Development of a multiplex qPCR assay for the simultaneous detection of *Mycoplasma bovis*, *Mycoplasma* species, and *Acholeplasma laidlawii* in milk

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Contagious bovine mastitis caused by *Mycoplasma bovis* and other *Mycoplasma* species including *Mycoplasma californicum*, *Mycoplasma bovigenitalium*, *Mycoplasma alkalescens*, *Mycoplasma arginini*, and *Mycoplasma canadense* is an economical obstacle affecting many dairy herds throughout California and elsewhere. Routine bacteriological culture-based assays for the pathogens are slow and subject to false-positive results due to the presence of the related, non-pathogenic species *Acholeplasma laidlawii.* To address the need for rapid and accurate detection methods, a new TaqMan multiplex, quantitative real-time PCR (qPCR) assay was developed that targets the 16S rRNA gene of *Mycoplasma*, *rpoB* gene of *M. bovis*, and the 16S to 23S rRNA intergenic transcribed spacer (ITS) region of *A. laidlawii*. qPCR amplification efficiency and range of detection were similar for individual assays in multiplex as when performed separately. The multiplex assay was able to distinguish between *M. bovis* and *A. laidlawii* as well as detect *Mycoplasma* spp. collectively, including *Mycoplasma californicum*, *Mycoplasma bovigenitalium*, *Mycoplasma canadense*, *Mycoplasma arginini* and *Mycoplasma alkalescens*. In milk, the lower limit of detection of *M. bovis*, *M. californicum,* and *A. laidlawii* with the multiplex assay was between 120 to 250 colony forming units (CFU) per mL. The assay was also able to simultaneously detect both *M. bovis* and *A. laidlawii* in milk when present in moderate (10^3 to 10^4 CFU/mL) to high (10^6 to 10^7 CFU/mL) quantities. Compared to laboratory culture-based methods, the multiplex qPCR diagnostic specificity (Sp) was 100% (95% CI 86.8, 100; n=26) and diagnostic sensitivity (Se) was 92.3% (95% CI: 74.9, 99.1; n=26) for *Mycoplasma* species in milk samples collected from California dairy farms. Similarly, the Sp was 100% (95% CI 90.5, 100; n=37) and Se was 93.3% (95% CI: 68.1, 99.8; n=15) for *M. bovis*. Our assay can detect and distinguish among *M. bovis*, other prevalent *Mycoplasma*

spp., and non-pathogenic *Acholeplasma laidlawii* for effective identification and control of Mycoplasma mastitis, ultimately supporting dairy cattle health and high-quality dairy products in California.

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2 ***bovis, Mycoplasma* species, and *Acholeplasma laidlawii* in milk**

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ABSTRACT

Contagious bovine mastitis caused by *Mycoplasma bovis* and other *Mycoplasma* species

including *Mycoplasma californicum, Mycoplasma bovigenitalium, Mycoplasma alkalescens,*

Mycoplasma arginini, and *Mycoplasma canadense* is an economical obstacle affecting many

dairy herds throughout California and elsewhere. Routine bacteriological culture-based assays

for the pathogens are slow and subject to false-positive results due to the presence of the related,

non-pathogenic species *Acholeplasma laidlawii*. To address the need for rapid and accurate

detection methods, a new TaqMan multiplex, quantitative real-time PCR (qPCR) assay was

developed that targets the 16S rRNA gene of *Mycoplasma, rpoB* gene of *M. bovis*, and the 16S to

23S rRNA intergenic transcribed spacer (ITS) region of *A. laidlawii*. qPCR amplification

efficiency and range of detection were similar for individual assays in multiplex as when

performed separately. The multiplex assay was able to distinguish between *M. bovis* and *A.*

laidlawii as well as detect *Mycoplasma* spp. collectively, including *Mycoplasma californicum,*

Mycoplasma bovigenitalium, Mycoplasma canadense, Mycoplasma arginini and *Mycoplasma*

alkalescens. In milk, the lower limit of detection of *M. bovis, M. californicum,* and *A. laidlawii*

with the multiplex assay was between 120 to 250 colony forming units (CFU) per mL. The assay

was also able to simultaneously detect both *M. bovis* and *A. laidlawii* in milk when present in

moderate (10^3 to 10^4 CFU/mL) to high (10^6 to 10^7 CFU/mL) quantities. Compared to laboratory

culture-based methods, the multiplex qPCR diagnostic specificity (Sp) was 100% (95% CI 86.8,

100; n=26) and diagnostic sensitivity (Se) was 92.3% (95% CI: 74.9, 99.1; n=26) for

Mycoplasma species in milk samples collected from California dairy farms. Similarly, the Sp

was 100% (95% CI 90.5, 100; n=37) and Se was 93.3% (95% CI: 68.1, 99.8; n=15) for *M. bovis*.

Our assay can detect and distinguish among *M. bovis*, other prevalent *Mycoplasma* spp., and

46   non-pathogenic *Acholeplasma laidlawii* for effective identification and control of Mycoplasma

47   mastitis, ultimately supporting dairy cattle health and high-quality dairy products in California.

48   **Key Words:** milk, bovine mastitis, qPCR, multiplex, Taqman, diagnostic, *Mycoplasma*

49

50   **INTRODUCTION**

51   Mastitis caused by *Mycoplasma* species is highly infectious in dairy cattle and causes

52   serious disease and economic burdens, especially in large dairy herds in the U.S. and other

53   countries worldwide (*Nicholas et al., 2016*). Pathogenic *Mycoplasma* spp. are unique compared

54   to most other bacterial causes of mastitis because they lack a cell wall and possess less genetic

55   code (*Fox et al., 2005*). These organisms infect the udder tissue of dairy cows, and the resultant

56   disease has long-term effects on milk quality, yield, and animal health (*Nicholas et al., 2016*). In

57   the US alone, the mean cost per clinical mastitis case caused by organisms other than Gram-

58   positive and Gram-negative bacteria, which included *Mycoplasma* spp., was estimated at US

59    $95.31 annually, with majority of the costs attributed to treatment (*Cha et al., 2011*). However,

60   because of the very contagious nature and recognized poor response to therapy, dairy cattle with

61   udder infections due to *Mycoplasma* spp. are often removed from the herd following

62   identification of infection which significantly adds to the economic burden of mycoplasma

63   mastitis (*Ball, 1999*; *Maunsell et al., 2011*; *Nicolas et al., 2016*). The most prevalent species

64   causing cattle mastitis is *Mycoplasma bovis*, but other *Mycoplasma* species including

65   *Mycoplasma californicum* and *Mycoplasma bovigenitalium* are also of diagnostic interest to

66   dairy cattle farmers in California (*Kirk et al., 1997*; *Infante-Martinez et al., 1999*; *Brenner et al.,*

67   *2009*).

68   Infectious *Mycoplasma* spp. are highly contagious, frequently resistant to antibiotic

69   treatment, and can result in chronic and subclinical states of infection. Because *Mycoplasma* can

70   spread quickly, this disease is particularly detrimental to large (>500 cow) dairy herds (*Jasper,*

71   *1982*; *Pfutzner and Sachse, 1996*). For these reasons, screening of bulk tank milk or pooled

72   animal milk samples is important to facilitate removal of infected animals thereby limiting the

73   spread of infection. Routine *Mycoplasma* investigations involve using laboratory-culture based

74   methods to enrich the bacteria from milk. This approach is challenging considering the numerous

75   nutrient requirements and specific incubation conditions required for *Mycoplasma* growth. Even

76   under optimal conditions, some mycoplasma strains are still difficult to grow on standard

77   laboratory culture media (*Razin, 1996*). Other challenges include the extended incubation time

78   (approximately 7 to 10 days) required before reaching sufficient cell numbers for analysis and

79   also the analyzing needed to identify *Mycoplasma* at the species level usually by serological

80   testing. Additionally, phenotypic similarities between *Mycoplasma* spp. and commensal

81   environmental contaminants, most predominantly, *Acholeplasma laidlawii,* can result in false

82   positives (*Pfutzner and Sachse, 1996*; *Clothier et al., 2010*; *Fox, 2012*).

83   Nucleic-acid based, culture-independent methods are now compelling alternatives for

84   *Mycoplasma* diagnosis because of their potential to be rapid, accurate, and cost effective. Use of

85   PCR and qPCR-based tests, in particular, have the most promise to be the most rapid and reliable

86   methods for *Mycoplasma* spp. detection, identification, and quantification. To this regard, several

87   end-point PCR and quantitative real-time PCR (qPCR) assays for *M. bovis* have been developed

88   targeting *M. bovis* 16S rRNA *(van Kuppeveld et al., 1992*; *Chavez Gonzalez et al., 1995*;

89   *Cornelissen et al., 2017*), *uvrC* (*Clothier et al., 2010*; *Rossetti et al., 2010*; *Naikare et al., 2015*;

90   *Gioia et al., 2016*; *Behera et al., 2018*), *oppD* (*Sachse et al., 2010*), *fusA* (*Boonyayatra et al.,*

91   *2012*), and *gltX* (*Appelt et al., 2019*) .

92   When assessing outbreaks of mycoplasma mastitis in dairies, especially for California's

93   large dairy herds, multiplex qPCR can be a favorable and practical approach for diagnostic

94   testing. The use of different primer and probe sets in a single reaction tube significantly shortens

95   the detection times and increases throughput capacity (*Chapela et al., 2015*). A multiplex qPCR

96   assay was developed to detect *M. bovis*, *M. californicum,* and *M. bovigenitalium* in milk samples

97   with a limit of detection of between 10^2 to 10^5 colony forming units (CFU) per mL (*Parker et al.,*

98   *2017*). There are also commercially-available, multiplex qPCR kits for milk such as PathoProof,

99   Mastitis Major-3 kit (Thermo Fisher Scientific, Finland), and *bactotype* Mastits HP3 PCR Kit

100 (Qiagen, Leipzig, Germany) designed to detect *M. bovis* along with the other contagious mastitis

101 pathogens *Staphylococcus aureus* and *Streptococcus agalactiae*. However, compared to

102 *Mycoplasma* spp., both *S. aureus* and *S. agalactiae* are relatively easy to isolate using

103 conventional aerobic culture methods with final results available within 48 hours.

104   In this study, we developed a multiplex qPCR assay for detection of *Mycoplasma* spp.,

105 *M. bovis,* and *A. laidlawii*. The specificity and quantification range were verified for the

106 individual primer sets as well as in multiplex. The multiplex assay was then tested for its

107 diagnostic capability compared to routine laboratory, culture-based methods.

108

109 **MATERIAL AND METHODS**

110 **Bacterial strains and milk samples**

111   *M. bovis* ATCC 25523, *M. californicum* ATCC 33461, *M. bovigenitalium* ATCC 19852,

112 *Mycoplasma canadense* ATCC 29410, *Mycoplasma arginine* ATCC 23838, *Mycoplasma*

113 *alkalescens* ATCC 29103 and *A. laidlawii* ATCC 23206 were purchased from the American

114   Type Culture Collection (ATCC, Manassas, VA) (**Table 1**). *M. bovis* ATCC 25523 and *A.*

115   *laidlawii* ATCC 23206 genomic DNA (gDNA) was also purchased from the ATCC.

116   Field isolates of *Mycoplasma* and *Acholeplasma* were obtained by routine bacteriological

117   testing of milk samples submitted by producers and milk processors to the Milk Quality

118   Laboratory (MQL) of the University of California, Davis Veterinary Medicine Teaching and

119   Research Center (VMTRC, Tulare, CA) from individual cows and bulk tanks located at dairies in

120   the Central Valley, California (**Table 1**). All *Mycoplasma* and *Acholeplasma* isolates were

121   recovered using VMTRC MQL protocols adapted from the National Mastitis Council (US,

122   2004). Briefly, milk samples were plated on modified mycoplasma agar (University of California

123   Davis Veterinary Medicine Biological Media Services) directly upon receipt and after

124   enrichment in mycoplasma specific broth (UC Davis VM Biological Media Services) for 48 h in

125   4% CO_2 at 37 °C. Putative *Mycoplasma* and *Acholeplasma* colonies were streaked for isolation

126   and subsequently identified to the species level by staining with fluorescent antibodies according

127   to previously described methods (*Baas and Jasper, 1972*). To discriminate between *A. laidlawii*

128   and *Mycoplasma* spp., a digitonin disk inhibition test was performed according to the method

129   previously described (*Thurmond et al., 1989*). All isolates were stored in 15% (v/v) glycerol at -

130   80 °C.

131   Additionally, a total of 33 isolates from 30 different bacterial species other than

132   mycoplasmas and commonly found in bovine milk were used for cross-specificity evaluation of

133   the qPCR assay (**Table 1**). These isolates were either obtained from the ATCC as type strains or

134   isolated at the MQL and Dairy Food Safety Laboratory (DFSL) of University of California,

135   Davis Veterinary Medicine Teaching and Research Center (VMTRC, Tulare, CA) from routine

136   diagnostic milk sample submissions from dairies by producers located in the Central Valley, CA.

137   Species identity was confirmed by 16S rRNA gene and *rpoB* sequencing (*Drancourt et al., 2000*;

138   *Drancourt and Raoult 2002*).

139

140   **Genomic DNA extraction**

141   The DNeasy Blood and Tissue Lysis kit (Qiagen, Carlsbad, CA) was used for bacterial

142   gDNA extraction and purification according to the manufacturer's instructions. The gDNA was

143   extracted from *Mycoplasma* and *Acholeplasma* strains after incubation in mycoplasma specific

144   broth (UC Davis VM Biological Media Services) at 37 °C in 4% CO_2 for between 24 to 72 h

145   (*Appelt et al., 2019*). The gDNA from other bacterial species (**Table 1**) was isolated from

146   colonies retrieved from bovine blood agar plates (UC Davis VM Biological Media Services)

147   after incubation at 37 °C for 24 to 48 h. The commercial kit was used for the isolation of gDNA

148   because raw milk may contain PCR inhibitors that affect the sensitivity of the assay or even lead

149   to false negative results (*Schrader et al., 2012*). For gDNA extractions from bacteria contained in

150   milk, 1 mL milk was centrifuged at 18,407 x *g* for 10 min. The resulting pellet was then

151   suspended in Buffer ATL (Qiagen, Carlsbad, CA) and Proteinase K (Qiagen, Carlsbad, CA) for

152   incubation at 56 °C for 1 h with continuous mixing prior to DNA extraction and purification. The

153   gDNA concentrations were measured on a Qubit 3.0 Fluorometer using the Qubit dsDNA HS

154   Assay Kit (Life Technologies, Eugene, OR). All gDNA was stored at -20 °C until analysis or at

155   4 °C for no longer than 2 to 4 weeks.

156

157   **qPCR primer design**

158   Clustal W (*Thompson et al., 1994*) was used to identify conserved and variable

159   *Mycoplasma* and *Acholeplasma* DNA targets for the development of new primer-probe pairs to

160   be used in the multiplex qPCR assay. DNA sequence alignments were performed for 16S rRNA,

161   *rpoB* and intergenic transcribed spacer (ITS) region between 16S and 23S rRNA genes in *M.*

162   *bovis, M. californicum, M. bovigenitalium, M. canadense M. alkalescens,* and *A. laidlawii.* Those

163   *Mycoplasma* species were selected for primer development because of their prevalence in

164   *Mycoplasma* mastitis reported in the US dairy herds (*Fox, 2012*; *Nicholas et al., 2016*) and by

165   the VMTRC MQL. The following strains were used for primer selection: *M. bovis* strain PG45

166   (ATCC 25523, NC_014760) (*Wise et al., 2011*), *M. californium* strain ST-6 (ATCC 33461,

167   NZ_CP007521) (*Calcutt et al., 2014*), *M. bovigenitalium* strain HAZ (ATCC 19852, AP017902)

168   (*Edward and Freundt, 1956*), *M. canadense* strain HAZ 360_1 (ATCC 29410, NZ_AP014631),

169   *M. alkalescens* 14918 (ATCC 29103, NZ_AMWK01000000), and *A. laidlawii* PG-8A (ATCC

170   23206, NC_010163) (*Lazarev et al., 2011*). DNA sequence alignments showed sufficient

171   nucleotide variation in the 16S rRNA and *rpoB* genes and ITS region for primer and Taqman

172   probe design (**Supplementary Figure 1**).

173   All qPCR primers and Taqman probes were designed using Primer-BLAST and Primer

174   Express software (Applied Biosystems) (**Table 2** and **Supplementary Figure 1**). Primers were

175   regarded to be acceptable if they contained at least two total mismatches to unintended targets,

176   including at least two mismatches within the last five base pairs of the 3' end. All primers were

177   designed to have a melting temperature at 59 °C to 62 °C and an amplicon size between 108 to

178   232 bp.

179

180   **qPCR assay parameters**

181   All primers and probes were optimized by testing concentrations in the range of 100 nM

182   to 400 nM and 75 nM to 400 nM, respectively. qPCR amplification was performed in 96-well

183   plates on an Applied Biosystems 7500 Fast thermal cycler (Applied Biosystems, Carlsbad, CA)

184   with an initial denaturation step of 5 min at 95 °C followed by 40 cycles of 45 s at 95 °C for

185   DNA denaturation and 45 s at 58 °C for primer and probe hybridization and DNA elongation

186   steps. The multiplex assay was carried out in a total volume of 20 µl, comprising 5 µl of the

187   gDNA, 10 µl of 2X QuantiFast Multiplex PCR master mix (Qiagen, CA), and between 75 and

188   200 nM of each primer and TaqMan probe (**Table 2**). qPCR assay efficiencies were calculated

189   using standard curves based on 10-fold serial dilutions of gDNA from *M. bovis* ATCC 25523 for

190   the *rpoB* and 16S rRNA assays and *A. laidlawii* ATCC 23206 for the ITS assay.

191

192   **Quantitative ranges of the qPCR assays**

193   Serial dilutions were prepared of gDNA from *M. bovis* ATCC 25523, *M. californicum*

194   ATCC 33461, *M. bovigenitalium* ATCC 19852, and *A. laidlawii* ATCC 23206. The serial

195   dilutions spanned a 10^6 - fold range, encompassing between approximately 5 fg to 5 ng gDNA.

196   The QuantiFast Multiplex PCR Master mix was used in combination with the primer and probe

197   concentrations described in **Table 2** with an annealing temperature of 58 °C. The assays were

198   tested in singleplex and multiplex in triplicate and in two separate runs and for each gDNA

199   primer/probe combination. To estimate genome equivalents, it was assumed that the average

200   base pair weighs 650 g/mol and the average genome sizes were 1 Mbp for *M. bovis* (*Wise et al.,*

201   *2011*), 0.79 Mbp for *M. californicum* (*Calcutt et al., 2014*), 0.86 Mbp for *M. bovigenitalium*

202   (*Hata et al., 2017*), and 1.5 Mbp for *A. laidlawii* (*Lazarev et al., 2011*). For verifying assay

203   specificity, a total of 1 to 25 ng gDNA from diverse bacterial strains (**Table 1**) was tested for

204   detection using the *Mycoplasma-M. bovis-A. laidlawii* multiplex assay.

205   To examine the quantitative range of the multiplex assay in milk, raw bovine milk

206   samples were first confirmed to be free of *Mycoplasma* spp. by plating on modified mycoplasma

207   agar and incubation at 37 °C in 4% CO_2 for 7 days. Next, 1×10^6 to 1×10^8 colony-forming units

208   (CFU)/mL of *M. bovis* ATCC 25523, *M. californicum* ATCC 33461, and *A. laidlawii* ATCC

209   23206 were inoculated into separate aliquots of that milk. The cell suspensions were immediately

210   used for serial dilutions in the same raw milk so that the final cell numbers spanned a 10^4-fold

211   (*M. californicum*) to 10^6 - fold (*M. bovis* and *A. laidlawii*) range. gDNA was then extracted and

212   tested with the *Mycoplasma-M. bovis-A. laidlawii* multiplex assay using conditions described in

213   **Table 2** before setting qPCR multiplex assay detection criteria (**Table 3**).

214

215   **Multi-species detection**

216   Mixtures of *M. bovis* ATCC 25523 and *A. laidlawii* ATCC 23206 gDNA were tested

217   with the multiplex assay to assess for the capacity for simultaneous, multi-species detection.

218   gDNA was mixed instead of intact cells because growth rate variations between the strains

219   limited our capacity to obtain sufficient cell numbers within the same period of time. To prepare

220   the gDNA mixtures, gDNA was extracted from a single strain immediately after inoculation into

221   separate aliquots of raw milk at either low (10^2 to 10^3 CFU/mL), medium (10^3 to 10^4 CFU/mL),

222   or high (10^6 to 10^7 CFU/mL) cell numbers. Next, gDNAs from those strains were mixed in equal

223   volumes (1:1 ratios) and tested with the multiplex assay using the assay conditions described in

224   Table 2. A total of nine combinations of gDNAs, representing low, medium, and high levels of

225   *M. bovis* and *A. laidlawii* cells were measured (**Table 4**).

226

227   **qPCR performance on field milk samples**

228   To compare the qPCR assay performance to conventional culture-based methods, a total

229 of 52 frozen (-20 °C) bulk tank and individual cow milk samples were first confirmed to be

230 either *Mycoplasma* spp. culture positive or negative. This was determined by plating the milk

231 samples directly on modified mycoplasma agar and after enrichment in mycoplasma specific

232 broth for 24 h in 4% CO_2 at 37 °C. Colonies were evaluated by the same person at 4 and 7 days

233 and verified as *M. bovis, M. bovigenitalium,* or *M. alkalescens* using staining with fluorescent

234 antibodies according to previously described methods (*Baas and Jasper, 1972*). The 52 milk

235 samples were anonymized for testing by the qPCR multiplex assay and 5 µl of total DNA

236 extracted from the milk was tested using assay parameters described in **Table 2**. Diagnostic

237 sensitivity (Se) and diagnostic specificity (Sp) of the multiplex qPCR assay was calculated as

238 described previously (Gioia et al., 2016). Using the results from laboratory culture as the

239 reference, the number of true positive (TP), true negative (TN), false positive (FP), and false

240 negative (FP) samples were combined into following formulas: Se (%) = TP / (TP + FN) and Sp

241 (%) = TN / (TN + FP). Kappa, an estimate of agreement beyond chance between laboratory

242 culture and qPCR was estimated and interpreted as <40%, poor; 41 to 75%, fair to good; >75%,

243 excellent (*Fleiss et al., 2003*).

244

245 **RESULTS**

246 **qPCR assay efficiency and range of detection for target gDNA**

247   The *Mycoplasma* 16S ribosomal RNA (16S rRNA) gene, *M. bovis rpoB,* and the *A.*

248 *laidlawii* ITS region were selected for qPCR primer design. These targets were previously shown

249 to be useful markers for detection of *Mycoplasma* and *Acholeplasma* in milk and clinical samples

250 (*van Kuppeveld et al., 1992*; *Cornelissen et al., 2017*; *Gioia et al., 2016*; *Parker et al., 2017*;

251 *Tang et al., 2000*). After testing between 75 and 200 nM of each primer and TaqMan probe, the

252 Taqman qPCR assay parameters (QuantiFast Multiplex PCR master mix; annealing temperature

253 of 58 °C) and assay primer and probe concentrations were set as described in **Table 2**. The qPCR

254 efficiencies for each of the individual qPCR assays on intended target gDNA ranged from 90 to

255 102.4% when tested separately (in singleplex) **(Table 2** and **Supplementary Figure 2)** and

256 combined into one *Mycoplasma-M. bovis-A. laidlawii* multiplex assay **(Table 2** and **Figure 1**).

257 For the 16S rRNA gene assay in multiplex, the qPCR amplification efficiency was high for *M.*

258 *bovis* (**Table 2**) (96.8%) and comparable to efficiencies found for *M. californicum* ATCC 33461

259 (102.4%) and *M. bovigenitalium* ATCC19852 (97.9%). The correlation coefficient of the straight

260 line, R^2 was higher than 0.98 for all assays over a 10^6-fold range in singleplex (**Supplementary**

261 **Figure 2**) and multiplex (**Figure 1**). The lower limit of detection (LLD) for each assay was

262 similar between the singleplex (**Supplementary Figure 2**) and multiplex (**Figure 1**) formats (*Ct*

263 values from 30 to 35). Both formats were able to detect low numbers of DNA copies per PCR

264 (50 fg DNA, approximately equal to 30 to 58 genome equivalents for the respective species

265 being targeted by the assay).

266

267 **Specificity of the multiplex qPCR assays**

268 We next examined the qPCR assays for cross-reactivity between the other species targets

269 contained in the *Mycoplasma-M. bovis-A. laidlawii* multiplex test. No amplification (*Ct* >37)

270 was found for the *A. laidlawii* 16-23S ITS assay when tested on either *M. bovis* ATCC25523*, M.*

271 *californicum* ATCC33461, or *M. bovigenitalium* ATCC19852 gDNA template (data not shown).

272 For the *M. bovis rpoB* assay, some amplification (C_t values 31 to 37) was observed for *M.*

273 *californicum* ATCC 33461 and *M. bovigenitalium* ATCC 19852 gDNA. However, that *Ct* range

274   of detection was approximately 17 *Ct* values higher than observed for equivalent quantities of the

275   *M. bovis* target (0.5 ng), comparable to a 10^5 to 10^6-fold difference in detection sensitivity. For

276   the *Mycoplasma* 16S rRNA assay, some amplification (C_t values 31 to 35) was observed for *A.*

277   *laidlawii* ATCC 23206 gDNA at the highest quantity of gDNA tested (0.5 ng). However, just as

278   found for the *rpoB* assay, those *Ct* values were also much higher (*Ct* difference of 17) than when

279   *A. laidlawii* was used as the target, comparable to a 10^5 to 10^6 - fold difference in detection.

280   No amplification (*Ct* >37) was observed with the *Mycoplasma-M. bovis-A. laidlawii*

281   multiplex assay for any non-*Mycoplasma* strains from 33 different bacterial species, including

282   representatives of the *Staphylococcus, Bacillus, Aerococcus, Enterococcus, Enterobacter,*

283   *Escherichia, Klebsiella, Proteus, Prototheca, Pseudomonas, Serratia,* and *Streptococcus* genera

284   (**Table 1**). As expected, gDNA from *Mycoplasma californicum, Mycoplasma bovigenitalium,*

285   *Mycoplasma canadense, Mycoplasma arginini,* and *Mycoplasma alkalescens* isolates (**Table 1**)

286   resulted in amplification with the 16S rRNA assay (*Ct* values < 28) but not with the *rpoB* and

287   ITS assays (*Ct* values > 37). Moreover, gDNA from *A. laidlawii* field isolates only resulted in

288   amplification with the ITS assay, while three other field isolates of *M. bovis* from milk were

289   accurately detected by the *rpoB* and 16S rRNA assays (*Ct* values ranged from between 21 to 24).

290

291   **Limit of Detection (LOD) of multiplex qPCR assay in milk**

292   Next, the qPCR multiplex assay range of detection was tested for *Mycoplasma* and *A.*

293   *laidlawii* in milk. A DNA amplification efficiency of 95 to 106% (R^2 > 0.98) was found for all

294   three gene targets spanning a 10^4 to 10^6 -fold range when the multiplex assay was applied on

295   gDNA extracted from serial dilutions of *M. californicum* ATCC33461, *M. bovis* ATCC25523, or

296   *A. laidlawii* ATCC23206 contained in milk (**Figure 2**). However, it was noted that milk alone

297   (no-template control or mycoplasma culture negative) sometimes resulted in some DNA

298   amplification (*Ct* cycles 33 to 37). Therefore, *Ct* values above 33 were reported as negative for

299   the multiplex qPCR assay. To avoid false positives, a LOD cut-off *Ct* value was set at <32 for

300   *Mycoplasma*, <33 for *M. bovis*, and <32 for *A. laidlawii* (**Table 3**). Those cut-off *Ct* values for

301   each target assay were selected based on a minimum 3-Ct difference between the lowest

302   quantities of the target organism in milk and the negative control *Ct*. Based on cell numbers

303   resulting in that *Ct,* the LOD was within the range of 120 to 250 CFU/mL.

304

305   **Multi-species detection with the multiplex qPCR assay**

306   Application of the multiplex qPCR assay on gDNA from *M. bovis* ATCC 25523 and *A.*

307   *laidlawii* ATCC 23206 mixed in different proportions showed that multiplex assay could

308   simultaneously detect *M. bovis* and *A. laidlawii* present in the same sample (**Table 4**). The 16S

309   rRNA and *rpoB* targeted assays were positive when gDNA from milk containing either moderate

310   (10^3 to 10^4 CFU/mL) to high (10^6 to 10^7 CFU/mL) numbers of *M. bovis* was applied, irrespective

311   of *A. laidlawii* gDNA abundance. Similarly, the *A. laidlawii* ITS qPCR assay results were

312   positive when gDNA from milk containing moderate or high numbers of *A. laidlawii* was used,

313   even in the presence of high quantities of *M. bovis* gDNA. Neither organism was detectable

314   according to qPCR when the gDNA was extracted from milk with low cell numbers (10^2 to 10^3

315   CFU/mL) (**Table 4**).

316

317   **qPCR performance on field milk samples**

318   To determine the extent to which the *Mycoplasma-M. bovis-A. laidlawii* multiplex qPCR

319   assay can reliably detect and differentiate between *M. bovis* from non-*bovis Mycoplasma* spp.

320   and *A laidlawii*, a total 52 milk samples collected from bulk tanks and individual cows on CA

321   dairy farms were analyzed with the assay and the results were compared to laboratory culture-

322   based detection methods (**Table 5**). No sample was shown to contain *A. laidlawii* and therefore

323   the presence of that organism in field milk samples was not evaluated. Using the *Mycoplasma*

324   spp. qPCR criteria we set for the multiplex assay (**Table 3**), no false-positive results, Sp 100%

325   (95% CI 86.8, 100; n = 26) were obtained for the milk samples found to lack *Mycoplasma* spp.

326   according to laboratory culture-based methods (**Table 5**). Similarly, using the *M. bovis* criteria,

327   no false positive results, Sp 100% (95% CI 90.5, 100; n=37) were obtained for the milk samples

328   found to lack *M. bovis* (**Table 5**). Notably, the assay parameters were discriminative because

329   even though the *rpoB* Ct value was relatively low (approximately 32) for sample ID# 482582, a

330   sample found to contain *M. alkalescens* according to laboratory culture-based methods, there was

331   an approximate five-Ct difference between the *rpoB* and 16S rRNA assays. That difference was

332   beyond the allowable limit for *M. bovis* by the multiplex assay (**Table 3**).

333   A total of 24 out of 26 milk samples that tested positive for *Mycoplasma* spp. according

334   to CFU isolation in laboratory culture medium were also positive according to qPCR, Se 92.3%

335   (95% CI: 74.9, 99.1; n=26). The remaining two samples (ID# 41672 and ID# 43960) were

336   negative by qPCR for all three gene targets but tested positive for *M. alkalescens* after

337   enrichment and plating for CFU identification. (**Table 5**).

338   The multiplex assay also accurately detected 14 out of 15 milk samples determined to

339   contain *M. bovis* according to culture-based methods, yielding a Se of 93.3% (95% CI: 68.1,

340   99.8; n=15) for *M. bovis*. The remaining milk sample (ID# 2927-3) tested positive for

341   *Mycoplasma* spp. but not *M. bovis* (**Table 5**). That sample resulted in only two presumptive *M.*

342   *bovis* colonies (approximately 100 CFU/ml) (**Table 5**). Notably the average *Ct* for that sample

343 (*Ct* of 33.76) was only slightly above the upper *Ct* value cut-off for *M. bovis* (*Ct* ≤ 33) (**Table 3**)

344 and the *Ct* for *Mycoplasma* detection with the 16S rRNA gene was near to the limit of the

345 acceptable range (*Ct* of 31.03 compared to *Ct* <32).

346   Both assays, laboratory culture and qPCR, had excellent agreement beyond chance as

347 measured by Kappa. Specifically, the agreement between laboratory culture and qPCR in

348 detecting milk samples test positive for *Mycoplasma* spp. was 92.31% (SE 13.83; P value <0.01)

349 and for *M. bovis* was 95.22% (SE 13.85; P value <0.01).

350

351 **DISCUSSION**

352   The TaqMan multiplex qPCR assay developed here was shown to be a rapid and reliable

353 diagnostic tool for detection of *Mycoplasma* spp. and discrimination between *Mycoplasma* and

354 *A. laidlawii* in milk. The assay was accurate and showed a large dynamic range for quantification

355 (10^6 fold range) while maintaining a comparable sensitivity to that of the individual assays for

356 each target. Simultaneous detection was achieved for *M. bovis* and *A. laidlawii* and the

357 diagnostic sensitivity and field performance of the multiplex qPCR assay was confirmed. The

358 assay is a significant advancement for mastitis diagnostics because it emphasizes *M. bovis*

359 detection while also affording the possibility for detection of other *Mycoplasma* genera and

360 simultaneous detection and differentiation of non-pathogenic *A. laidlawii* contaminants.

361   The individual qPCR assays used here target genes that have also previously shown to be

362 useful for *Mycoplasma* and *A. laidlawii* detection by other methods. Conventional PCR assays

363 targeting *Mycoplasma* 16S rRNA genes were used for detection (*Hirose et al., 2001*) and both

364 detection and differentiation of different *Mycoplasma* spp., including *M. bovigenitalium* and *M.*

365 *californicum* (*McAuliffe et al., 2005*). For developing genus specific primers, target sequences

366   used should have the greatest sequence divergence between genera but be conserved for species

367   within the same genus. The 16S rRNA gene is an ideal target in this regard since the presence of

368   two gene copies also increases the detection limit for PCR. In comparisons between the 16S

369   rRNA genes of *Mycoplasma* and other related species including *M. californicum*, *M.*

370   *bovigenitalium*, *M. alkalescens*, *M. arginini* and *M. canadense*, we observed significant sequence

371   similarity in the V7 variable region. This finding agreed with a previous study which concluded

372   that mycoplasma genus-specific sequences occur between the 16S rRNA V6 and V7 regions,

373   although some cross reactivity was found with *Acholeplasma* species (*van Kuppeveld et al.,*

374   *1992*). The alignment of the V7 regions showed a 118-bp sequence which distinguished *A.*

375   *laidlawii* from *Mycoplasma* spp., while being conserved among *Mycoplasma* species.

376   A variety of *M. bovis* genes including, *uvrC, gltX, fusA* and *oppD* have been targets for

377   species detection by PCR methods (*Clothier et al., 2010*; *Rossetti et al., 2010*; *Sachse et al.,*

378   *2010*; *Boonyayatra et al., 2012*; *Naikare et al., 2015*; *Gioia et al., 2016*; *Behera et al., 2018*;

379   *Appelt et al., 2019*). We selected *rpoB*, the gene encoding the highly conserved beta-subunit of

380   the bacterial RNA polymerase, for assay development because there were more nucleotide

381   variations in this gene between *M. bovis* and other *Mycoplasma* species. *rpoB* was also found to

382   be a suitable target for phylogenetic analysis of *Mycoplasma* spp. including *M. bovis* (*Kim et al.,*

383   *2003*). The use of the *rpoB* gene for *M californicum* detection in milk has already been explored

384   in a probe-based, real-time PCR assay (*Boonyayatra et al., 2012*; *Parker et al., 2017*). Therefore,

385   in this study, we used a 232-bp sequence in *rpoB* gene for the design of primers and probe that

386   could as well detect *M. bovis* in milk samples. Additionally, the *rpoB* gene assay reached a

387   higher amplification efficiency compared to the qPCR assay we previously developed for *M.*

388   *bovis* targeting *gltX* (*Appelt et al., 2019*).

389   For the detection of *A. laidlawii*, the ITS region (108-bp; separating the two 16S-23S

390   rRNA operons in *A. laidlawii*) was used for species-specific primers and probe development.

391   The ITS region was previously shown to have sufficient heterogeneity to differentiate between

392   *Mycoplasma* spp. and *A. laidlawii* (*Tang et al.,* 2000; Gioia *et al., 2016*). This finding is

393   consistent with the lack of amplification observed for *M. bovis* or the other *Mycoplasma* tested

394   with that assay.

395   The individual qPCR assays performed well when combined in multiplex qPCR, with no

396   observed cross-reactivity between primer pairs and impacts on the dynamic range of target

397   detection. The multiplex assay was also capable of detecting gDNA from as few as 210 to 250

398   CFU/mL in milk for each of the targets. The sensitivity of this qPCR method is comparable to

399   other assays which reported a detection limit for *M. bovis* in a range of between 10 and 240

400   CFU/mL reported in milk (*Clothier et al., 2010*; *Sachse et al., 2010*; *Boonyayatra et al., 2012*).

401   Our assay was also more sensitive than the LOD reported for a multiplex qPCR assay targeting

402   *uvrC* in *M. bovis*, *rpoB* in *M. californicum*, and the ITS region in *M. bovigenitalium* (*Parker et*

403   *al., 2017*). In that study, the detection limit was 130 CFU/mL, 600 CFU/mL, and 10^5 CFU/mL

404   for *M. bovis*, *M californicum,* and *M. bovigenitalium*, respectively. Sensitivity decreased by 100-

405   to 1000-fold when all three species were present at the same time to simulate a multispecies

406   infection (*Parker et al., 2017*). Our prior qPCR assay and methods targeting the *M. bovis gltX*

407   was less sensitive to detect low quantities of *M. bovis* cells. The detection limit was between 10

408   to 100 gDNA copies per reaction, corresponding to 10^4 to 10^5 cells per mL (*Appelt et al., 2019*).

409   However, it should be noted that a rapid DNA extraction method was used along with other Taq

410   polymerase reagents which may have impacted estimates of *M. bovis* detection limits.

411   Recently, a survey study in 95 New York dairy farms detected both *M. bovis* and *A.*

412 *laidlawii* in the same sample in 6% of the farms and mixed mycoplasma infections in 14% of the

413 farms (*Gioia et al., 2016*). Multispecies detection could be useful when testing for mastitis

414 because the occurrence of mixed mycoplasma spp. infection has been reported previously, with

415 *M. bovis* being isolated in approximately 90% of those cases and other species of mycoplasma

416 are isolated as secondary pathogens or contaminants (*Kirk et al., 1997*; *Szacawa et al., 2015*).

417 The multiplex qPCR assay reliably detected *M. bovis* and *A. laidlawii* gDNA when mixed

418 together. This indicated that *M. bovis* would be detectable, even with high levels of *A. laidlawii*

419 contaminants. Future studies will investigate the sensitivity of the multiplex qPCR assay for

420 mixed *Mycoplasma* species present in milk samples.

421   The multiplex assay showed a high level of Sp and Se when tested on milk samples

422 collected from different dairy farms in CA. Multiplex qPCR yielded a Se of 92.3% for 16S

423 rRNA and 93.3% for *rpoB* and a Sp of 100% for each of the targets compared with milk samples

424 confirmed by traditional laboratory bacterial culture methods. These results are comparable to

425 those obtained by others examining for *M. bovis* in milk (*Clothier et al., 2010*; *Parker et al.,*

426 *2017*; *Appelt et al., 2019*). Even though the sample size for the field validation test was small, no

427 false positive results were obtained. Instead, such samples were correctly identified as negatives

428 by qPCR as they did not meet the assay criteria for species/genera identification even when

429 culture results were positive. We suggest that qPCR negative results can be associated with the

430 presence of PCR inhibitors or low *Mycoplasma* cell numbers which might have led to a general

431 decrease in PCR sensitivity. In the former case the utilization of a DNA internal control in the

432 same tube as the target can eliminate false-negative PCR results caused by PCR inhibiting

433 substances, whereas in the latter case the multiplex assay cannot be used for direct detection in

434 milk samples containing bacterial numbers below the detection limit as it requires these samples

435 to be enriched before detection. Estimation of the diagnostic sensitivity and specificity estimates

436 for *A. laidlawii* was not possible given that none of the study samples tested positive for *A.*

437 *laidlawii* by laboratory culture. In addition, estimates of diagnostic accuracy for *Mycoplasma*

438 spp. and *Mycoplasma bovis* reported here should be interpreted with caution given the small

439 sample size used in their diagnostic test evaluation.

440

441 **CONCLUSIONS**

442 Because the multiplex assay results in amplifying all three target DNA sequences within

443 the same tube, the costs, labor, and time required for sample preparation are reduced. This assay

444 therefore provides an opportunity for more rapid diagnosis of contagious *Mycoplasma* pathogens

445 and the reduction of risk for false positive results caused by *A. laidlawii*. In particular, the assay

446 can be used as a first pass identification method for making preliminary decisions on

447 mycoplasma diagnosis, thereby minimizing the risk of pathogen spread within dairy herds.

448

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601 **Table 1**. **Bacterial strains used in this study.**

602

Organism	Strain/Source [a]
Mycoplasma bovis	ATCC 25523 and field isolates (n=3)
Mycoplasma californicum	ATCC 33461 and field isolates (n=3)
Mycoplasma bovigenitalium	ATCC 19852 and field isolates (n=3)
Mycoplasma canadense	ATCC 29410 and field isolates (n=3)
Mycoplasma arginini	ATCC 23838 and field isolates (n=3)
Mycoplasma alkalescens	ATCC 29103 and field isolates (n=3)
Acholeplasma laidlawii	ATCC 23206 and field isolate (n=3)
Aerococcus viridans	ATCC 700406
Bacillus cereus	MQ 17
Enterobacter cloacae subsp *cloacae*	ATCC 13847
Enterobacter sp.	MQ 14M0100-3
Enterococccus faecalis	ATCC 29212
Enterococcus durans	MQ 15M0470-1
Enterococcus faecium	ATCC 35667
Enterococcus gallinarium	ATCC 700425
Escherichia coli	ATCC 25922
Klebsiella pneumoniae	MQ 14
Proteus hauseri	ATCC 13315
Prototheca species	MQ 16M1258-4
Pseudomonas aeruginosa	ATCC 10145
Serratia marcescens	MQ 15M0394-3
Staphylococcus aureus	MQ 16M1264-9; ATCC 25923
Staphylococcus capitis subsp *capitis*	ATCC 35661
Staphylococcus chromogenes	DFSL 8435
Staphylococcus cohnii	ATCC 35662
Staphylococcus epidermidis	DFSL 1780
Staphylococcus haemolyticus	DFSL 8043
Staphylococcus pasteuri	DFSL 8109
Staphylococcus warneri	MQ 15M0945
Staphylococcus xylosus	ATCC 35663
Streptococcus agalactiae	ATCC 27956
Streptococcus dysgalactiae subsp *dysgalactiae*	ATCC 27957
Streptococcus dysgalactiae subsp *equisimilis*	ATCC 35666
Streptococcus equi subsp *zooepidemicus*	ATCC 700400
Streptococcus equinus	MQ 15M0757-12
Streptococcus infantarius subsp *coli*	ATCC 27960
Streptococcus mutans	MQ 15M1463-3
Streptococcus uberis	MQ 16M1263-101
Streptococcus uberis	ATCC 27958
Streptococcus uberis	ATCC 700407

603

604   [a]ATCC = American Type Culture Collection (Manassas, VA); MQ = Milk Quality Laboratory (VMTRC,

605   UC Davis, Tulare, CA); DFSL = Dairy Food Safety Laboratory (VMTRC, UC Davis, Tulare, CA).

606   **Table 2. Sequences of primers, probes, PCR product sizes and amplification efficiency of the qPCR assays [a]**

Organism	Target	Primer and Probe[b]	Primer and Probe Sequence (5' to 3')	nM	Product (bp)	qPCR efficiency (%) [c]	
						Singleplex	Multiplex
Mycoplasma spp[d]	16S rRNA	Myco_F Myco_R Myco_Probe	CGAGCGCAACCCTTATCCTT CCCCACTCGTAAGAGGCATGA VIC-TCGTCCCCACCTTCCTCCCG-QSY	100 100 75	118	88.8 - 100.9	96.8 - 102.4
M. bovis	*rpoB*	M.bovis_F M.bovis_R M.bovis_Probe	TTTCAGCCGCTAACTTCAGAGC GCAAGTTCCCCATCCTTGAAG ABY-TCGCCTTTAGCAACTTCTTGACCAA-QSY	200 200 200	232	87.1	95.6
A. laidlawii	ITS	Achol_F Achol_R Achol_Probe	AAGTGGGCAATACCCAACGC ACGTTCCCGTAGGGATACCTTG 6-FAM-ACGGCTCCCTCCCTTTCGGG-QSY	15015 075	108	91.9	91.2

607

608   [a] The QuantiFast Multiplex PCR master mix (Qiagen, CA) was used for all assays and with an annealing temperature of 58 °C.

609   [b] F and R indicate forward and reverse primers, respectively. . TaqMan probes were designed with 6-FAM (6-carboxyfluorescein) VIC (2'-

610   chloro-7phenyl-1,4-dichloro-6-carboxy-fluorescein) and ABY (Thermo Fisher) as reporter dyes on the 5′ end and QSY 7 (QSY7 succinimidyl

611   ester) as the quencher dye on the 3′ end.

612   [c] Amplification efficiencies were determined using gDNA from *M. bovis* ATCC25523, *M. californicum* ATCC 33461, and *M. bovigenitalium*

613   ATCC19852 (16S rRNA), *M. bovis* ATCC25523 (*rpoB*), and *A. laidlawii* ATCC 23206 (ITS).

614   [d] The PCR amplification efficiencies for *M. bovis*, *M. californicum* ATCC 33461, and *M. bovigenitalium* ATCC19852 were measured as

615   88.8%, 97.1%, and 100.9% in singleplex and 96.8, 102.4% and 97.9% in multiplex, respectively.

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619

620 **Table 3. Criteria for detection of *Mycoplasma* spp., *M. bovis, and A. laidlawii* in milk with**

621 **the TaqMan multiplex qPCR assay.**

	Gene target (Cut-off *Ct* value) [a]		
Organism [b]	**16S rRNA** $(C_t \leq 32)$	***rpoB*** $(C_t \leq 33)$	**ITS** $(C_t \leq 32)$
Mycoplasma **spp.**	+	-	-
M. bovis [c]	+	+	-
A. laidlawii	-	-	+

628 [a] Ct = Threshold Cycle value; positive (+) and negative (-) symbols indicate a positive and

629 negative result for that group of bacteria by the multiplex qPCR assay.

630 [b]*M. californicum* ATCC33461 *M, bovis* ATCC25523, and *A. laidlawii* ATCC23206 were used

631 for determining LOD levels of *Mycoplasma, M. bovis,* and *A. laidlawii*, respectively.

632 [c]*M. bovis* gDNA was expected to be detected by both the 16S rRNA and *rpoB* assays, therefore a

633 positive *M. bovis* sample is expected to have < 3 *Ct* value difference for those targets.

634

635   **Table 4**. **Detection of *M. bovis* and *A. laidlawii* mixtures in milk with the multiplex qPCR**

636   **assay [a].**

		Target	**Ct (avg ± stdev) [b]**
M. bovis (low)	*A. laidlawii* **(low)**	**16S rRNA**	33.47 ± 0.24
	A. laidlawii **(intermediate)**		33.32 ± 0.12
	A. laidlawii **(high)**		33.41 ± 0.41
	A. laidlawii **(low)**	***rpoB***	33.80 ± 0.52
	A. laidlawii **(intermediate)**		33.47 ± 0.21
	A. laidlawii **(high)**		33.49 ± 0.66
	A. laidlawii **(low)**	**ITS**	32.91 ± 0.38
	A. laidlawii **(intermediate)**		25.65 ± 0.07
	A. laidlawii **(high)**		15.79 ± 0.11
M. bovis (intermediate)	*A. laidlawii* **(low)**	**16S rRNA**	27.44 ± 0.11
	A. laidlawii **(intermediate)**		27.42 ± 0.12
	A. laidlawii **(high)**		27.22 ± 0.16
	A. laidlawii **(low)**	***rpoB***	27.05 ± 0.23
	A. laidlawii **(intermediate)**		26.97 ± 0.10
	A. laidlawii **(high)**		26.83 ± 0.31
	A. laidlawii **(low)**	**ITS**	32.87 ± 0.51
	A. laidlawii **(intermediate)**		25.71 ± 0.07
	A. laidlawii **(high)**		15.77 ± 0.07
M. bovis (high)	*A. laidlawii* **(low)**	**16S rRNA**	16.68 ± 0.15
	A. laidlawii **(intermediate)**		16.63 ± 0.18
	A. laidlawii **(high)**		16.90 ± 0.05
	A. laidlawii **(low)**	***rpoB***	16.38 ± 0.19
	A. laidlawii **(intermediate)**		16.42 ± 0.22
	A. laidlawii **(high)**		16.58 ± 0.14
	A. laidlawii **(low)**	**ITS**	36.70 ± 0.71
	A. laidlawii **(intermediate)**		26.02 ± 0.15
	A. laidlawii **(high)**		15.69 ± 0.05

637

638   [a] gDNA was extracted from milk inoculated with *M. bovis* ATCC 25523 and *A. laidlawii* ATCC

639   23206 at either low (10^2 to 10^3 CFU/mL), medium (10^3 to 10^4 CFU/mL), or high (10^6 to 10^7

640   CFU/mL).

641   [b] Shading indicates average *Ct* values within range for detection (n = 3) using qPCR criteria for

642   multiplex assay given in Table 3.

643

644

645 **Table 5. Laboratory culture and multiplex qPCR results for field milk samples.**
646

ID#	Sample Type [a]	Laboratory Culture [b]			Multiplex qPCR Result (*Ct*) [c]			
		DP	MB	Organism	Gene Target			Organism
					16S rRNA	*rpoB*	ITS	
4750	C	≥400	+	*M. bovis*	+(15.10)	+(17.18)	-	*M. bovis*
1331-2	T	4	+	*M. bovis*	+(29.79)	+(31.43)	-	*M. bovis*
1985-1	T	85	+	*M. bovis*	+ (24.60)	+(25.90)	-	*M. bovis*
2927-3	T	2	-	*M. bovis*	+ (31.03)	-(33.76)	-	*Mycoplasma sp.*
3145-1	T	+	+	*M. bovis*	+ (27.70)	+(32.35)	-	*M. bovis*
3606-1	T	+	+	*M. bovis*	+ (26.97)	+(28.34)	-	*M. bovis*
3642-1	T	≥100	+	*M. bovis*	+ (25.16)	+(27.80)	-	*M. bovis*
3915-1	T	+	+	*M. bovis*	+ (26.17)	+(27.32)	-	*M. bovis*
43722-1	T	≥200	+	*M. bovis*	+ (23.19)	+(24.91)	-	*M. bovis*
43897-1	T	4	+	*M. bovis*	+ (28.81)	+(31.46)	-(34.4)	*M. bovis*
4395-1	T	≥600	+	*M. bovis*	+ (26.27)	+(27.55)	-	*M. bovis*
43967-2	T	1	+	*M. bovis*	+ (29.42)	+(31.45)	-(34.52)	*M. bovis*
4435-1	T	9	+	*M. bovis*	+ (29.44)	+(30.62)	-(36.74)	*M. bovis*
4437-1	T	≥200	+	*M. bovis*	+ (23.94)	+(24.95)	-(35.99)	*M. bovis*
4784-1	T	9	+	*M. bovis*	+ (27.63)	+(29.77)	-(34.96)	*M. bovis*
8229	C	-	+	*M. bovigenitalium*	+ (17.84)	-(34.89)	-(37.21)	*Mycoplasma sp.*
180	C	-	+	*M. alkalescens*	+ (31.16)	-	-	*Mycoplasma sp.*
6141	C	-	+	*M. alkalescens*	+ (29.31)	-(38.47)	-	*Mycoplasma sp.*
33257	C	-	+	*M. alkalescens*	+ (26.78)	-(35.35)	-	*Mycoplasma sp.*
39494	C	-	+	*M. alkalescens*	+ (25.85)	-(38.41)	-	*Mycoplasma sp.*
41672	C	-	+	*M. alkalescens*	-	-	-	-
42275	C	6	+	*M. alkalescens*	+ (25.25)	-(36.52)	-(34.61)	*Mycoplasma sp.*
43960	C	-	+	*M. alkalescens*	-(34.67)	-	-(32.43)	-
482582	C	1	+	*M. alkalescens*	+(26.46)	-(31.54)	-	*Mycoplasma sp.*
749	C	85	+	*M. alkalescens*	+(19.99)	-(35.69)	-	*Mycoplasma sp.*
750	C	-	+	*M. alkalescens*	+(26.76)	-(34.34)	-	*Mycoplasma sp.*
829	C	-	-	-	-(32.42)	-(34.28)	-(34.89)	-
818	C	-	-	-	-(33.07)	-(35.25)	-(35.56)	-
802	C	-	-	-	-(33.18)	-(33.88)	-	-
828	C	-	-	-	-(33.43)	-(34.23)	-(34.07)	-
809	C	-	-	-	-(33.57)	-(34.22)	-	-
819	C	-	-	-	-(34.19)	-(34.09)	-(34.71)	-
815	C	-	-	-	-(34.54)	-	-	-
810	C	-	-	-	-(34.57)	-(34.58)	-	-
826	C	-	-	-	-(34.83)	-(34.35)	-(36.11)	-
823	C	-	-	-	-(34.96)	-	-(33.46)	-
804	C	-	-	-	-(35.26)	-	-(36.09)	-
822	C	-	-	-	-(35.40)	-	-	-
814	C	-	-	-	-(35.85)	-	-	-

824	C	-	-	-	-(36.02)	-(38.96)	-(35.90)	-
801	C	-	-	-	-(36.03)	-	-	-
808	C	-	-	-	-(36.06)	-(34.92)	-(35.19)	-
803	C	-	-	-	-(36.17)	-(39.70)	-	-
820	C	-	-	-	-(36.94)	-	-(34.57)	-
812	C	-	-	-	-(37.08)	-(34.66)	-	-
817	C	-	-	-	-(37.37)	-	-	-
806	C	-	-	-	-(37.65)	-(35.19)	-(34.94)	-
811	C	-	-	-	-(37.90)	-	-	-
813	C	-	-	-	-(38.00)	-(35.11)	-(34.94)	-
821	C	-	-	-	-(38.25)	-	-	-
807	C	-	-	-	-	-	-(34.88)	-
816	C	-	-	-	-	-	-(34.84)	-

647 Cow (C) or Bulk Tank (T) milk sample

648 DP = Direct plating. Based on results from direct plating of milk samples on modified mycoplasma

649 agar using cotton swabs; MB = Mycoplasma Broth. Based on culture results of 24 h enriched broth

650 samples; Plates with bacterial growth are indicated by a plus (+) and no growth by a minus (-);

651 *Mycoplasma* spp. were identified by fluorescently labeled, species-specific antibody staining method.

652 The positive qPCR results are indicated by a plus (+) and negative results by a minus (-); *Mycoplasma*

653 spp. were identified by *Ct* cut-off criteria (**Table 3**).

654

656 **Figure Legends**

657

658 **Figure 1**. **Standard curves for the 16S rRNA, *rpoB* and ITS TaqMan qPCR assays**

659 **performed in multiplex.** The standard curves were constructed with 10-fold serial dilutions of

660 *M. bovis* ATCC 25523 (16S rRNA and rpoB assays)*, M. bovigenitalium* ATCC 19852 (16S

661 rRNA assay)*, M. californicum* ATCC 33461 (16S rRNA assay), and *A. laidlawii* ATCC 23206

662 (ITS assay) gDNA, ranging from between approximately 5 fg to 5 ng gDNA. Results shown are

663 from a single run with each dilution tested in triplicate. The R^2 value was 0.99 for the standard

664 curve of each target (16S rRNA, *rpoB* and ITS). Error bars indicate standard deviation (±) based

665 on the results for three replicates.

666

667 **Figure 2. Limit of detection of multiplex assay for *M. bovis*, *M. californicum*, and *A.***

668 ***laidlawii* in milk.** Serial dilutions of *M. bovis* ATCC 25523 (starting cell number of 2.5 X 10^8

669 CFU/mL)*, M. californicum* ATCC 33461 (starting cell number of 2.3 X 10^6 CFU/mL), and *A.*

670 *laidlawii* ATCC 23206 (1.22 X 10^8 CFU/mL) were performed in *Mycoplasma* - negative raw

671 milk prior to gDNA extraction and target detection with the multiplex qPCR assay. Results

672 shown are from a single run with each dilution tested in duplicate. The R^2 value was 0.99 for the

673 standard curve of each target (16S rRNA, *rpoB* and ITS). Error bars indicate standard deviation

674 (±) based on the results for all replicates.

Figure 1

Standard curves for the 16S rRNA, *rpoB* and ITS TaqMan qPCR assays performed in multiplex.

The standard curves were constructed with 10-fold serial dilutions of *M. bovis* ATCC 25523 (16S rRNA and rpoB assays)*, M. bovigenitalium* ATCC 19852 (16S rRNA assay), *M. californicum* ATCC 33461 (16S rRNA assay), and *A. laidlawii* ATCC 23206 (ITS assay) gDNA, ranging from between approximately 5 fg to 5 ng gDNA. Results shown are from a single run with each dilution tested in triplicate. The R^2 value was 0.99 for the standard curve of each target (16S rRNA, *rpoB* and ITS). Error bars indicate standard deviation (±) based on the results for three replicates.
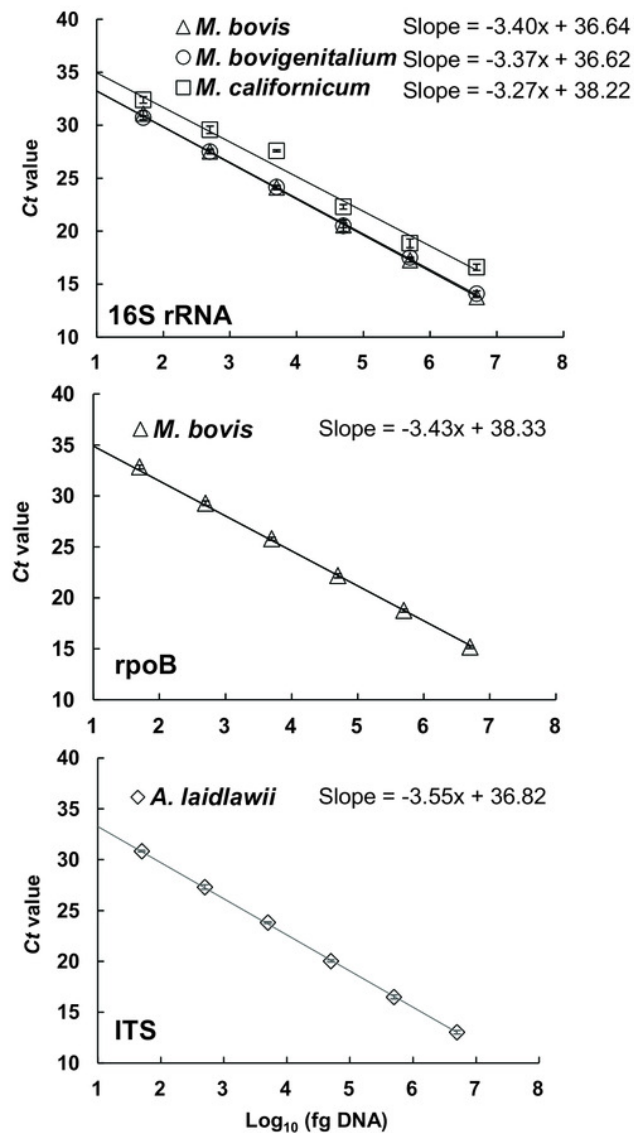
Figure 2

Limit of detection of multiplex assay for *M. bovis*, *M. californicum,* and *A. laidlawii* in milk.

Serial dilutions of *M. bovis* ATCC 25523 (starting cell number of 2.5 X 10^8 CFU/mL), *M. californicum* ATCC 33461 (starting cell number of 2.3 X 10^6 CFU/mL), and *A. laidlawii* ATCC 23206 (1.22 X 10^8 CFU/mL) were performed in *Mycoplasma* - negative raw milk prior to gDNA extraction and target detection with the multiplex qPCR assay. Results shown are from a single run with each dilution tested in duplicate. The R^2 value was 0.99 for the standard curve of each target (16S rRNA, *rpoB* and ITS). Error bars indicate standard deviation (±) based on the results for all replicates.