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

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## ABSTRACT

Recommendations for bovine mycoplasma culture $CO_2$ 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 $CO_2$ concentrations of 10 or 5% or in candle jars (2.7 ± 0.2% $CO_2$). 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 $10^8$ cfu/mL and serially diluted in pasteurized whole milk to achieve test suspensions of $10^2$ and $10^6$ 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 $CO_2$ 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 ($\log_{10}$ 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 $CO_2$ 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 $CO_2$ 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 bovigenitalium*, *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% $CO_2$) 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% $CO_2$ 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% $CO_2$

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(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., 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 $CO_2$ concentrations of 10 or 5% or in candle jars (2.7 ± 0.2% $CO_2$). 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. bovigenitalium* (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. bovigenitalium* 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 μL) 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 μL of culture stock was transferred to 40 mL of sterile pleuropneumonia-like organisms (**PPLO**) broth. Inoculants were incubated at 37°C in 10% $CO_2$ for 5 d. Broth cultures of the mycoplasma strains were then centrifuged at 5,000 × $g$ 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% $CO_2$. To enumerate colony growth, a 100-μL portion of inoculated samples was serially diluted and 100-μL 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 $CO_2$ concentrations: incubators set at 10 and 5% $CO_2$ 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 $CO_2$ atmosphere of 2.7 ± 0.2%. To verify $CO_2$ concentrations, a 0 to 30% $CO_2$, 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× 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 μL 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% $CO_2$. Genomic DNA was extracted with the PureLink 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 μL containing 1× PCR buffer (20 m$M$ Tris-HCl, 2 m$M$ $MgCl_2$, and 50 m$M$ 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

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