bars) CO₂ for 7 d.

Specifically, all *M. bovis* laboratory isolates were detected by 3 d of incubation regardless of CO₂ concentration. All *M. bovis* laboratory isolates were detected by 3 d of incubation regardless of CO₂ concentration. After 3 d of incubation, 90.9% of *M. californicum* laboratory isolates were detected when incubated within a candle jar or 5% CO₂ compared with 81.8% of isolates incubated in 10% CO₂. The percentage of isolates detected after 7 d of incubation increased to 100% regardless of CO₂ concentration. The average enumeration of *M. californicum* isolates ($\pm$ SEM) was 4.34 ± 0.12 , 4.59 ± 0.12 , and 4.45 ± 0.12 log₁₀ cfu/mL when incubated for 7 d within a candle jar, 5% CO₂, or 10% CO₂, respectively, and differences were not significant ($P = 0.33$; Figure 1). After 3 d of incubation, 90.9% of isolates were detected when incubated within a candle jar or 5% CO₂ compared with 81.8% of isolates incubated in 10% CO₂. The percentage of isolates detected after 7 d of incubation increased to 100% regardless of CO₂ concentration, and the number of colonies counted was significantly greater after 7 d of incubation ($P < 0.0001$; Figure 2). Across all CO₂ concentrations, enumerations ($\pm$ SEM) increased from 3.98 ± 0.12 log₁₀ cfu/mL after 3 d of incubation to 4.74 ± 0.12 log₁₀ cfu/mL after 7 d of incubation.

Field Isolates

After freezer storage for 4 to 558 d, 98 of the 304 field samples (32.24%) yielded growth for subsequent species identification (data not shown). Isolate species identi-

fied by PCR-RFLP were as follows: *A. axanthum* ($n = 4$), *M. bovis* ($n = 64$), *M. californicum* ($n = 26$), and other *Mycoplasma* spp. ($n = 4$). Isolates identified as other *Mycoplasma* spp. were presumptively *M. arginini* or *M. canadense*; however, due to similar amplicon sizes produced by chromosomal digests, a definitive species classification could not be made.

The proportion of isolates detected for each species was not significantly affected by the 3 tested atmospheric CO₂ concentrations ($P = 0.74$). Overall, growth detection was significantly influenced by different species ($P < 0.001$). Not all isolates were detected on all days of incubation under all CO₂ conditions: 85.26% of *M. californicum*, 82.61% of *A. axanthum*, and 68.83% of *M. bovis* isolates could be enumerated on all days and under all CO₂ conditions. *Mycoplasma* spp. isolates were detected on all days at all CO₂ conditions.

The percentage of *A. axanthum* isolates ($\pm$ SEM) that could be detected on both d 3 and 7 of incubation was 87 ± 12.5 , 75 ± 25 , and $87 \pm 12.5\%$ within a candle jar and under 5 or 10% CO₂, respectively (Figure 3). Across all CO₂ concentrations, the percentage of isolates identified increased 17% after d 7 of incubation (from $75 \pm 25\%$ to $92 \pm 8.3\%$; Figure 4). One *A. axanthum* isolate was not detected when incubated for 7 d under 5% CO₂.

For *M. bovis*, the percentage of isolates ($\pm$ SEM) that could be detected on both d 3 and 7 was 68 ± 5.2 , 71 ± 4.5 , and $67 \pm 4.7\%$ within a candle jar and under 5 and 10% CO₂, respectively (Figure 3). Additionally, $48 \pm 6.3\%$ of *M. bovis* field isolates were detectable by 3 d of incubation, which increased to $92 \pm 3.4\%$ after 7 d of incubation (Figure 4). Six *M. bovis* isolates were

Figure 2. Growth (log₁₀ cfu/mL) $\pm$ SEM of each laboratory isolate *Mycoplasma* species after 3 (black bars), 5 (hatched bars), and 7 (gray bars) days of incubation. Bars with different letters (a–c) within species are significantly different ($P < 0.05$).

Figure 3. The percentage of isolates $\pm$ SEM of each *Acholeplasma* or *Mycoplasma* species detected when field isolates were incubated within a candle jar with $2.7 \pm 0.2\%$ CO₂ (black bars) or in incubators of 5% (hatched bars) or 10% (gray bars) CO₂ for 7 d.

not detected when incubated for 7 d within a candle jar but were detected under the other CO₂ concentrations; 2 isolates were not detected when incubated in 10% CO₂ for 7 d but were detected under 5% CO₂ and when incubated within a candle jar. One isolate was detected only when incubated under 5% CO₂ for 7 d, whereas another was detected only under 10% CO₂ and another only when incubated within a candle jar for 7 d.

For *M. californicum* isolates, 88 ± 6.3 , 87 ± 4.4 , and $81 \pm 7.3\%$ were detected on both d 3 and 7 of incubation within a candle jar and under 5 or 10% CO₂, respectively (Figure 3). After 3 d of incubation, $76 \pm 8.4\%$ of *M. californicum* field isolates were detectable, which increased to $95 \pm 3.6\%$ after 7 d of incubation (Figure 4). Two *M. californicum* isolates were never detected when incubated for 7 d within a candle jar, whereas another 2 isolates were never detected when incubated under 10% CO₂ for 7 d.

DISCUSSION

CO₂ Concentrations

Recommendations for atmospheric conditions when culturing mycoplasmas vary across disciplines, with conflicting reports of the efficacy of these concentrations (Edward, 1950; Fabricant et al., 1962; Polak-Vogelzang et al., 1983; Middleton et al., 2017). Perhaps most common is the use of a candle jar, 5% CO₂, and 10% CO₂. Work from Polak-Vogelzang et al. (1983) demonstrated that mycoplasma could be successfully cultured in a wide range of atmospheric conditions. They observed *Mycoplasma hyorhinis* subcultivation rates exceeding

70% when isolates were incubated within a candle jar with 3% atmospheric CO₂ or an incubator with a mixture of 6% CO₂ and hydrogen. Likewise, the American Type Culture Collection recommends cultivating *Mycoplasma* species within a candle jar or under 5% atmospheric CO₂. Nicholas and Baker (1998) recommend a 5% CO₂ atmosphere when culturing *Mycoplasma* spp. from animal sources. On the other hand, Middleton et al. (2017) advocates culturing bovine mycoplasma isolates in a 10% CO₂ atmosphere for 7 to 10 d, a recommendation derived from the first reported case of *M. bovis* mastitis by Hale et al. (1962). In their study, they did not detect growth when they used a conventional culture technique (blood agar, 37°C, 48 h) but did when they used a 10% CO₂ environment for 5 d. The utilization of a 10% CO₂ atmosphere was not empirically determined. Thus, the goal of the study was to determine whether growth of bovine *Mycoplasma* species differed under different CO₂ concentrations so that veterinarians and diagnostic laboratories could select culture atmospheres that optimize mycoplasma isolation. Under the various CO₂ concentrations (candle jar, 5% CO₂, or 10% CO₂), growth for both laboratory and field isolates was not significantly different for any species, suggesting that other factors may have a greater influence on growth of the mycoplasma isolates. It should be noted that growth of *Mycoplasma* species associated with mastitis was not tested under ambient atmospheric conditions. Such tests would have to be made in future studies to determine the extent of the advantage in detection of these pathogens under ambient as opposed to enriched CO₂ conditions.

In this series of experiments, we inoculated pasteurized bulk tank milk with dilutions of isolates tested; milk was then incubated at 24 h before culture under test CO₂ conditions. This preincubation step was done to acclimatize the various isolates to the milk medium and to partially emulate what might happen in vivo, where mycoplasma isolates are in the mammary gland milk at a similar temperature. This procedure appeared to result in increasing growth over time as the trend indicates that the longer the incubation time, the greater the enumeration (Figures 2 and 4). Parker et al. (2016) found that with a similar inoculation, the *M. bovis* recovery decreased in milk when held at 23°C and 37°C.

Factors Affecting Growth

The isolates obtained from the field milk samples used in the study were first cultured by the veterinary diagnostic laboratory and frozen for up to 19 mo before they were donated to the Washington State University Field Disease Investigative Unit laboratory. Only 32%

Figure 4. The percentage of isolates $\pm$ SEM of each *Acholeplasma* or *Mycoplasma* species detected after 3 (black bars) and 7 (gray bars) days of incubation. Bars with different letters (a, b) within species are significantly different ($P < 0.05$).

of all field isolates yielded growth by 7 d of incubation. A low concentration of cells coupled with freezer morbidity may have increased the likelihood of no growth. Biddle et al. (2004) observed that freezing mycoplasma samples at -20°C for 4 wk resulted in a 3 to 4 $\log_{10}$ cfu/mL reduction in viable cells, and recovery decreased with increasing episodes of freezing and thawing. The effects of repeated freeze–thawing and prolonged storage times may have reduced the number of viable cells within the original sample that were in some cases stored for more than 1 yr. Cai et al. (2005) suggested that a minimum of 100 cfu/mL of milk can be reliably detected using bacterial culture. The limit of detection in this study was 100 cfu/mL, and thus any samples containing organisms below this threshold would not have yielded observable growth. Although Sachse et al. (2010) noted that in practice a positive culture below 1,000 cfu/mL of milk was rarely found, a 4-log reduction due to storage would make detecting that level unlikely.

Beyond sample handling, the concentration of viable cells, and storage time, the growth of *Mycoplasma* spp. is dependent on culture conditions. Mycoplasmas have special growth requirements that are distinct from most mastitis pathogens, requiring specialized media, nonatmospheric CO_2 concentrations, and prolonged incubation periods of up to 10 d (Bushnell, 1984). For all isolates observed in this study, growth was significantly greater after 7 d of incubation compared with 3 d of incubation ($P < 0.05$). *Mycoplasma* spp., *M. bovis*, and *M. californicum* isolates increased by 0.65, 0.17, and 0.76 $\log_{10}$ cfu/mL, respectively, when incubated for 7 d. Perhaps more important, all *M. bovis* and *M. bovis genitalium* isolates were detected by 3 d of incubation and all *M. californicum* isolates were detected by 5 d of incubation regardless of CO_2 concentration. It is possible that the subculturing protocol applied to the banked isolates selected for faster growing isolates when the 3-d culture was spread onto the surface of a modified Hayflick's agar plate. To determine whether this selection effect had occurred, the effect of incubation length was evaluated in the field isolates as well.

Middleton et al. (2017) and Maunsell et al. (2011) recommend incubation periods of 7 to 10 d before samples are considered negative. Our results support this recommendation, as the likelihood of detecting field isolates from d 3 to 7 increased 17% for *A. axanthum*, 46% for *M. bovis*, and 19% for *M. californicum* field isolates. Strain differences within species were present, which would explain why 100% detection was not achieved. Overall, after 7 d of incubation, 4% (4/98) of field isolates were not detected when incubated under 5% CO_2 , 7% (7/98) were not detected when incubated under

10% CO_2 , and 10% (10/98) were not detected when incubated within a candle jar. This suggests that no single CO_2 concentration yields 100% detection of field isolates. Significant differences in growth and growth detection for bovine mycoplasma isolates under the CO_2 concentrations were not found; however, the use of candle jars slightly increases the risk of false negatives as 10% of field isolates were not detected after 7 d of incubation. For the producer and the diagnostic laboratories, allowing 7 d of incubation increases the test sensitivity, resulting in fewer false negatives and, subsequently, missing fewer cows infected with mycoplasma. The fact that many isolates could be detected in as few as 3 d is important. For such isolates, the reduced incubation time leads to more timely reporting of results to the producer, who can then make more timely management decisions with respect to the infected animals. On the other hand, approximately twice as many *M. bovis* isolates were detected after 7 d than after 3 d. Anecdotal comments from local diagnostic laboratories indicate that there is a preference for examining growth of suspected *Mycoplasma* spp.—positive cultures at 3 and 7 d. In addition, in our study of laboratory isolates it was observed that there was no difference in growth between d 3 and 5 and no difference in growth between d 5 and 7, but there was a significant difference in growth of *Mycoplasma* spp. in general when growth at d 3 was compared with growth at d 7. For these reasons, we chose to compare growth at d 3 and 7 in our study of field isolates. Therefore, mollicute cultures might be best incubated for at least 7 d in the absence of growth and this absence reported as indeterminate rather than negative until after 7 d have passed.

CONCLUSIONS

Mycoplasma infections in dairy cattle are often persistent, difficult to cure, and challenging to diagnose. Due to the contagious nature, identification of infected cows is important in the control of the disease. These results suggest that no significant growth difference occurs between candle jar, 5% CO_2 , or 10% CO_2 atmospheres. Laboratories using candle jars may not be at a significant disadvantage for detecting mycoplasma mastitis pathogens compared with laboratories using CO_2 incubators. Consistent with the literature, we found that colonies may be detected by 3 d of incubation but that 7 d of incubation resulted in a greater proportion of positive plates. In summary, the range of CO_2 concentrations and incubation times suitable for cultivating and identifying bovine mycoplasma mastitis pathogens may be broader than those recommended by the National Mastitis Council.

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