Improved diagnostics and further investigations of bovine mastitis caused by mollicutes

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Abstract

The ultimate aims of this thesis were to improve the diagnostics of mollicute-associated mastitis and confirm their pathogenic role in bovine mastitis.

Three types of experiments were carried out on improving diagnostics of mollicute associated mastitis (PCR, real time PCR-high resolution melting curve analysis (HRM), and Enzyme linked immunosorbent assay (ELISA)) on 368 milk samples from a single commercial dairy farm in South Australia (Farm 1), and were compared to conventional culture. Samples were collected purposively only from cows with high somatic cell count and/or mastitis treatment failure. For some tests (e.g. ELISA) samples from a second farm (n=40) in South Australia were also used (Farm 2). Novel primers of 16S ribosomal RNA were used in the PCR and HRM methodologies. A phylogenetic relationship among field isolates of mycoplasmas and acholeplasmas was created based on 16S rRNA sequences. An indirect ELISA, based on a recombinant fragment of the *Mycoplasma* immunogenic lipase A (MilA) protein was tested in milk for *M. bovis* antibodies, and compared to PCR and culture. For estimation of the pathogenic role of mollicutes associated with bovine mastitis, milk herd test data was analysed from 7,560 cow-tests.

Four types of media (glycerol (GLY) + dimethyl sulphoxide (DMSO), gelatine + DMSO, foetal bovine serum (FBS) + DMSO, and original milk (CON)) were used to test the survivability of *Mycoplasma bovis* over time at 1, 2, 4, 8 and 16 weeks freezing in domestic and -80°C freezers.

Very high prevalence of mollicutes was detected in the 288 purposively sampled cows (76.7%) using species-specific PCR. Culture was inferior in detecting infected milk samples (25.1%). The novel universal PCR demonstrated best concordance with species-specific PCR (Cohen's Kappa= 0.747 ± 0.031). The novel HRM analysis was able to discriminate between four of the

field isolates of *Mycoplasma* spp. and *Acholelasma laidlawii*. *Mycoplasma bovis* antibodies were detected only in 68/291 samples (23.4%).

The co-infection with two or more mollicutes had a similar effect on milk composition to other major mastitis pathogens. Long-term stored milk samples should be enriched with some of the cryopreservatives used in this thesis. All cryopreservatives improved the survivability of *M. bovis* in milk samples stored under freezing conditions. The combination of GLY and DMSO resulted in significantly higher recovery rates at week 16, compared to CON with 57.1% (95% CI = 21.43–133.34) and 19.1% (95% CI = 11.73–60.27), respectively. The use of GLY and DMSO should therefore be encouraged for use as a cryoprotectant for *M. bovis* at − 20 and − 80 °C.

Microbiological and molecular techniques used in this thesis should result in improved diagnostics of mollicute-associated mastitis providing rapid and accurate screening techniques. This should become a cornerstone in control strategies of mollicute-associated bovine mastitis.

List of Abbreviations:

AU: Antibody unit

CFU: Colony forming unit

CON: Control

CoNS: Coagulase negative staphylococci

CoPS: Coagulase positive staphylococci

DMSO: Dimethyl sulphoxide

ELISA: Enzyme linked immunosorbent assay

FBS: Foetal Bovine Serum

GEL: Gelatin

GLY: Glycerol

GST: Glutathione s transferase

HRM: High resolution melt

IgG: Immunoglobulin G

LAMP: Loop-mediated isothermal amplification

MilA: *Mycoplasma* immunogenic lipase

PCR: Polymerase chain reaction

PPCR-HRM: Polymerase chain reaction-High Resolution Melt

rRNA: Ribosomal ribonucleic acid

SAS: Statistical analysis software

SCC: Somatic cell count

SCS: Somatic cell score

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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List of publications by candidate included in the thesis

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Conferences:

Al-Farha A, Subclinical mastitis caused by Mycoplasma-like bacteria in in dairy cattle in South Australia, 2nd International Conference on Livestock Nutrition, 2016 Brisbane, Australia. Oral presentation

Al-Farha A, Evaluation of somatic cell counts and milk productions in cattle affected by Mycoplasma mastitis College Science Week Scientific Conference, 2017, Gold Coast, Australia. Oral presentation

Al-Farha A, Mycoplasma subclinical mastitis EMBL Australia Postgraduate Symposium 2016, Adelaide, Australia. Oral Presentation

Al-Farha, A, Rapid screening method for identification of bovine milk mycoplasmas and *Acholeplasma laidlawii.* The 30th World Buiatrics Congress 2018 Sapporo, Japan. Oral presentation

Al-Farha A, Survivability of Mycoplasma spp. Isolated from milk samples from South Australia in different storage conditions The 29th World Buiatrics Congress (WBC), 2016 Dublin, Ireland. Poster

Al-Farha A, Comparison of PCRs to culture results in detection of Mycoplasma spp. from milk samples from South Australia, The 29th World Buiatrics Congress (WBC), 2016 Dublin, Ireland. Poster

Al-Farha A, Microbiology and molecular tools for detection of *Mycoplasma* mastitis in South Australia Animal Production Congress / Australian Society of Animal Production, 2016. Poster

Al-Farha, A, Hemmatzadeh, F, Trott, D, Hoare, A and Petrovski K The effect of *Mycoplasma* mastitis on somatic cell counts patterns and bovine milk production in South Australia, Australian Society of Animal Production annual meeting. 2016, Adelaide, South Australia. Poster

Al-Farha, A, Hemmatzadeh, F, Trott, D, Hoare, A and Petrovski K Evaluation of Recoverability of *Mycoplasma*-like organisms causing mastitis in dairy cattle in South Australia under different freezing conditions. , Australian Society of Animal Production annual meeting. 2016. Adelaide, South Australia. Poster

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1 **Chapter 1: General introduction**

2 Mycoplasmas and acholeplasmas are the smallest ubiquitous bacteria related to the mollicutes
3 class. *Mycoplasma* spp are well-known to be a responsible pathogen for many diseases in cattle
4 including respiratory disorders, arthritis, mastitis, urogenital tract disorders, meningitis,
5 keratoconjuctivitis (Calcutt et al., 2018, Stipkovits et al., 1993) and have recently been isolated
6 from post-surgical seromas (Gille et al., 2016). *Mycoplasma* mastitis has received much
7 attention over the last two decades as several outbreaks have occurred in Australia, USA,
8 Europe, and sporadically elsewhere (Pothmann et al., 2015, Aebi et al., 2012, Radaelli et al.,
9 2011, Punyapornwithaya et al., 2010, Infante-Martinez et al., 1999). *Mycoplasma* mastitis can
10 be clinical or sub-clinical, and imposes severe economic impact to the dairy industry.

11 The economic consequences of *Mycoplasma* infection in cattle are enormous. They include the
12 cost of loss of beef weight, the cost of culling of diseased cattle, significant decrease in milk
13 production, the cost of the implementation of control procedures as well as the cost of diagnosis
14 and treatment for associated disorders such as mastitis and arthritis (Nicholas et al., 2016,
15 Maunsell et al., 2011). In the United States, for instance, the cost of *M. bovis* associated diseases
16 is more than $140 million each year and more than €150 million in Europe annually (Amram
17 et al., 2013).

18 The importance of *Mycoplasma* in veterinary medicine and the variety of the diseases caused
19 by this organism have led to excessive classification studies. Mycoplasmas are mainly
20 classified as "mollicutes" which is derived from the Latin meaning "soft skin" (Razin and
21 Hayflick, 2010). These bacteria are characterised by lack of their cell wall as well as
22 peptidoglycan synthesis. According to 16S rRNA sequence analysis, the genus *Mycoplasma*
23 has been classified in the phylum Firmcutes (Razin and Rottem, 1978, Steim et al., 1969), and
24 belongs to the Mycoplasmaceae family which is a single family of the order *Mycoplasmatales*.

25 In veterinary medicine, two genera are important, *Mycoplasma* and *Ureaplasma*. It has been
26 suggested that *Mycoplasma* species might be better identified based on their genomic features
27 (Thompson et al., 2011). These features include Multilocus Sequence Analysis, Karlin
28 Genomic Signature and the Average Amino Acid Identity. Using Denaturing Gradient Gel
29 Electrophoresis (PCR-DGGE) technique, several species of *Mycoplasma* that affect cattle have
30 been reported (Nicholas and Ayling, 2016, Nicholas et al., 2008). These species include *M.*
31 *alkalescence, M. alvi, M. bovigenetalium, M. bovirhinis, M. bovis, M. californicum, M.*
32 *canadense M. canis, M. dispar, M. leachii, M. mycoides* subsp. *mycoides* small colony type,
33 and *M. verecundum,*. Other *Mycoplasma* species isolated from bulk tank milk samples such as
34 *M. arginini* and *M. gateae* have been reported (Hata, 2015, Justice-Allen et al., 2011).
35 Additionally, *M. wenyonii* has been detected in cattle-derived samples using the loop-mediated
36 isothermal amplification (LAMP) assay (Song et al., 2013). The presence of *M. canadense* has
37 been confirmed in Argentinean cattle (Tamiozzo et al., 2014). *M. bovis*, *M arginini* and *M.*
38 *bovirhinis* have been reported as causes of bovine mastitis (Kokotovic et al., 2007, Gonzalez
39 and Wilson, 2003).

40 *Mycoplasma* mastitis was firstly reported in England (Davidson and Stuart, 1960), and the first
41 report from Australia was in 1967 (Connole et al., 1967). In the last few years, *Mycoplasma*
42 mastitis gained importance in Australian dairy herds (Hazelton et al., 2017, Parker et al., 2017a,
43 Morton et al., 2014). Therefore, highlighting different strategies for detection of these
44 pathogens is important for farmers, veterinarians and laboratory staff. Thus, the ultimate aims
45 of this thesis were to improve diagnostics of mollicute-associated mastitis and confirm their
46 pathogenic role in bovine mastitis. These aims will be addressed in a series of five chapters
47 (Chapter 2-6) within this thesis.

48 Chapter 2 describes research which aimed to develop a rapid, accurate and reliable screening
49 method for identification of mollicutes, and analyses the concordance between the universal
50 PCR, species-specific PCR and conventional culture isolation from bovine milk samples from
51 a single commercial dairy farm in South Australia. Conventional culture has been widely
52 accepted as the gold standard for detection of *Mycoplasma* mastitis (D'Inzeo et al., 2017).
53 However, the fastidiousness of these bacteria and the special lab requirements including
54 *Mycoplasma* specific media and 10% CO2 incubation for 2-3 weeks may hinder the microbial
55 isolation (Hogan et al., 1999). Thus, mycoplasmas can be a missing component from the
56 routine mastitis culture of most diagnostic labs (Ghadersohi et al., 1997). Furthermore, there
57 are individual species requirements for different mollicutes including nutrients and
58 atmospheric carbon dioxide (Lowe et al., 2018, Boonyayatra et al., 2012), which can affect the
59 growth of these organisms particularly when co-infection with two or more species/genera of
60 these mollicutes are involved. Therefore, a rapid screening detection of these mollicutes rather
61 than conventional culture was needed. In the study described in Chapter 2, polymerase chain
62 reaction (PCR) system targeting 16S rRNA has been developed. This preliminary stage of
63 screening mollicutes in milk is clinically important as it can reveal the existence of mollicutes
64 on a farm, covering the *M. bovis* infection, but not neglecting the other potentially involved
65 mycoplasmas and acholeplasmas. As *Mycoplasma* mastitis can be caused by few genera and
66 species, an essential step to discriminate between different mollicutes is an efficient assay. In
67 fact, possibly due to the limitation of detection of multiplex PCR, previous milk PCR studies
68 have focused on limited *Mycoplasma* species. For instance, the multiplex PCR probes
69 developed by Parker et. al. (2017), target only *M. bovis*, *M. californicum* and *M.*
70 *bovigenetalium*. Similarly, the multiplex PCR developed by Gioia et. al. (2016) omits *A.*
71 *axanthum* and *M. bovirhinis.*

72 Chapter 3 resolves the question raised in Chapter 2, and describes a suitable diagnostic real
73 time PCR-high resolution melting curve analysis (PCR-HRM), which identifies and
74 distinguishes between five different mollicutes isolated at cow-level from a single commercial
75 dairy farm in South Australia. Previous work has been limited to biochemical and multiplex
76 PCR differentiation of the mollicutes of interest (Boonyayatra et al., 2012, Jang et al., 2009),
77 which is most likely to be time consuming and costly. The PCR-HRM assay was first described
78 in 2003 (Wittwer et al., 2003), and has been widely used for various microbiological
79 applications (Liu et al., 2018, Ren et al., 2017, Sacks et al., 2017). However, there is a dearth
80 of knowledge in using HRM profile to discriminate between milk mollicutes. Although melting
81 profile has been previously used with some mycoplasmas (Rebelo et al., 2011, Ghorashi et al.,
82 2010), to our knowledge, no study has shown the ability of this technique to discriminate
83 between milk mollicutes. Using real time PCR-HRM analysis can provide a rapid, accurate and
84 cost-effective assay in *Mycoplasma* mastitis detection. The variety of milk mollicutes does not
85 mean they are all pathogenic. The crucial factor that can discriminate between pathogenic and
86 saprophytic mollicutes is the effects of these organisms on milk composition. Thus, one of the
87 major objectives of this thesis was to estimate the effect of the various mollicutes on milk
88 composition.

89 Chapter 4 identifies the genetic relationship between field isolates described in Chapter 2 in
90 addition to two other isolates of *M. alkalescens* and *A. axanthum* based on 16S ribosomal RNA
91 sequencing. The usefulness of 16S rRNA sequencing, due to the high copy numbers of
92 sequenced data, has been proven and it is an efficacious discriminatory tool with high power,
93 sensitivity and accuracy for studying epidemiology of pathogens (van Kuppeveld et al., 1994a).

94 The intermittent shedding of *M. bovis* through milk can hinder the routine detection of milk
95 mollicutes via culture or PCR. Hence, investigating *M. bovis* antibodies in milk could

96 potentially explain the false-negative results of culture or PCR. In addition, *M. bovis* carrier
97 cows are considered to be the main source of infection, particularly if they have been introduced
98 to native farms. Therefore, investigating *M. bovis* antibodies status could contribute in
99 biosecurity management. The diagnostic procedures are extended in Chapter 5 with the aim to
100 investigate another aspect of the disease through highlighting the potential use of an indirect
101 ELISA in the detection of milk *M. bovis* antibodies. Some immunogenic proteins from *M. bovis*
102 have been evaluated previously for their capacity in detection of *M. bovis* antibodies (Sun et
103 al., 2014, Byrne et al., 2000), mainly at bulk tank milk level (Parker et al., 2017a, Arede et al.,
104 2016, Nielsen et al., 2015). However, the recombinant *Mycoplasma* immunogenic lipase
105 (MilA) Immunoglobulin G (IgG) indirect ELISA has not been evaluated in milk but only
106 evaluated in experimentally infected calves with *M. bovis* in serum (Wawegama et al., 2016,
107 Wawegama et al., 2014). As a result, a study aimed to detect *M. bovis* antibodies with the MilA
108 ELISAs compared to the presence of *M. bovis* by conventional culture and PCR in milk was
109 designed.
110 Chapter 6 builds on the findings of Chapter 2 and Chapter 3, and determines the effects of
111 different *Mycoplasma* spp. and *A. laidlawii* compared to conventional mastitis pathogens on
112 milk yield and other milk components in cattle from a single dairy herd in South Australia with
113 high somatic cell count (SCC), which was used as an indicator of sub-clinical mastitis.
114 Additionally, there was a low response rate to conventional antimicrobial therapy in cows with
115 clinical mastitis on this farm. The increase in individual cow milk SCC and decrease in milk
116 yield have been widely demonstrated with major mastitis pathogens, but information on SCC,
117 milk yield and effects on other milk components such as total milk solids (TMS), total milk
118 protein and fat percentage in mollicute-associated bovine mastitis is lacking in the literature
119 and warrants further investigations. SCC's are mainly composed of epithelial and white blood
120 cells in milk, and are considered as a gold standard to discriminate between healthy and

121 diseased cows, particularly in sub-clinical mastitis (Ruegg and Pantoja, 2013, Pillai et al.,
122 2001). Changes in milk composition associated with mastitis (e.g. protein and fat percentage)
123 have also been associated with specific mastitis pathogens (Petrovski, 2006). While the role of
124 *M. bovis* in bovine mastitis has been described in the literature as the major mastitis causing
125 *Mycoplasma* (Nicholas et al., 2016), controversial reports have shown the role of some other
126 mycoplasmas and *A. laidlawii* in bovine mastitis either to be pathogenic or saprophytic
127 organisms. The significance of each of these mollicutes in bovine mastitis needs more
128 clarifications. Understanding the role of each of these mollicutes can lead to improved
129 diagnostic, and in turn, control strategies of the disease can be improved. Moreover, there is a
130 lack of knowledge about the roles of these mollicutes in sub-clinical mastitis as individual or
131 co-invaders either with different species/genera of mollicutes or with other mastitis pathogens.
132 Thus, in Chapter 6, herd test data and infection status were used to determine the effect of
133 various mollicutes on milk quality and quantity. The effect of mollicutes-affected cows were
134 compared to other cows in the herd.

135 Finally, all the aforementioned detection techniques cannot be achieved without an effective
136 strategy for storage these bacteria. Hence, Chapter 7 evaluates the survival of *M. bovis* in
137 bovine milk following various storage times under three different freezing conditions (4ºC, -
138 20ºC and -80ºC) using milk only as a control (CON) or three different storage media (milk
139 supplemented with dimethyl sulphoxide (DMSO) and either foetal bovine serum (FBS), gelatin
140 (GEL) or glycerol (GLY)). There is limited literature regarding the storage of mycoplasmas in
141 milk, and no literature regarding the use of GLY/DMSO, GEL/DMSO or FBS/DMSO as milk
142 additives for storage of samples.

143 Throughout this thesis, the term "isolate" refers to field milk mollicutes that is confirmed
144 through axenization of bacteria. Axenization of bacteria was performed by selecting 3-5

145 colonies from each plate which were then subcultured into the enriched *Mycoplasma* broth and
146 cultured per routine mollucites microbiological methods. An important step in mollicutes
147 culture is monitoring of plates for change in colour due to pH alterations. As soon as the phenol
148 red indicator changed to yellow, the subculture onto a fresh broth and agar was carried out.
149 Furthermore, the DNA was extracted directly from milk and *Mycoplasma* broth. All procedures
150 were carried out in duplicate using the same PCRs, HRM analysis and sequencing with no
151 differences observed between them.

152

153

154 **Chapter 2: Comparison of PCR with culture for detection of field isolates of bovine milk**
155 **mollicutes**

156 **Aims:** To develop a rapid and accurate screening method for identification of *Mycoplasma* spp.
157 and *Acholeplasma laidlawii* and investigate relative merits of conventional culture-based
158 method versus DNA amplification for detecting mycoplasmas and *A. laidlawii* in bovine milk.

159 **Null Hypothesis:** There was no correlation between culture-based method versus DNA
160 amplification for detecting mycoplasmas and *A. laidlawii* in bovine milk.

161 **Note**: Raw data available in Appendix 1

162

Statement of Authorship

Title of Paper	Comparison of PCR with culture for detection of field isolates of bovine milk mollicutes
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Abd Al-Bar Ahmed Noori Al-Farha		
Contribution to the Paper	Assisted in conceptualisation of the study, designed the study, prepared the samples, performed culture and conventional PCR for samples, wrote the manuscript, and acted as a corresponding author.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	2/5/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Farhid Hemmatzadeh		
Contribution to the Paper	Supervised all steps of the experiment, assisted in the study design, edited and approved the manuscript.		
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166 **Comparison of PCR with culture for detection of field isolates of bovine milk mollicutes**

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179 **Abstract**

180 Background:

181 *Mycoplasma* mastitis raises significant concerns in the dairy industry worldwide. The study

182 objective was to develop a rapid and accurate screening method for identification of field

183 isolates of *Mycoplasma* and *Acholeplasma* spp. and investigate relative merits of conventional

184 culture-based method versus DNA amplification for detecting mycoplasmas and acholeplasmas

185 in bovine milk.

186 Methods:

187 A total of 368 individual cow milk samples were collected from a single commercial dairy farm

188 in South Australia. These samples subjected to conventional *Mycoplasma* culture, species-

189 specific PCR and our specifically designed universal mollicutes PCR.

Results:

Of 368 milk samples collected at individual cow level from a single dairy farm in South Australia, 192 (52%) tested positive for mollicutes using a conventional culture-based method, compared to 269 (73%) using our specifically designed universal mollicutes PCR-based method for testing DNA extracted directly from milk. A subset of 30 samples were sequenced targeting the 16S rRNA gene for confirmation. Sequencing results showed five different mollicutes species involved, including *Acholeplasma laidlawii, A. axanthum*, *M. arginini*, *M. bovirhinis*, and *Mycoplasma bovis*. According to these results, species-specific PCR was conducted on all samples. DNA amplifications using species-specific PCR yielded 256 (70%) positive mollicutes samples. Our developed universal PCR demonstrated best concordance with species-specific PCR (Cohen's Kappa= 0.747 ± 0.031). Co-infection by two or more of the above mentioned mollicutes showed highest prevalence.

Conclusion: Due to rapidity and higher sensitivity compared to the conventional culture method for surveying mycoplasmas and acholeplasmas in dairy herds, we recommend screening using our universal PCR method.

Keywords: *Mycoplasma*, mastitis, dairy cattle, milk, mollicutes

Introduction

Mycoplasmas and acholeplasmas are the smallest bacteria, belonging to the mollicutes class, some of these mollicutes are responsible for many infections in cattle. *Mycoplasma*-associated diseases occur worldwide causing serious problems for the dairy and beef feedlot industries and impose significant economic impact (Pfutzner and Sachse, 1996). Specific concerns regarding mycoplasmas arise from difficulty of detection, long persistence in affected herds, a variety of transmission methods, the tendency to cause mixed infections, and poor response to a wide range of antimicrobials (Sulyok et al., 2017, Lysnyansky and Ayling, 2016). In dairy herds,

214 mycoplasmas may cause clinical, subclinical or chronic mastitis (González and Wilson, 2003).
215 Among 200 of *Mycoplasma* species discovered, several have been reported to be involved in
216 bovine mastitis or isolated occasionally from milk including *M. bovis*, *M. bovigenitalium, M.*
217 *californium, M. bovirhinis, M. arginini, M. dispar, M. canadense*, *Mycoplasma* species bovine
218 group 7 and F-38 (Fox, 2012). Among mycoplasmas, *M. bovis* is the most common pathogen
219 causing mastitis (George et al., 2007). Some studies claim that *Acholeplasma* spp., another
220 genus of the mollicutes class, can be a milk contaminant or non-pathogenic saprophyte
221 (Boonyayatra et al., 2012, Jasper et al., 1979). However, others have reported that isolation of
222 *A. laidlawii* from clinical and subclinical bovine mastitis cannot be excluded (Al-Farha et al.,
223 2017a, Windsor et al., 2010, Kirk et al., 1997, Watts, 1988). Additionally, *A. axanthum* has
224 been isolated from bovine milk harvested from cows suffering from mastitis (Roy et al., 2008,
225 Gonzalez and Wilson, 2003).

226 Identification of milk mycoplasmas is often achieved using conventional microscopic culture
227 or through serological determination methods. However, both detection methods have the
228 significant limitation of a prolonged sampling to results timeframe (Hotzel et al., 2003, Pinnow
229 et al., 2001). *Mycoplasma* species can cause bovine mastitis cases either individually or as co-
230 *Mycoplasma* infection (Schnee et al., 2012). Currently, most molecular studies focus on a single
231 *Mycoplasma* species invader (usually *M. bovis*) and disregard potential co-infection. Few recent
232 studies have included multiple mycoplasmas and acholeplasmas in milk using multiplex PCR
233 (Parker et al., 2017b, Gioia et al., 2016). However, the universal PCR detailed in this thesis
234 expands to few more common milk mollicutes including species not been reported previously,
235 e.g. *A. axanthum* and *M. bovirhinis*. Previous studies have reported co-infections of limited
236 variety. Current knowledge does not inform the reader of the potential combinations and their
237 effect on milk composition and yield.

238 Detection of *Mycoplasma* infection using 16S rRNA as a molecular marker has been previously
239 evaluated (Bashiruddin et al., 2005, Kobayashi et al., 1998). The usefulness of 16S rRNA gene
240 was demonstrated in detecting slow-growing bacteria (Srinivasan et al., 2015). However, most
241 previous studies targeted species-specific oligonucleotides and disregarded co-infection of
242 *Mycoplasma* and *Acholeplasma* species. The clinical importance of co-infection with
243 mycoplasmas has been reported (Al-Farha et al., 2017, Szacawa et al., 2015). Given that
244 mollicutes have a small genome and low G-C content (Nicholas et al., 2008), a sensitive,
245 accurate and broad-species detection is required. Implementation of a rapid, reliable and
246 affordable screening method can be used in eradication strategies of this disease at quarter, cow,
247 herd and national level.

248 The aim of our study was to develop a rapid, accurate and reliable screening method for
249 identification of mollicutes, and analyse the concordance between our universal PCR, species-
250 specific PCR and conventional culture isolation in bovine milk samples from a single
251 commercial dairy farm in South Australia.

252 Materials and methods

253 Samples collection

254 A total of 368 individual cow milk samples were collected from a single commercial dairy farm
255 in South Australia. At the time of collection, cows had high somatic cell counts and the farm
256 had experienced repeated failure of mastitis treatment. Milk samples were aseptically collected
257 from each functional quarter in sterile 50 mL tubes. Samples were kept on ice and sent
258 immediately to the PC2 laboratory at the School of Animal and Veterinary Sciences, The
259 University of Adelaide, Roseworthy, South Australia. Milk samples were subjected to
260 conventional *Mycoplasma* culture, and remaining sample contents were frozen at -20ºC and
261 retained for molecular analysis.

262 ***Mycoplasma* Culture**

263 To enrich *Mycoplasma* growth, 200 µL of each fresh milk sample was aseptically added to a 5
264 mL enriched *Mycoplasma* broth and *Mycoplasma* supplement G, containing penicillin and
265 thallus acetate (Oxoid, Sydney, NSW, Australia) (Thurmond et al., 1989). *Mycoplasma*-
266 enriched samples were incubated at 37±1°C for seven days under 10% CO_2-enriched
267 conditions. Next, samples were filtered through a 0.45 µm filter (Merck Millipore, Bayswater,
268 Vic, Australia). Two hundreds µL of each filtered product was plated onto *Mycoplasma*-
269 selective agar (Oxoid, Sydney, Australia) and incubated at 37±1°C for 14 days under 10% CO_2-
270 enriched conditions. *Mycoplasma*-like colonies were detected using a stereomicroscope
271 (Olympus SZ30, Vic, Australia) at 10x magnification. Cultures were considered positive when
272 growth of at least one *Mycoplasma*-like colony was detected (Markey et al., 2013). To confirm
273 isolation of *Mycoplasma*, 3-5 selected colonies from each plate were subcultured into the
274 enriched *Mycoplasma* broth and inoculated under the same conditions, checked for colour
275 change of broth and typical *Mycoplasma* colonies on the agar plate. As soon as the phenol red
276 indicator changed to yellow, the subculture onto a fresh broth and agar was carried out. In fact,
277 this is the process of axenization of mollicutes.

278 **DNA extraction**

279 The DNA was extracted from both frozen milk or enriched samples and all tests were repeated
280 on both type of samples. After thawing milk samples at ambient temperature, 2 mL of each
281 milk sample was centrifuged at 8,000 g for 20 min to remove supernatant fat and excess liquid.
282 The enriched samples in broth were used directly for DNA extraction. DNA extraction was
283 performed using QIAmp DNA extraction kit (Qiagen, Germany) according to manufacturer's
284 instructions. Genomic DMA concentration was measured using Nanodrop l000c (Thermofisher
285 Scientific Inc., Waltham, MA, USA) and DMA was stored at -20° C until further use.

286 **PCR probes and protocol**

287 Five separate PCR reactions with five different primers pairs were used in our study. The first
288 universal primers, Mol-F: GGCGAAYGGGTGAGTAACAC and Mol-R:
289 CATHGYCTTGGTRRGCYNTTA were designed to target 16S rRNA gene at genus-level and
290 generate amplicon (180 bp). Multiple sequence alignment of 16S rRNA gene was conducted
291 on number of cattle-associated mycoplasmas and acholeplasmas using Clustal Omega (Sievers
292 and Higgins, 2014). A block containing highly variable region, flanking by two conserve
293 regions in upstream and downstream of the sequences, was selected. Based on general criteria
294 for primer designing, forward and reverse primers were selected from conserved region of
295 multiple blocks. The accuracy of the different primer sets for different blocks was checked by
296 ATCC strains, PCR and sequencing. *Acholeplasma laidlawii* (Sabin) Edward and Freundt
297 (ATCC® 23206-MINI-PACK™) and *Mycoplasma bovis* (ATCC® 25025™) were used as
298 positive controls. *M. bovis*-specific 16S rRNA primers (442 bp), composed of PpSM5-1: 5'-
299 CCAGCTCACCCTTATACATGAGCGC-3' and PpSM5-2: 5'-
300 TGACTCACCAATTAGACCGACTATTTCACC-3' were used for *M. bovis* detection (Hotzel
301 et al., 1993); while the other three primers for *A. laidlawii*, *M. arginini* and *M. bovirhinis* were
302 previously published elsewhere and cited in our previous work (Al-Farha et al., 2017). In-vitro
303 amplification of DNA to detect mycoplasmas and acholepasmas was conducted for each primer
304 separately. Amplifications were carried out in 25 µL containing 0.25 µL Taq DNA polymerase,
305 5 µL of 5x reaction buffer (Bioline, UK), 1 µL (0.5 µM) of each forward and reverse primers,
306 1 µL (approximately 20 ng) of template, and 16.75 µL of DEPC-treated water. The negative
307 control was prepared from the same reagents of Master Mix, except DNA template, and the
308 volume was compensated with DEPC water (Lorenz, 2012). Amplifications were performed
309 for 35 PCR cycles conditions using T100™ Thermal Cycler (Biorad thermocycler, Australia),
310 and consisted of pre-heating activation for 5 minutes at 95ºC, denaturation at 95ºC for 30 sec,
311 annealing at 60ºC for the universal primer, *M. bovis* and A. *laidlawii*; 55ºC for *M. arginini* and

312 64ºC for *M. bovirhini,* and primer extension at 72ºC for 45 seconds. The final extension step
313 was performed at 72ºC for 10 minutes. The PCR products were analysed by 1.5% agarose gel
314 electrophoresis and visualised by staining with Gel Red. Selected species for our study were
315 nominated based on the 16S rRNA sequencing of the universal PCR. The same PCR methods
316 have been done on all isolated mycoplasmas to identify the isolate in sequencing of the PCR
317 products. All tests were carried out in duplicate.

318 **16S rRNA sequencing**

319 The PCR products from 16S rRNA gene were submitted to the Australian Genome Research
320 Facility Ltd (AGRF, Adelaide, South Australia) for Sanger sequencing (six samples for each
321 positive PCR detected species). Each fragment was sequenced in forward and reverse
322 directions. To reconstitute the sequence, forward and reverse sequences were edited and
323 assembled using BioEdit package v.7.0.4.1. Edited sequences were blasted against existing
324 sequences in GeneBank using the basic local alignment search tool (BLAST)
325 (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and nucleotide sequences from relevant *Mycoplasma*
326 strains were used as reference strains for nucleotide alignments using ClustalW program
327 version 2 (Larkin et al., 2007).

328 **Statistical analysis**

329 Positive results of conventional culture method, universal PCR and species-specific PCR were
330 reported as number and percentage. Agreement between sets of data for the aforementioned
331 detection methods was identified using Cohen's Kappa coefficient test (R version 3.1.1, R
332 Development Core Team, New Zealand). Raw data used for the analyses are presented in the
333 Appendix 1.

334 **Results**

335 Of 368 milk samples collected at individual cow level from a single dairy farm in South
336 Australia, our universal PCR showed higher prevalence of mollicutes in milk (73%) as

337 compared to the conventional culture method (52%) Table 1. Samples were considered as
338 positive for culture growth when at least a single colony of mollicutes was identified. PCR
339 results were confirmed using species-specific primers (according to 16S rRNA sequencing
340 results) for *A. laidlawii, M. arginini* and *M. bovirhinis*. Using species-specific primers, co-
341 infection with two or more of the aforementioned mollicutes was detected in 165 (45%); *A.*
342 *laidlawii* was the highest individual species detected followed by *M. bovis* and *M. bovirhinis*,
343 *M. arginini* while *A. axanthum* had the lowest prevalence (Figure 1). In addition, 34% of
344 samples were negative for culture and positive for either or both PCR methods (universal and
345 species-specific). However, approximately 7% of positive samples were identified by culture
346 but not by PCRs, 36 samples tested positive using the universal PCR, but negative using
347 species-specific primers for *A. laidlawii*, *M. bovis*, *M. bovirhinis* and *M. arginini* (Figure 2).
348 These were confirmed as *A. axanthum* via 16S rRNA sequencing. Cohen's Kappa coefficients
349 showed good agreement between our universal PCR and species-specific PCRs and fair
350 agreement between culture and both PCR tests (Table 2).

351 **Discussion**

352 The study objective was to develop a rapid, accurate and reliable screening method for
353 identification of *Mycoplasma* and *Acholeplasma* spp. and investigate the relative merits of
354 conventional culture-based method versus DNA amplification for detection of mollicutes in
355 bovine milk.

356 The studied farm had a history of treatment failure of mastitis with high somatic cell counts
357 (~300,000 cells/mL at bulk tank level). *Mycoplasma* mastitis has a variety of transmission
358 methods including direct contact through milking machines and other fomites (Radaelli et al.,
359 2011, Justice-Allen et al., 2010). Intermittent shedding of the pathogen from cows suffering
360 chronic mastitis may be another important reason for the relatively high prevalence of

361 *Mycoplasma* mastitis (Maunsell et al., 2011). It is understood that chronic mastitis results from
362 the capability of *Mycoplasma* spp. to form multiple micro-abscesses within the infected
363 mammary gland (Jasper, 1982). Results of our study may raise awareness of the importance of
364 *Mycoplasma*-induced mastitis for the dairy industry. The association of these mollicutes and
365 mastitis in addition to their pathogenic significance have previously studied by our group (Al-
366 Farha et al., 2017). We found that the co-infection with mycoplasmas and acholeplasmas has
367 similar effects on milk composition to other major mastitis pathogens. Therefore, our developed
368 universal PCR in this study is useful for milk mollicutes screening. Further research in affected
369 herds is required to establish the current prevalence of this disease in Australia. Our study found
370 that sensitivity of mollicutes detection using the novel universal 16S rRNA amplification was
371 significantly higher than detection using the culture-based method. Naturally, 16S rRNA
372 demonstrates high copy numbers and low sequence diversity which can enhance sensitivity of
373 PCR based tests (Peredeltchouk et al., 2011, Waters and McCuthan, 1990). Results of our study
374 show that one third of samples returned negative *Mycoplasma* results for culture and positive
375 for both PCR methods (Figure 1). This difference can be explained by the fastidious nature of
376 mycoplasmas, as failure to culture may occur due to lack of a cell wall (Nicholas et al., 2008),
377 or due to involvement of multiple *Mycoplasma* species in a single case of mastitis that may
378 have affected the growth requirements of each individual *Mycoplasma* colony. However,
379 approximately 7% of positive samples were identified by culture, but not by PCR methods. This
380 may be attributed to failure of DNA amplification due to existing inhibitors in milk samples or
381 due to failure of the developed universal 16S rRNA PCR to detect some of the species.

382 Although culture-based methods is still considered as a gold standard in the detection of
383 *Mycoplasma* infection (D'Inzeo et al., 2017), the specificity of this test particularly for various
384 genera and species of mollicutes is challenging. Morphologically, detected colonies which grew
385 on the specialised media were characterised by the typical fried egg appearance. However,

discrimination between different mollicutes genus and species using culture alone was not possible, *i.e.* morphology and sizes of all detected colonies appeared to be similar for most of the identified species. Indistinguishable *Mycoplasma* and *Acholeplasma* colonies has also been observed previously (Boonyayatra et al., 2012). Hence, these authors developed biochemical and molecular differentiation techniques. In our study, we have confirmed the different species using PCR/sequencing tests but not using the biochemical properties.

Conclusion

In conclusion, our newly developed universal PCR of 16S rRNA showed significant sensitivity to detect various *Mycoplasma* and *Acholeplasma* at genus-level in milk. Direct extraction of DNA from milk for detection of mycoplasmas can save time and money. Consequently, implementation of our methodology may be a cornerstone for further surveys at cow, farm, regional and state level by providing a rapid, reliable and accurate method to identify milk *Mycoplasma* and *Acholeplasma* spp. for farmers and laboratory staff.

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408 **References**

409 AL-FARHA, A., HEMMATZADEH, F., KHAZANDI, M., HOARE, A. & PETROVSKI, K.
410 2017. Evaluation of effects of Mycoplasma mastitis on milk composition in dairy cattle
411 from South Australia. *BMC Veterinary Research,* 13, 351.
412 BASHIRUDDIN, J. B., FREY, J., KONIGSSON, M. H., JOHANSSON, K. E., HOTZEL, H.,
413 DILLER, R., DE SANTIS, P., BOTELHO, A., AYLING, R. D., NICHOLAS, R. A.,
414 THIAUCOURT, F. & SACHSE, K. 2005. Evaluation of PCR systems for the
415 identification and differentiation of Mycoplasma agalactiae and Mycoplasma bovis: a
416 collaborative trial. *Vet J,* 169**,** 268-75.
417 BOONYAYATRA, S., FOX, L. K., GAY, J. M., SAWANT, A. & BESSER, T. E. 2012.
418 Discrimination between Mycoplasma and Acholeplasma species of bovine origin using
419 digitonin disc diffusion assay, nisin disc diffusion assay, and conventional polymerase
420 chain reaction. *J Vet Diagn Invest,* 24**,** 7-13.
421 D'INZEO, T., DE ANGELIS, G., FIORI, B., MENCHINELLI, G., LIOTTI, F. M.,
422 MORANDOTTI, G. A., DE MAIO, F., NAGEL, D., ANTONACI, M. &
423 SANGUINETTI, M. 2017. Comparison of Mycoplasma IES, Mycofast Revolution and
424 Mycoplasma IST2 to detect genital mycoplasmas in clinical samples. *The Journal of*
425 *Infection in Developing Countries,* 11**,** 98-101.
426 FOX, L. K. 2012. Mycoplasma mastitis: causes, transmission, and control. *Vet Clin North Am*
427 *Food Anim Pract,* 28**,** 225-37.
428 GEORGE, L. W., DIVERS, T. J., DUCHARME, N. & WELCOME, F. L. 2007. Diseases of
429 the Teats and Udder. *Rebhun's diseases of dairy cattle.* Second Edition ed.: Elsevier
430 Health Sciences.
431 GIOIA, G., WERNER, B., NYDAM, D. V. & MORONI, P. 2016. Validation of a mycoplasma
432 molecular diagnostic test and distribution of mycoplasma species in bovine milk among
433 New York State dairy farms. J Dairy Sci, 99, 4668-4677.
434 GONZÁLEZ, R. N. & WILSON, D. J. 2003. Mycoplasmal mastitis in dairy herds. *Veterinary*
435 *Clinics of North America: Food Animal Practice,* 19**,** 199-221.
436 HOTZEL, H., DEMUTH, B., SACHSE, K., PFLITSCH, A. & PFUTZNER, H. 1993. Detection
437 of Mycoplasma bovis using in vitro deoxyribonucleic acid amplification. *Rev Sci Tech,*
438 12**,** 581-91.
439 HOTZEL, H., FREY, J., BASHIRUDDIN, J. & SACHSE, K. 2003. Detection and
440 differentiation of ruminant mycoplasmas. *PCR Detection of Microbial Pathogens***,** 231-
441 245.
442 JASPER, D., DELLINGER, J., ROLLINS, M. & HAKANSON, H. 1979. Prevalence of
443 mycoplasmal bovine mastitis in California. *American journal of veterinary research,*
444 40**,** 1043-1047.
445 JASPER, D. E. 1982. The role of Mycoplasma in bovine mastitis. *J Am Vet Med Assoc,* 181**,**
446 158-62.
447 JUSTICE-ALLEN, A., TRUJILLO, J., CORBETT, R., HARDING, R., GOODELL, G. &
448 WILSON, D. 2010. Survival and replication of Mycoplasma species in recycled
449 bedding sand and association with mastitis on dairy farms in Utah. *J Dairy Sci,* 93**,** 192-
450 202.
451 KIRK, J. H., GLENN, K., RUIZ, L. & SMITH, E. 1997. Epidemiologic analysis of
452 Mycoplasma spp isolated from bulk-tank milk samples obtained from dairy herds that
453 were members of a milk cooperative. *J Am Vet Med Assoc,* 211**,** 1036-8.
454 KOBAYASHI, H., HIROSE, K., WORARACH, A., PAUGTES, P., ITO, N., MOROZUMI, T.
455 & YAMAMOTO, K. 1998. In vitro amplification of the 16S rRNA genes from

456 Mycoplasma bovirhinis, Mycoplasma alkalescens and Mycoplasma bovigenitalium by
457 PCR. *J Vet Med Sci,* 60**,** 1299-303.
458 LARKIN, M. A., BLACKSHIELDS, G., BROWN, N., CHENNA, R., MCGETTIGAN, P. A.,
459 MCWILLIAM, H., VALENTIN, F., WALLACE, I. M., WILM, A. & LOPEZ, R. 2007.
460 Clustal W and Clustal X version 2.0. *bioinformatics,* 23**,** 2947-2948.
461 LORENZ, T. C. 2012. Polymerase chain reaction: basic protocol plus troubleshooting and
462 optimization strategies. Journal of visualized experiments: JoVE.
463 LYSNYANSKY, I. & AYLING, R. D. 2016. Mycoplasma bovis: Mechanisms of Resistance
464 and Trends in Antimicrobial Susceptibility. *Front Microbiol,* 7**,** 595.
465 MARKEY, B., LEONARD, F., ARCHAMBAULT, M., CULLINANE, A. & MAGUIRE, D.
466 2013. *Clinical veterinary microbiology*, Elsevier Health Sciences.
467 MAUNSELL, F. P., WOOLUMS, A. R., FRANCOZ, D., ROSENBUSCH, R. F., STEP, D. L.,
468 WILSON, D. J. & JANZEN, E. D. 2011. Mycoplasma bovis infections in cattle. *J Vet*
469 *Intern Med,* 25**,** 772-83.
470 NICHOLAS, R., AYLING, R. & MCAULIFFE, L. 2008. *Mycoplasma diseases of ruminants*,
471 CABI.
472 PARKER, A. M., HOUSE, J. K., HAZELTON, M. S., BOSWARD, K. L. & SHEEHY, P. A.
473 2017b. Comparison of culture and a multiplex probe PCR for identifying Mycoplasma
474 species in bovine milk, semen and swab samples. PloS one, 12, e0173422.
475 PEREDELTCHOUK, M., DAVID, S. A., BHATTACHARYA, B., VOLOKHOV, D. V. &
476 CHIZHIKOV, V. 2011. Detection of mycoplasma contamination in cell substrates using
477 reverse transcription-PCR assays. *J Appl Microbiol,* 110**,** 54-60.
478 PFUTZNER, H. & SACHSE, K. 1996. Mycoplasma bovis as an agent of mastitis, pneumonia,
479 arthritis and genital disorders in cattle. *Rev Sci Tech,* 15**,** 1477-94.
480 PINNOW, C. C., BUTLER, J. A., SACHSE, K., HOTZEL, H., TIMMS, L. L. &
481 ROSENBUSCH, R. F. 2001. Detection of Mycoplasma bovis in preservative-treated
482 field milk samples. *J Dairy Sci,* 84**,** 1640-5.
483 RADAELLI, E., CASTIGLIONI, V., LOSA, M., BENEDETTI, V., PICCININI, R.,
484 NICHOLAS, R. A., SCANZIANI, E. & LUINI, M. 2011. Outbreak of bovine clinical
485 mastitis caused by Mycoplasma bovis in a North Italian herd. *Res Vet Sci,* 91**,** 251-3.
486 SCHNEE, C., SCHULSSE, S., HOTZEL, H., AYLING, R. D., NICHOLAS, R. A.,
487 SCHUBERT, E., HELLER, M., EHRICHT, R. & SACHSE, K. 2012. A novel rapid
488 DNA microarray assay enables identification of 37 Mycoplasma species and highlights
489 multiple Mycoplasma infections. *PLoS One,* 7**,** e33237.
490 SIEVERS, F. & HIGGINS, D. G. 2014. Clustal omega. Current protocols in bioinformatics,
491 48, 3.13. 1-3.13. 16.
492 SRINIVASAN, R., KARAOZ, U., VOLEGOVA, M., MACKICHAN, J., KATO-MAEDA, M.,
493 MILLER, S., NADARAJAN, R., BRODIE, E. L. & LYNCH, S. V. 2015. Use of 16S
494 rRNA gene for identification of a broad range of clinically relevant bacterial pathogens.
495 *PloS one,* 10**,** e0117617.
496 SULYOK, K. M., KREIZINGER, Z., WEHMANN, E., LYSNYANSKY, I., BANYAI, K.,
497 MARTON, S., JERZSELE, A., RONAI, Z., TURCSANYI, I., MAKRAI, L., JANOSI,
498 S., NAGY, S. A. & GYURANECZ, M. 2017. Mutations Associated with Decreased
499 Susceptibility to Seven Antimicrobial Families in Field and Laboratory-Derived
500 Mycoplasma bovis Strains. *Antimicrob Agents Chemother,* 61.
501 SZACAWA, E., NIEMCZUK, K., DUDEK, K., BEDNAREK, D., ROSALES, R. & AYLING,
502 R. 2015. Mycoplasma bovis infections and co-infections with other Mycoplasma spp.
503 with different clinical manifestations in affected cattle herds in eastern region of Poland.
504 *Bulletin of the Veterinary Institute in Pulawy,* 59**,** 331-338.

505 WATERS, A. & MCCUTHAN, T. 1990. Ribosomal RNA: nature's own polymerase-amplified
506 target for diagnosis. *Parasitology Today,* 6**,** 56-59.
507 WATTS, J. L. 1988. Etiological agents of bovine mastitis. *Veterinary microbiology,* 16**,** 41-66.
508 WINDSOR, H. M., WINDSOR, G. D. & NOORDERGRAAF, J. 2010. The growth and long
509 term survival of Acholeplasma laidlawii in media products used in biopharmaceutical
510 manufacturing. *Biologicals,* 38**,** 204-210.

511

512

513 *Table 2-1. Count of samples positive for detected mollicutes using conventional culture method,*
514 *universal PCR and species-specific PCR.*

Test	Positive	Negative	Percent +ve
Conventional culture Method	**192**	**176**	**52%**
Universal PCR	**269**	**99**	**73%**
Species-specific PCR	**256**	**112**	**70%**

515

516

517

518 *Table 2-2. Concordance between three detecting tests for identification of Mycoplasma and*
519 *Acholeplasma spp. from 368 bovine milk samples from a single farm in South Australia*

Tests	Concordant	discordant	Percent concordant	Cohen's Kappa (95% CE)	Concordance
Culture vs. Universal PCR	**225**	**143**	**61%**	**0.298 ± 0.049**	**Fair**
Culture vs. Species-specific PCR	**212**	**156**	**58%**	**0.213 ± 0.048**	**Fair**
Universal PCR vs. Species-specific PCR	**313**	**55**	**85%**	**0.747 ± 0.031**	**Good**

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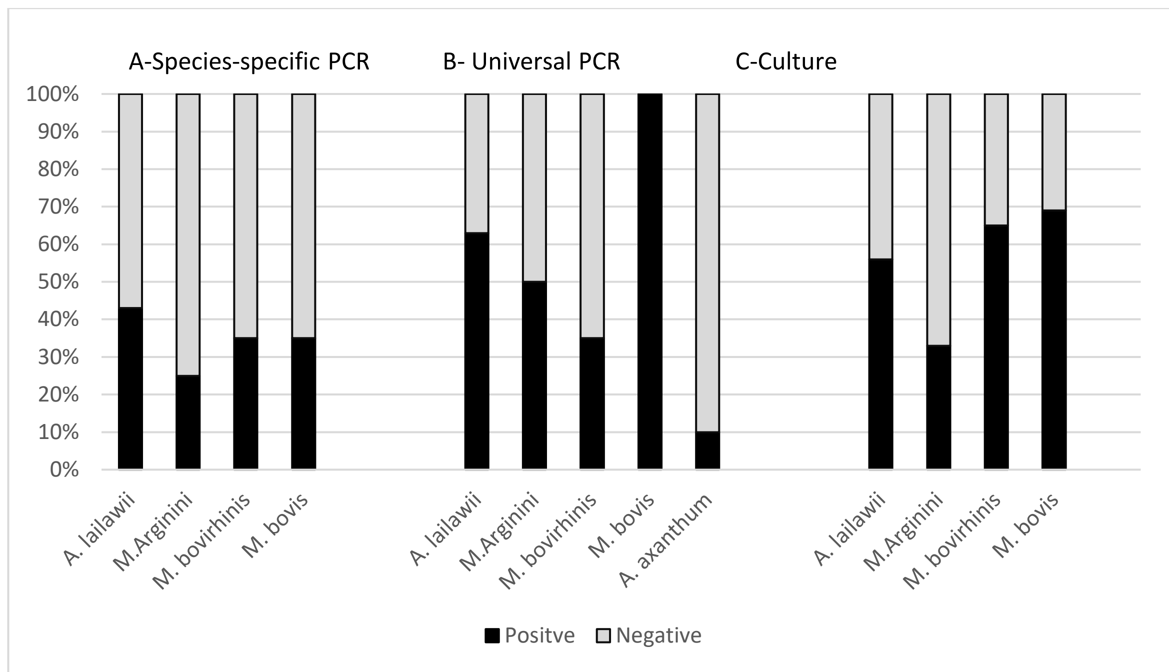

523 *Figure 2-1. Percentage of positive/negative samples detected by species-specific PCR,*
524 *universal PCR and culture for each species from 368 bovine milk samples from a single farm*
525 *in South Australia*

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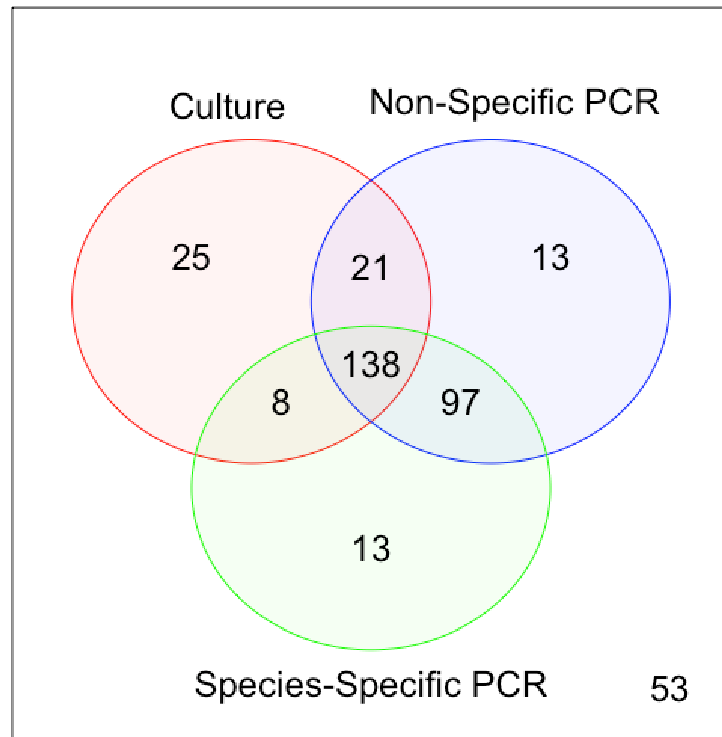

529

530 *Figure 2-2 Venn diagram of the positive and negative samples of the three detection methods*
531 *from 368 bovine milk samples from a single farm in South Australia*

532

533

Chapter 3 Discrimination between some *Mycoplasma* spp. and *Acholeplasma laidlawii* in bovine milk using high resolution melting curve analysis

Aim: This study aimed to provide a rapid, accurate and cost-effective diagnostic real time polymerase chain reaction-high resolution melting curve assay (PCR-HRM) to identify and distinguish between four different mycoplasmas *and Acholeplasma laidlawii* isolated at cow-level from a single commercial dairy farm in South Australia.

Null Hypothesis: real time PCR-HRM analysis is unable to discriminate between mycoplasmas and *A. laidlawii*

Note: 1-Raw data presented in Appendices 1 and 3.

2- Identification of *Mycoplasma bovis* was carried out by axenization as stated in Chapter 2

3- Primers design was clarified in Chapter 2

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Statement of Authorship

Title of Paper	Discrimination between some *Mycoplasma* spp. and *Acholeplasma laidlawii* in bovine milk using high resolution melting curve analysis
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Name of Principal Author (Candidate)	Abd Al-Bar Ahmed Noori Al-Farha		
Contribution to the Paper	Assisted in conceptualisation of the study, designed the study, prepared the samples, performed real time-HRM, analysed data, wrote the manuscript, and acted as a corresponding author.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	9/5/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kiro Petrovski		
Contribution to the Paper	Supervised the experiment, assisted in the conceptualisation of the study, and the study design, edited and approved the manuscript.		
Signature		Date	02 May 18

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Name of Co-Author	Razi Jozani		
Contribution to the Paper	Designed the universal primer, edited and approved the manuscript.		
Signature		Date	9/5/18

Name of Co-Author	Andrew Hoare		
Contribution to the Paper	Assisted in sampling, herd test data collection and coordination with the farm.		
Signature		Date	7/5/18

Name of Co-Author	Farhid Hemmatzadeh		
Contribution to the Paper	Supervised the experiment, assisted in conceptualisation of the study, contributed in study design, edited and approved the manuscript.		
Signature		Date	09.05.18

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BMC Research Notes

RESEARCH NOTE

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Discrimination between some *Mycoplasma* spp. and *Acholeplasma laidlawii* in bovine milk using high resolution melting curve analysis

Abd Al-Bar Al-Farha[1,2*] , Kiro Petrovski[1,3,4], Razi Jozani[5], Andrew Hoare[6] and Farhid Hemmatzadeh[1,3]

Abstract

Objectives: This study aimed to provide a rapid, accurate and cost-effective diagnostic real time polymerase chain reaction-high resolution melting curve assay (PCR-HRM) to identify and distinguish between four different mycoplasmas and *Acholeplasma laidlawii* isolated at cow-level from a single commercial dairy farm in South Australia. One set of genus-level universal primers was designed targeting the 16S ribosomal RNA gene.

Results: Real time PCR-HRM analysis was able to identify and distinguish between five different mollicutes, namely *A. laidlawii*, *M. arginini*, *M. bovirhinis*, *M. bovis* and uncultured *Mycoplasma*. Results were confirmed through sequencing. Our developed assay provides rapid and accurate screening for *Mycoplasma* mastitis detection.

Keywords: *Mycoplasma*, Mastitis, Cattle, Milk, *Acholeplasma*

Introduction

Mycoplasma mastitis is of emerging significance worldwide, posing significant economic impacts on the dairy industry. Early detection of *Mycoplasma* mastitis is important to disease control strategies [1]. Several *Mycoplasma* spp. are mainly responsible for mastitis, including *M. bovis*, *M. bovoculi*, *M. alkalescence*, *M. alvi*, *M. bovigenetalium*, *M. bovirhinis*, *Mycoplasma species bovine group 7*, *M. californicum*, *M. dispar*, *M. canis*, *M. verecundum*, *M. canadense* and *M. mycoides* subsp. *mycoides* [2]. *Acholeplasma* spp. may be isolated from milk either as a contaminant [3] or as a co-invader with other mycoplasmas [4, 5]. Conventional microbial culture of mollicutes can be laborious and time-consuming with a variety of species-specific growth requirements [6]. Misdiagnosis of *Mycoplasma* using serological detection is common due to the lag period required for antibody formation. Therefore, a rapid and accurate diagnostic assay is required for screening of mycoplasma in dairy herds. High resolution melting curve analysis (HRM) has been recently developed and widely used for phenotyping at strain or species-level of various organisms including mycoplasmas [7, 8]. However, field isolates of mastitis related mycoplasmas and other milk environmental mollicutes have not been assessed previously using this method.

The aim of this study was to provide a suitable diagnostic real time polymerase chain reaction-high resolution melting curve analysis (PCR-HRM) to identify and distinguish between five different mollicutes isolated at cow-level from a single commercial dairy farms in South Australia.

Main text

Methods

Samples were selected based on conventional PCR findings of a previous study conducted on single commercial dairy farm in South Australia. This farm had a history of repeated mastitis treatment failure with high somatic cell

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count (SCC) and poor response to antimicrobials [4]. Six isolates for each of the following species were selected in this study: *A. laidlawii*, *M. arginini*, *M. bovirhinis*, *M. bovis* and uncultured mollicutes. One set of genus-level universal primers targeting the 16S rRNA gene was designed for real time-PCR. Forward primer Mol-F: GGC GAAYGGGTGAGTAACAC and reverse primer Mol-R: CATHGYCTTGGTRRGCYNTTA. The real time PCR mixture was prepared using HRM kit AccuMelt HRM SuperMix (Quantabio, Australia). DNA amplification was conducted in a 96 microplate (Illumina, San Diego, CA, USA). Each well contained 10 µL reaction solution of 5 µL HRM SuperMix, 1 µL DNA template (approximately 20 ng), 1 µL each primer (0.2 nmol) and 2 µL nuclease free water (Qiagen, Germany). The reaction was conducted using an Illumina Thermal Cycler with preheating activation for 2 min followed by 40 PCR cycles of three steps: denaturation at 95 °C for 15 s, annealing at 60 °C for 45 s, then extension at 72 °C for 15 s. HRM was performed at 55–95 °C at the rate of 0.1 °C. Results were analysed via EcoStudy software (version 5.0, Illumina). PCR products were subject to electrophoresis in 1.5% agarose gels and visualised by staining with Gel Red. PCR products from the 16S rRNA gene were submitted to the Australian Genome Research Facility Ltd (AGRF, Adelaide, South Australia) for Sanger sequencing. Each fragment was sequenced in forward and reverse directions. To reconstitute the sequence, forward and reverse sequences were edited and assembled using BioEdit package v.7.0.4.1. Edited sequences were blasted against existing sequences in GeneBank using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences from relevant *Mycoplasma* strains were used as reference strains for nucleotide alignments using ClustalW program version 2.

Results

Five different mollicutes, *A. laidlawii, M. arginini, M. bovirhinis, M. bovis* and uncultured mollicutes, produced normalised and derivative melt curves (Fig. 1). *A. laidlawii* (Accession No. LC201977.1) generated one melting peak at 81.2 °C, *M. arginini* (Accession No. LC158832.1) generated two melting peaks at 88.5 and 84.7 °C. *M. bovirhinis* (Accession No. AP018135.1) generated three melting peaks at 85.7, 77.6 and 88.2 °C. *M. bovis* (Accession No. KX462439.1) generated two melting peaks at 77.6 and 85.2 °C. Uncultured *Mycoplasma* spp. (Accession No. LT679634.1) generated one melting peak at 83.9 °C.

Discussion

While conventional culture, the traditional method for mollicute detection, imposes technical challenges in distinguishing between milk pathogenic and saprophytic mollicutes, our study indicated that real time PCR-HRM assay provides a sensitive, rapid and cost-effective screening method to identify and discriminate between

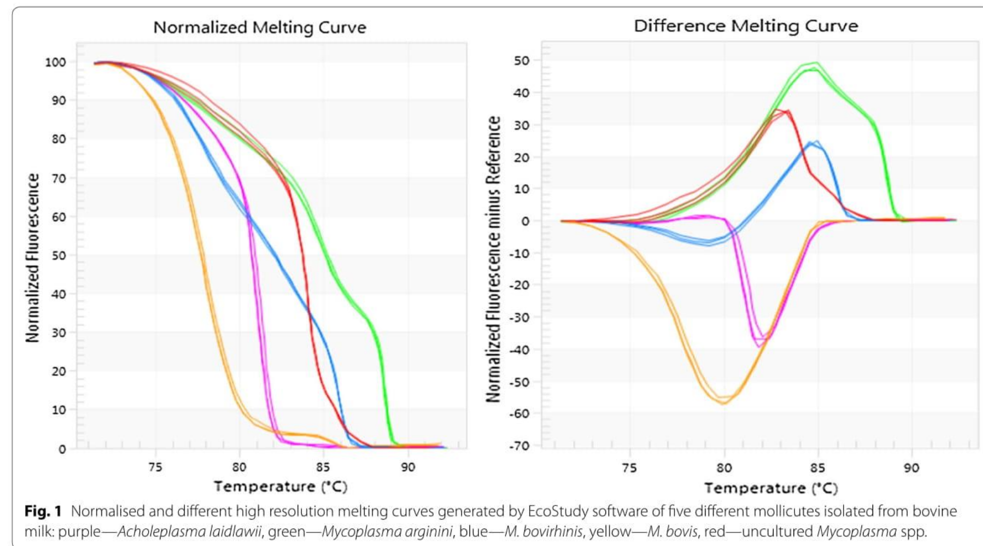

Fig. 1 Normalised and different high resolution melting curves generated by EcoStudy software of five different mollicutes isolated from bovine milk: purple—*Acholeplasma laidlawii*, green—*Mycoplasma arginini*, blue—*M. bovirhinis*, yellow—*M. bovis*, red—uncultured *Mycoplasma* spp.

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some pathogenic and environmental mollicutes isolated from milk DNA. Effects of some of these mollicutes on SCC and milk composition have been previously studied [4]. We considered SCC as the crucial factor that discriminates between contagious and environmental mollicutes. *M. bovis* was widely reported as a main mastitis causing *Mycoplasma* [9, 10]. Inconsistent results have been reported regarding involvement of *M. arginini, M. bovirhinis* and *A. laidlawii* in bovine mastitis, particularly with co-infection *Mycoplasma* mastitis [5, 11, 12]. However, several studies indicate these mollicutes are not significant pathogens [3, 13].

Melting profile, introduced in 2002, is widely used for genotyping a wide range of microorganisms [14–17]. HRM-based assay describes correlation between temperature and DNA extent of denaturation [18]. The variety of melting temperatures for different species is attributed to DNA length, sequencing and GC content [14]. In summary, as an alternative to sequencing, our developed real time PCR-HRM assay offered a rapid, low-cost and simple discriminative method to distinguish between some mastitis causing pathogenic mycoplasmas and other saprophytic mollicutes in bovine milk. This method was useful for screening of *Mycoplasma* mastitis and can be extended to identify more mollicutes species.

Limitations

One of the limitations of HRM-based analysis in *Mycoplasma* mastitis detection is the inability to detect co-infection cases due to amplimer concentration differences and the requirement of separation each individual amplicon Tm [18]. Primers used in this study were designed to target more spp. of major *Mycoplasma*-causing mastitis in dairy herds. However, in this study, we used only field isolates of five different mollicutes.

Abbreviations

HRM: high resolution melt; PCR: polymerase chain reaction; SCC: somatic cell count.

Authors' contributions

AAA, FH and KP participated in the study design and coordination. AAA, KP and AH contributed to sample collection. AAA and FH contributed in sample processing and real time PCR in the PC2 laboratory. RJ designed the primers for HRM analysis All authors were involved in drafting the manuscript, corrected. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent to publish

Not applicable.

Ethics approval and consent to participate

Not applicable. Samples were collected by field veterinary support as a part of the mastitis investigation as per farmer request and no Animal Ethics application was required (Australian code for the care and use of animals for scientific purposes, 8th edition, 2013).

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References

1. Kirk JH, Lauerman LH, Roberts C. *Mycoplasma* mastitis in dairy cows. Compend Contin Educ Pract Vet. 1994;16(4):541–52.
2. Nicholas R, Ayling R, McAuliffe L. *Mycoplasma* mastitis. Vet Rec. 2007;160(11):382 **(author reply 383)**.
3. Boonyayatra S, Fox LK, Gay JM, Sawant A, Besser TE. Discrimination between *Mycoplasma* and *Acholeplasma* species of bovine origin using digitonin disc diffusion assay, nisin disc diffusion assay, and conventional polymerase chain reaction. J Vet Diagn Investig. 2012;24(1):7–13.
4. Al-Farha AAB, Hemmatzadeh F, Khazandi M, Hoare A, Petrovski K. Evaluation of effects of *Mycoplasma* mastitis on milk composition in dairy cattle from South Australia. BMC Vet Res. 2017;13(1):351.
5. Counter DE. A severe outbreak of bovine mastitis associated with *Mycoplasma bovigenitalium* and *Acholeplasma laidlawii*. Vet Rec. 1978;103(7):130–1.
6. Hahn RG, Kenny GE. Differences in arginine requirement for growth among arginine-utilizing *Mycoplasma* species. J Bacteriol. 1974;117(2):611–8.
7. Ghorashi SA, Noormohammadi AH, Markham PF. Differentiation of *Mycoplasma gallisepticum* strains using PCR and high-resolution melting curve analysis. Microbiology. 2010;156(Pt 4):1019–29.
8. Rebelo AR, Parker L, Cai HY. Use of high-resolution melting curve analysis to identify *Mycoplasma* species commonly isolated from ruminant, avian, and canine samples. J Vet Diagn Investig. 2011;23(5):932–6.
9. Aebi M, van den Borne BH, Raemy A, Steiner A, Pilo P, Bodmer M. *Mycoplasma bovis* infections in Swiss dairy cattle: a clinical investigation. Acta Vet Scand. 2015;57:10.
10. Kunkel JR. Isolation of *Mycoplasma bovis* from bulk milk. Cornell Vet. 1985;75(3):398–400.
11. Szacawa E, Niemczuk K, Dudek K, Bednarek D, Rosales R, Ayling R. *Mycoplasma bovis* infections and co-infections with other *Mycoplasma* spp. with different clinical manifestations in affected cattle herds in eastern region of Poland. Bull Vet Inst Pulawy. 2015;59(3):331–8.
12. González RN, Wilson DJ. Mycoplasmal mastitis in dairy herds. Vet Clin N Am Food Anim Pract. 2003;19(1):199–221.
13. Jasper D, Dellinger J, Rollins M, Hakanson H. Prevalence of mycoplasmal bovine mastitis in California. Am J Vet Res. 1979;40(7):1043–7.

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14. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics. 2007;8(6):597.
15. Ren X, Fu Y, Xu C, Feng Z, Li M, Zhang L, Zhang J, Liao M. High resolution melting (HRM) analysis as a new tool for rapid identification of *Salmonella enterica* serovar *Gallinarum* biovars *Pullorum* and *Gallinarum*. Poult Sci. 2017;96(5):1088–93.
16. Yong TB, Hashim R, Noor AM, Hamzah SH, Ahmad N. Identification of *Brucella* spp. isolated from human brucellosis in Malaysia using high-resolution melt (HRM) analysis. Diagn Microbiol Infect Dis. 2015;81(4):227–33.
17. Gago S, Zaragoza Ó, Cuesta I, Rodríguez-Tudela JL, Cuenca-Estrella M, Buitrago MJ. High-resolution melting analysis for identification of the *Cryptococcus neoformans–Cryptococcus gattii* complex. J Clin Microbiol. 2011;49(10):3663–6.
18. Tong SY, Giffard PM. Microbiological applications of high-resolution melting analysis. J Clin Microbiol. 2012;50(11):3418–21.

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567 **Chapter 4: Phylogenetic relationship among field isolates of mycoplasmas and**
568 **acholeplasmas in two South Australian dairy herds based on sequencing of a short 16S**
569 **rRNA gene fragment**

570 **Aim:** to determine the genotype distribution of *Mycoplasma* spp and other milk mollicutes
571 isolated from two commercial dairy farms in South Australia, and compare their evolutionary
572 relationship to isolates collected elsewhere.

573 **Null Hypothesis:** There is no correlation between mollicutes isolated in different South
574 Australian dairy herds and those isolated elsewhere.

575 **Note**: Raw data available in Appendix; Accession numbers available online
576 http://www.ncbi.nlm.nih.gov

577

578 ## Statement of Authorship

Title of Paper	**Phylogenetic relationship among field isolates of mycoplasmas and acholeplasmas in two South Australian dairy herds based on sequencing of a short 16S rRNA gene fragment**
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	09/05/2018

580 ## Co-Author Contributions

581 By signing the Statement of Authorship, each author certifies that:

582 i. the candidate's stated contribution to the publication is accurate (as detailed above);

583 ii. permission is granted for the candidate in include the publication in the thesis; and

584 iii. the sum of all co-author contributions is equal to 100% less the candidate's stated
585 contribution.

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Contribution to the Paper	Assisted in the conceptualisation of the study and the study design, supervised, edited and approved the manuscript.		
Signature		Date	9 May 18

Name of Co-Author	Jamie Moffat		
Contribution to the Paper	Assisted with collection of samples, edited and approved the manuscript.		
Signature		Date	9 / 5 / 18

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Contribution to the Paper	Contributed in the study design, analysed data, edited and approved the manuscript.		
Signature		Date	2, 5, 2018

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Contribution to the Paper	Contributed in the study design, sampling, edited and approved the manuscript.		
Signature		Date	2/05/2018

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Contribution to the Paper	Designed the 16S rRNA primers, edited and approved the manuscript.		
Signature		Date	9/5/18

Name of Co-Author	Eman Taher		
Contribution to the Paper	Involved in the PCR and gel electrophoresis, edited and approved the manuscript.		
Signature		Date	9-5-2018

Name of Co-Author	Andrew Hoare		
Contribution to the Paper	Contributed in sample herd test data collection, coordination with the farm, edited and approved the manuscript.		
Signature		Date	7/5/18

587

Name of Co-Author	Farhid Hemmatzadeh		
Contribution to the Paper	Assisted in the conceptualisation of the study and the study design, supervised the whole experiment sequences alignment, edited and approved the manuscript.		
Signature		Date	09.05.18

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591 ***Short communication***

592 **Phylogenetic relationship among field isolates of mycoplasmas and acholeplasmas in two**
593 **South Australian dairy herds based on sequencing of a short 16S rRNA gene fragment**

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610 **Abstract**

611 *Mycoplasma* mastitis has been recognised as an emerging disease with a significant impact on
612 the dairy industry. This study aimed to determine the genotypic distribution of some
613 *Mycoplasma* and *Acoleplasma* spp isolated from two commercial dairy farms in South Australia
614 based on 16S rRNA sequencing, and compare their evolutionary relationship to some similar
615 isolates from elsewhere. Neighbour-joining phylogeny of 16S rRNA demonstrated involvement
616 of four different spp. isolated from Farm 1 including*: M. bovis*, *M. bovirhinis*, *Acholeplasma*
617 *laidlawii* and *A. axanthum* while *M. alkalescens* was isolated from Farm 2. Two strains of *M.*
618 *bovis* showed similarity to Egyptian, Hungarian and Chinese strains. *M. bovirhinis* clustered
619 with the Egyptian group. *Mycoplasma alkalescens* grouped with Swedish and Japanese strains.
620 The *Acholeplasma* group showed two distinct clusters of *A. laidlawii* and *A. axanthum*.
621 Determination of the species/genera involved in mastitis may enhance the molecular
622 epidemiology and in-turn, can contribute to control strategies of the disease.

623 Keywords: *Mycoplasma, Acholeplasma*, mastitis, phylogeny, 16S rRNA, cattle

624 **Introduction**

625 Mastitis caused by *Mycoplasma* spp is among the emerging challenges facing the dairy industry
626 worldwide, resulting in significant economic consequences. Similarly to the more common
627 mastitis pathogens, *Mycoplasma* mastitis can affect milk quantity and quality (Al-Farha et al.,
628 2017, Pothmann et al., 2015, Aebi et al., 2012). More than 200 spp. of mycoplasmas,
629 acholeplasmas and other related mollicutes have been discovered (Nicholas et al., 2008).
630 Several of these mollicutes are of great concern in the dairy industry including *M. bovis, M.*
631 *bovigenetalium, M. alkalescens, M. californium,* and *M. canadense.* Other isolates that are less
632 frequently involved in bovine mastitis include: *M. arginini, M bovirhinis, M. leachii,* and *M.*
633 *dispar* (Parker et al., 2018). In addition, *A. laidlawii* has been reported to be less significant

634 (Nicholas et al., 2008) or of equal significance (Al-Farha et al., 2017, Counter, 1978a) to
635 mastitis cases caused by mycoplasmas. Determining the species and strains involved should
636 contribute to diagnostic and control strategies, in addition to providing better understanding of
637 the epidemiology of *Mycoplasma* mastitis in Australian dairy herds. The usefulness of 16S
638 rRNA sequencing, due to the high copy numbers of sequenced data, has been proven and it is
639 an efficacious, discriminatory, sensitive and accurate tool for studying the epidemiology of
640 pathogens (van Kuppeveld et al., 1994a). Copy numbers of some mollicutes have been
641 estimated for the 16S rRNA gene to vary between 400 and 2000 (Peredeltchouk et al., 2011).

642 Although the genotyping of Australian strains of *M. bovis* as the major pathogenic *Mycoplasma*
643 in cattle has been reported recently (Parker et al., 2016), other mastitis causing mycoplasmas
644 and non-pathogenic milk mollicutes are yet to be studied. Moreover, co-infection of
645 mycoplasmas and *Acholeplasma laidlawii* has been recently reported to cause similar mastitis
646 to other major mastitis pathogens (Al-Farha et al., 2017). This study aimed to determine the
647 genotype distribution of *Mycoplasma* spp and other milk mollicutes isolated from two
648 commercial dairy farms in South Australia, and compare their evolutionary relationship to
649 isolates collected elsewhere.

650 **Materials and Methods**

651 Milk samples originated from two commercial dairy farms from the South East and Mid North
652 regions of South Australia. A total of 368 milk samples were collected from Farm 1. Of them,
653 one representative sample for each individual identified species was selected based on
654 sequencing results (Al-Farha et al., 2017). The remaining 40 milk samples were collected from
655 Farm 2. One sample of *M. alkalescens* isolated from Farm 2 was selected based on sequencing
656 and 16S rRNA universal primer results (Appendix 2). All samples were positive for
657 *Mycoplasma* on conventional culture. Axenization of these bacteria was carried out (see

658 Chapter 2). DNA was extracted directly from milk samples using DNA extraction kit (Qiagene,
659 Germany). One set of universal primers included Mol-F: 5'-
660 GGCGAAYGGGTGAGTAACAC-3' and reverse primer Mol-R: 5'-
661 CATHGYCTTGGTRRGCYNTTA-3' (see Chapter 2). We have developed HRM analysis and
662 qPCR assays to cover the variable parts of the 16S rRNA from mollicutes with the conserved
663 primer binding sites. The PCR product is a 180 nucleotide and our submitted sequence for
664 *Acholeplasma* has >700bp. We have amplified short part of the sequence to compare wider
665 ranges of mollicutes; i.e., the primers and the sequences have been extracted from submitted
666 sequences with different lengths but the assay has run on short 180bp sequence for test
667 development and screening the archive. Real time PCR-HRM analysis was performed to
668 discriminate between some of these isolates using two pairs of genus level universal primers
669 targeting 16S rRNA (Al-Farha et al., 2018b). Based on their HRM profile, one representative
670 sample from each different profile was selected for 16S rRNA sequencing. Subsequently, DNA
671 of the specific band (bp=180) was purified from gel using (Qiagene, Germany) for sanger
672 sequencing. Sanger sequencing was performed at the Australian Genome Research Facility
673 (AGRF, Adelaide, Australia). Assembling of sequences was performed using (ClustalX, 2.1)
674 and compared with selected closest neighbours references identified by NCBI
675 (www.ncbi.nlm.nih.gov) (Appendix 2). Evolutionary analyses at cluster level were conducted
676 in MEGA7 (Molecular Evolutionary Genetic Analysis, version 7.0). The evolutionary history
677 at cluster level was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The
678 optimal tree with the sum of branch length = 1.97698025 is shown. To build a reliable tree,
679 huge length sequences were trimmed. The tree is drawn to scale, with branch lengths in the
680 same units as those of the evolutionary distances used to infer the phylogenetic tree. The
681 evolutionary distances were computed using the Poisson correction method and are in the units
682 of the number of amino acid substitutions per site. All ambiguous positions were removed for

683 each sequence pair. There were a total of 1738 positions in the final dataset. Evolutionary

684 analyses were conducted in MEGA7 (Kumar et al., 2016).

685 **Results and discussion**

686 In this study, six different field isolates of mycoplasmas and acholeplasmas have clustered in

687 three distinct groups (Appendix 2). The *Acholeplasma* group compromises two distinct clusters

688 of field isolates of acholeplasmas. First, *A. laidlawii* (MH259813.1) showed 100% similarity to

689 other bovine strains of this spp. isolated in The United States and South Korea (JN935887.1,

690 NR074448.2 and LC201977.1), and were clearly distinct from other environmental *A. laidlawii*

691 (FJ226559.1, J65556.1 and JN935888) presented in Figure 1. The presence of *A. laidlawii* in

692 milk samples from mastitis cows remains a controversial subject however, with some authors

693 considering it as pathogenic and others suggesting that it acts as an environmental milk

694 saprophyte (Boonyayatra et al., 2012, Counter, 1978b, Seffner et al., 1983, Singh et al., 1990,

695 Kirk et al., 1997, Al-Farha et al., 2017b). Second, *A. axanthum* (MH259849.1) showed 99%

696 similarity to US (FJ876270.1) and Swedish (NR 028827.1) strains. *Acholepllasma axanthum*

697 was firstly reported in 1970 (Tully and Razin, 1970), and its pathogenicity in pneumonic swine

698 has been studied (Stipkovits et al., 1974). *A. axanthum* has been isolated from mastitic milk

699 (Roy et al., 2008, Gonzalez and Wilson, 2003). However, its role in mastitis is still unknown.

700 The effects of some of the aforementioned mollicutes on milk quality and quantity have already

701 been testified (Al-Farha et al., 2017b). Co-infection with more than one species has severe

702 consequences on milk compositions, similar to major conventional mastitis pathogens.

703 Studying the correlation between genetic, observational and pathological effects of these

704 genera/species may contribute in determination of their role in bovine mastitis.

705 *Mycoplasma alkalescens* (MH259845.1) was isolated from Farm 2, a 400 cow herd in the Mid

706 North region of South Australia (Appendix 2). The farm had a rising SCC over a period of four

707 weeks with multiple antimicrobial treatment failures. *Mycoplasma alkalescens* clustered into

708 the same clades of Swedish strain PG51 (Pettersson et al., 1996) and other Japanese PG51
709 strains with reference number LC158831.1 (unpublished). *Mycoplasma alkalescens* was firstly
710 identified in Australian cattle in 1963 (Hudson and Etheridge, 1963), and has been isolated from
711 the nasal cavity, joints affected by arthritis, mammary gland affected by mastitis, cows with
712 dystocia and cows with endometritis (Sosa et al., 2018, Manso-Silvan et al., 2013, Ghanem et
713 al., 2013, Bennett and Jasper, 1978). This reflects the ability of this pathogen for colonisation
714 in a range of tissues. Further studies on the pathogenicity of these pathogens in mastitis cases
715 are required.

716 The phylogenetic position of the *M. bovirhinis* strain in this study (MH266037.1) showed high
717 similarity to an Egyptian strain: Fay. Bu1-10 (Fig 1; Appendix 2). *Mycoplasma bovirhinis* is
718 well known to be isolated from the respiratory organs, and causes secondary infections in cattle
719 (Hata et al., 2017). However, this pathogen has also been isolated from cows with mastitis (Al-
720 Farha et al., 2017, Pettersson et al., 1996). *Mycoplasma bovirhinis* strain PG51 has been
721 reported previously as a secondary invader in mastitic cows in more than 70% of Australian
722 dairy herds, which can be explained by the ascending transmission from the nasal secretions of
723 calves during suckling (Hirose et al., 2001). *Mycoplasma bovirhinis* sequences in this study
724 clustered with the *M. arginini* group which may clinically explain the usual co-invasion of these
725 genetically related mycoplasmas in bovine diseases (Al-Farha et al., 2017, Szacawa et al.,
726 2015).

727 Two different strains of *M. bovis* has been recorded in this study (Appendix 2). The first strain
728 (40) clustered with *M. bovis* strains: 08M, Ningxia-1 and JF4278. The first two of these strains
729 have been isolated in China (Sun et al., 2018, Chen et al., 2017), while the third strain was
730 identified in Switzerland (Aebi et al., 2012). The second field strain of *M. bovis* in this study
731 (9029) was grouped with Egyptian and Hungarian strains (Sulyok et al., 2017, Sahar et al.,

732 2013). The genetic relatedness between some of the strains isolated in this study and those
733 previously isolated elsewhere indicate a correlation of the molecular epidemiologic distribution
734 of mycoplasmas, and it is obviously influenced by live animal movement interstate or foreign
735 trade. Although results of this study are not representative for all dairy herds in South Australia,
736 the existing strains of pathogenic mycoplasmas may raise the awareness to include them in
737 perspective epidemiological surveys and control strategies.

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743

744 **References**

745 Aebi, M., Bodmer, M., Frey, J., Pilo, P., 2012. Herd-specific strains of Mycoplasma bovis in
746 outbreaks of mycoplasmal mastitis and pneumonia. Vet Microbiol 157, 363-368.
747 Al-Farha, A.A., Hemmatzadeh, F., Khazandi, M., Hoare, A., Petrovski, K., 2017. Evaluation
748 of effects of Mycoplasma mastitis on milk composition in dairy cattle from South Australia.
749 BMC veterinary research 13, 351.
750 Al-Farha, A.A., Petrovski, K., Jozani, R., Hoare, A., Hemmatzadeh, F., 2018. Discrimination
751 between some Mycoplasma spp. and Acholeplasma laidlawii in bovine milk using high
752 resolution melting curve analysis. BMC Res Notes 11, 107.
753 Bennett, R.H., Jasper, D.E., 1978. Mycoplasma alkalescens-induced arthritis in dairy calves. J
754 Am Vet Med Assoc 172, 484-488.
755 Boonyayatra, S., Fox, L.K., Gay, J.M., Sawant, A., Besser, T.E., 2012. Discrimination between
756 Mycoplasma and Acholeplasma species of bovine origin using digitonin disc diffusion assay,
757 nisin disc diffusion assay, and conventional polymerase chain reaction. J Vet Diagn Invest 24,
758 7-13.
759 Chen, S., Hao, H., Zhao, P., Gao, P., He, Y., Ji, W., Wang, Z., Lu, Z., Liu, Y., Chu, Y., 2017.
760 Complete Genome Sequence of Mycoplasma bovis Strain 08M. Genome Announc 5.
761 Counter, D., 1978a. A severe outbreak of bovine mastitis assoicated with Mycoplasma
762 bovigenitalium and Acholeplasma laidlawii. The Veterinary record 103, 130-131.
763 Counter, D.E., 1978b. A severe outbreak of bovine mastitis assoicated with Mycoplasma
764 bovigenitalium and Acholeplasma laidlawii. Vet Rec 103, 130-131.
765 Ghanem, M.E., Higuchi, H., Tezuka, E., Ito, H., Devkota, B., Izaike, Y., Osawa, T., 2013.
766 Mycoplasma infection in the uterus of early postpartum dairy cows and its relation to dystocia
767 and endometritis. Theriogenology 79, 180-185.

768 Hata, E., Nagai, K., Murakami, K., 2017. Complete Genome Sequence of Mycoplasma
769 bovirhinis Strain HAZ141_2 from Bovine Nasal Discharge in Japan. Genome Announc 5.
770 Hirose, K., Kawasaki, Y., Kotani, K., Tanaka, A., Abiko, K., Ogawa, H., 2001. Detection of
771 mycoplasma in mastitic milk by PCR analysis and culture method. J Vet Med Sci 63, 691-693.
772 Hudson, J., Etheridge, J., 1963. A NEW TYPE OF PLEUROPNEUMONIA-LIKE
773 ORGANISM (PPLO) FROM THE NOSE OF CATTLE. Australian Veterinary Journal 39, 1-
774 5.
775 Kirk, J.H., Glenn, K., Ruiz, L., Smith, E., 1997. Epidemiologic analysis of Mycoplasma spp
776 isolated from bulk-tank milk samples obtained from dairy herds that were members of a milk
777 cooperative. J Am Vet Med Assoc 211, 1036-1038.
778 Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis
779 version 7.0 for bigger datasets. Molecular biology and evolution 33, 1870-1874.
780 Manso-Silvan, L., Tardy, F., Baranowski, E., Barre, A., Blanchard, A., Breton, M., Couture,
781 C., Citti, C., Dordet-Frisoni, E., Dupuy, V., Gaurivaud, P., Jacob, D., Lemaitre, C., Nikolski,
782 M., Nouvel, L.X., Poumarat, F., Thebault, P., Theil, S., Thiaucourt, F., Sirand-Pugnet, P., 2013.
783 Draft Genome Sequences of Mycoplasma alkalescens, Mycoplasma arginini, and Mycoplasma
784 bovigenitalium, Three Species with Equivocal Pathogenic Status for Cattle. Genome Announc
785 1.
786 Nicholas, R., Ayling, R., McAuliffe, L., 2008. Mycoplasma diseases of ruminants. CABI.
787 Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K., A review of
788 mycoplasma diagnostics in cattle. Journal of Veterinary Internal Medicine 0.
789 Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K., 2018. A review of
790 mycoplasma diagnostics in cattle. Journal of veterinary internal medicine.

791 Parker, A.M., Shukla, A., House, J.K., Hazelton, M.S., Bosward, K.L., Kokotovic, B., Sheehy,
792 P.A., 2016. Genetic characterization of Australian Mycoplasma bovis isolates through whole
793 genome sequencing analysis. Vet Microbiol 196, 118-125.
794 Peredeltchouk, M., David, S. A., Bhattacharya, B., Volokhov, D. V. Chizhikov, V. 2011.
795 Detection of mycoplasma contamination in cell substrates using reverse transcription-
796 PCR assays. J Appl Microbiol, 110, 54-60.
797 Pettersson, B., Leitner, T., Ronaghi, M., Bolske, G., Uhlen, M., Johansson, K.E., 1996.
798 Phylogeny of the Mycoplasma mycoides cluster as determined by sequence analysis of the 16S
799 rRNA genes from the two rRNA operons. J Bacteriol 178, 4131-4142.
800 Pothmann, H., Spergser, J., Elmer, J., Prunner, I., Iwersen, M., Klein-Jobstl, D., Drillich, M.,
801 2015. Severe Mycoplasma bovis outbreak in an Austrian dairy herd. J Vet Diagn Invest.
802 Sahar, E., Metwally, A., Al-Saud, N., Ibrahim, M., 2013. Molecular typing of different isolates
803 of Mycoplasma bovis, Proceedings of the 6th Scientific Conference of Animal Wealth Research
804 in the Middle East and North Africa, Hurghada, Egypt, 27-30 September 2013. Massive
805 Conferences and Trade Fairs, pp. 277-290.
806 Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing
807 phylogenetic trees. Molecular biology and evolution 4, 406-425.
808 Seffner, W., Pfutzner, H., Wehnert, C., 1983. [Mycoplasma mastitis of cattle. 11. Histological
809 udder findings after experimental infection with Mycoplasma bovigenitalium and
810 Acholeplasma laidlawii]. Arch Exp Veterinarmed 37, 375-382.
811 Singh, A., Gupta, P., Banga, H., 1990. Pathogenicity of Acholeplasma laidlawii for the goat
812 udder. Australian veterinary journal 67, 155-156.
813 Sosa, C., Tirante, L., Chaves, J., Pol, M., Ambrogi, A., Giraudo, J.A., Tamiozzo, P., 2018.
814 [Identification of species of Mycoplasma and Ureaplasma diversum from Argentinian dairy
815 herds]. Rev Argent Microbiol 50, 31-35.

816 Stipkovits, L., Romvary, J., Nagy, Z., Bodon, L., Varga, L., 1974. Studies on the pathogenicity
817 of Acholeplasma axanthum in swine. Epidemiology & Infection 72, 289-296.
818 Sulyok, K.M., Kreizinger, Z., Wehmann, E., Lysnyansky, I., Banyai, K., Marton, S., Jerzsele,
819 A., Ronai, Z., Turcsanyi, I., Makrai, L., Janosi, S., Nagy, S.A., Gyuranecz, M., 2017. Mutations
820 Associated with Decreased Susceptibility to Seven Antimicrobial Families in Field and
821 Laboratory-Derived Mycoplasma bovis Strains. Antimicrob Agents Chemother 61.
822 Sun, P., Luo, H., Zhang, X., Xu, J., Guo, Y., He, S., 2018. Whole-Genome Sequence of
823 Mycoplasma bovis Strain Ningxia-1. Genome Announc 6.
824 Szacawa, E., Niemczuk, K., Dudek, K., Bednarek, D., Rosales, R., Ayling, R., 2015.
825 Mycoplasma bovis infections and co-infections with other Mycoplasma spp. with different
826 clinical manifestations in affected cattle herds in eastern region of Poland. Bulletin of the
827 Veterinary Institute in Pulawy 59, 331-338.
828 Tully, J.G., Razin, S., 1970. Acholeplasma axanthum, sp. n.: a new sterol-nonrequiring member
829 of the Mycoplasmatales. Journal of bacteriology 103, 751-754.
830 van Kuppeveld, F.J., Johansson, K.E., Galama, J.M., Kissing, J., Bolske, G., Hjelm, E., van der
831 Logt, J.T., Melchers, W.J., 1994. 16S rRNA based polymerase chain reaction compared with
832 culture and serological methods for diagnosis of Mycoplasma pneumoniae infection. Eur J Clin
833 Microbiol Infect Dis 13, 401-405.

834

835 *Figure 4-1. Neighbour-joining consensus phylogenetic tree of six bovine milk mollicutes*
836 *created by Mega 7 software based on 16S rRNA sequencing, and their relationship to*
837 *referenced mollicutes.*

838

Chapter 5 Application of an indirect MilA ELISA for the detection of *Mycoplasma bovis* antibodies in bovine milk

Aim: to evaluate an indirect IgG ELISA for the detection of *M. bovis* antibodies in bovine milk.

Null Hypothesis: Milk *M. bovis* antibodies cannot be detected by indirect IgG ELISA.

Note: Raw data available in Appendix 1

Statement of Authorship

Title of Paper	Application of an indirect MilA ELISA for the detection of *Mycoplasma bovis* antibodies in bovine milk
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

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Contribution to the Paper	Assisted in the conceptualisation of the study and the study design, performed literature search, prepared the samples, performed MilA ELISA, culture and qPCR for all samples, analysed data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	05/05/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Nadeeka Wawegama		
Contribution to the Paper	Provided MilA ELISA Kit, contributed to data analysis, edited and approved the manuscript		
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Contribution to the Paper	Contributed in sampling, herd test data collection and coordination with the farm, edited and approved the manuscript.		
Signature		Date	7/5/18

864

Name of Co-Author	Kiro Petrovski		
Contribution to the Paper	Assisted in the conceptualisation of the study and the study design, assisted in first farm sampling, supervised the experimental phase, edited and approved the manuscript.		
Signature		Date	05 May 18

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866

867 *Short communications*

868 **Application of an indirect MilA ELISA for the detection of *Mycoplasma bovis* antibodies**
869 **in bovine milk**

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886 **Abstract**

887 The objective of this study was to detect *Mycoplasma bovis* antibodies using the MilA ELISA
888 with aim to detect ‘carrier cows’. An indirect ELISA, based on a recombinant fragment of the
889 *Mycoplasma* immunogenic lipase A (MilA) protein, was conducted on 291 milk samples for
890 the detection of *M. bovis* antibodies. Samples were also tested with conventional *Mycoplasma*
891 culture and *M. bovis* PCR. Samples were collected at cow level from two commercial dairy
892 herds in South Australia. Out of 291 samples tested, 68 (23.4%) were detected positive for *M.*
893 *bovis* antibodies, 150 (51.5%) were positive for *M. bovis* PCR and 166 (57.0%) for culture.
894 These results indicate that MilA indirect ELISA can be utilised for the detection of milk *M.*
895 *bovis* antibodies.

896 Keywords: *Mycoplasma bovis*; mastitis; ELISA; PCR; Milk

897 **Introduction**

898 *Mycoplasma bovis* is increasingly raising concerns in the dairy industry as a mastitis-causing
899 pathogen. It can cause significant economic losses as a result of a decrease in milk production,
900 an increase in somatic cell counts (SCC), the cost of associated treatments (Timonen et al.,
901 2017) and eradication strategies (Nicholas et al., 2016). As a result, early and accurate detection
902 of *M. bovis* in milk has attracted the attention of recent research, to better improve the strategic
903 control of the disease (Parker et al., 2017b). Cattle subclinically infected with *M. bovis*,
904 otherwise known as “carrier cows”, pose significant challenges, particularly if these are being
905 introduced to an uninfected herd as newly purchased stock (Hazelton et al., 2017, Parker et al.,
906 2017a). Among these challenges, for instance, is recognition of ‘carrier cows’ at the time of
907 sampling.

908 The misdiagnosis of *M. bovis* from milk samples, via culture or Polymerase Chain Reaction
909 (PCR), is commonly attributed to the intermittent shedding of the pathogen in milk (Biddle et
910 al., 2003) or the inability of current diagnostic tests to identify the pathogen during the
911 convalescent phase of the disease. Hence, identifying a diagnostic test, capable of accurately
912 detecting *M. bovis,* in milk samples, from carrier cows is necessary.

913 Indeed, some immunogenic proteins from *M. bovis* have been evaluated previously for their
914 capacity in detection of *M. bovis* antibodies (Sun et al., 2014, Byrne et al., 2000). Other
915 commercial ELISA kits have been used for *M. bovis* antibodies screening at bulk tank milk
916 level in Danish and some Australian dairy herds (Parker et al., 2017, Arede et al., 2016, Nielsen
917 et al., 2015). However, the recombinant *Mycoplasma* immunogenic lipase (MilA)
918 Immunoglobulin G (IgG) indirect ELISA has not been evaluated in milk. It has been developed
919 and evaluated in experimentally infected calves with *M. bovis* on serum. MilA has shown
920 greater sensitivity and comparable specificity to other commercial ELISA kits (BIO K302 and
921 BIO K260 from BioX Diagnostics (Belgium)). Therefore, it has been recommended for
922 serological screening for *M. bovis* in cattle (Wawegama et al., 2016, Wawegama et al., 2014).
923 As a result, this study aimed to detect *M. bovis* antibodies with the MilA ELISAs compared to
924 the presence of *M. bovis* by conventional culture and PCR with aim to find an accurate method
925 for detection of 'carrier cows' in milk.

926 **Materials and Methods**

927 A total of 291 milk samples were collected aseptically at cow-level once from two commercial
928 dairy farms in South Australia. Of these, 251 were collected from Farm 1, a 2,500 cow dairy
929 located in the South East region of South Australia and were previously used in another study
930 (Al-Farha et al., 2017). The remaining 40 samples were collected from Farm 2, a 400 cow dairy
931 located in the Mid-North region of South Australia. Both farms had a history of repeated

932 mastitis treatment failure and high SCC, however no clinical signs were observed at the time of
933 sampling for any of the sampled cows. The problem of high SCC has been historic on both
934 farms, being previously detected and dealt with on Farm 1 (using a commercial laboratory for
935 testing of milk for presence of *M. bovis*) but there was no previous detection of *M. bovis* on
936 Farm 2.

937 All 291 samples were analysed by ELISA, culture and PCR. MilA ELISA was conducted to
938 test for *M. bovis* antibodies in milk following the procedure described by (Wawegama et al.,
939 2014). The ELISA cut-off value was calculated using Bayesian latent class modelling in
940 multiple populations (Wawegama et al., 2016) and was estimated at 105 antibody unit (AU).
941 All samples were subjected to purified glutathione-S-transferase (GST) ELISA.

942 For culture analysis, an aliquot of each milk sample (250 µL) was added to a *Mycoplasma* broth
943 (Oxoid, Australia) and left for five days, before plating on a *Mycoplasma* media (Oxoid,
944 Australia). Plates were incubated for 15 days at 37º C using 10% CO_2 jars. The plates were then
945 examined for colonies using a stereomicroscope (Olympus SZ30, Australia). Samples were
946 considered positive when at least a single *Mycoplasma* colony was detected (Markey et al.,
947 2013). To confirm isolation of *Mycoplasma*, 3-5 selected colonies from each plate subcultured
948 into the enriched *Mycoplasma* broth and inoculated at the same condition and checked for
949 colour change of broth and typical *Mycoplasma* colonies on agar. As soon as the phenol red
950 indicator changed to yellow, the subculture onto the fresh broth and agar were carried out.

951 DNA extraction was performed directly on each milk sample (Qiagene, Germany), and a DNA
952 concentration measurement was performed using Nanodrop 1000c (Thermofisher Scientific
953 Inc., Waltham, MA, USA). HRM-real time PCR was conducted on each of these samples (Al-
954 Farha et al., 2018) using *M. bovis* specific primers targeting 16S rRNA including primer

955 forward: 5'-CCAGCTCACCCTTATACATGAGCGC-3' and primer reverse: 5'-
956 TGACTCACCAATTAGACCGACTATTTCACC-3' (Sachse and Frey, 2003). The qPCR cut-
957 off value was estimated at 30 copy number following recommendations by (Behera et al., 2018).

958 Raw data used for analyses are included in Appendix 1.

959 **Results and discussion**

960 Results of the ELISA indicated that 68/291 samples (23.4%) were positive for *M. bovis*
961 antibodies (≥ 140 AU). By farm, 63/251 (25.1%) and 5/40 (12.5%) samples were positive from
962 Farm 1 and 2, respectively. In comparison, a total of 166/291 samples (57.0%) were positive
963 for *Mycoplasma*-like colonies by conventional culture. By farm, 144/251 (57.4%) and 22/40
964 (55.0%) samples were positive from Farm 1 and 2, respectively. Finally, 150/291 (51.5%)
965 samples were positive for *M. bovis* by PCR, all of which were from Farm 1 (Table 1).

966 Another previously described ELISA designed with similar conditions, using GST protein as
967 coating antigen was also carried out to ensure the signals in MilA ELISA were due to the *M.*
968 *bovis* antigen and not the result of the GST fusion antigen. All samples tested in the MilA
969 ELISA negative to GST protein (data not shown) (Byrne et al., 2005).

970 A total of 24/68 (35.3%) and 23/68 (33.8%) samples, positive by indirect ELISA were negative
971 on culture and PCR, respectively. This may have occurred due to several reasons. Firstly,
972 indirect ELISAs not only detect animals undergoing current infections but also those which
973 have undergone past exposure to the pathogen and secondly, the sensitivity of the indirect
974 ELISA to detect antibodies during the convalescent phase of the disease, when the cow has
975 seroconverted and cleared the organism. Another reason for positive ELISA detection versus
976 negative culture and PCR can be explained by the intermittent shedding of the pathogen through

977 milk (Maunsell et al., 2011). Moreover, in advanced stages of *M. bovis* infection, the
978 impermeability of mammary gland epithelium to the antibodies detected by ELISA can be
979 another reason for the discrepancies between detection of antigen and antibody (Stelwagen and
980 Singh, 2014). In this study, PCR had higher rate of positive samples than ELISA. In fact, this
981 is not an unusual finding. ELISA detects antibodies and PCR detect the antigen. The topic of
982 interest is why there is a high discrepancy. One possible explanation may be the incubation or
983 prodromal period of the majority of sampled cows when antibodies are not present yet but cows
984 are shedding the antigen found by the PCR. Impermeable mammary gland may result in
985 prevention of sero-conversion that may have resulted in the low prevalence of antibodies in
986 milk. After recovery, the persistence of the antibodies to *M. bovis* in the mammary gland may
987 be very short, that have resulted in a transient finding of antibodies in milk only. Not all media
988 are suitable for testing of antibodies by ELISA. Milk may be unsuitable media and this needs
989 further research. It is also possible that the cut-off used in the MilA ELISA was too high for
990 bovine milk, as the cut-off used in this study had not been the same as one used previously on
991 bovine serum samples (Wawegama et al., 2014). Testing the sensitivity and specificity at
992 different ELISA cut-offs indicated that the cut off value for ELISA at 140 was the
993 recommended threshold; however, it did give a low sensitivity (30.0%) but relatively good
994 specificity (83.7%). It should be noted that the PCR was probably not the appropriate 'gold
995 standard' test for testing the prevalence of antibodies in milk. A more appropriate 'gold
996 standard' would be a commercially available *M. bovis* antibody testing ELISA and this should
997 be area of a future research.

998 **Conclusion**

999 This is the first study where MilA ELISA was used to detect *M. bovis* specific antibodies in
1000 bovine milk. The results indicate that this ELISA could be utilised for improving biosecurity

1001 on dairy farms, not for detecting positive cows (low sensitivity) but for confirming positive
1002 cows as they were likely true positive (high specificity). MilA ELISA in milk may need further
1003 validation compared to commercial ELISA kits approved for use on milk. The cut-off value for
1004 the MilA ELISA may need to be re-evaluated for bovine milk and this would require further
1005 studies on farms with known *M. bovis* outbreaks. Indeed, it would be more beneficial to
1006 repeatedly test cows with known timing of infection (e.g. by challenge model) and estimate the
1007 timing of appearance and waning of the antibodies in bovine milk.

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1013 **References**

1014 AL-FARHA, A. A.-B., PETROVSKI, K., JOZANI, R., HOARE, A. & HEMMATZADEH, F.
1015 2018. Discrimination between some Mycoplasma spp. and Acholeplasma laidlawii in
1016 bovine milk using high resolution melting curve analysis. *BMC Research Notes,* 11**,**
1017 107.
1018 AL-FARHA, A. A., HEMMATZADEH, F., KHAZANDI, M., HOARE, A. & PETROVSKI,
1019 K. 2017. Evaluation of effects of Mycoplasma mastitis on milk composition in dairy
1020 cattle from South Australia. *BMC Vet Res,* 13**,** 351.
1021 AREDE, M., NIELSEN, P. K., AHMED, S. S., HALASA, T., NIELSEN, L. R. & TOFT, N.
1022 2016. A space-time analysis of Mycoplasma bovis: bulk tank milk antibody screening
1023 results from all Danish dairy herds in 2013-2014. Acta Vet Scand, 58, 16.
1024 BEHERA, S., RANA, R., GUPTA, P. K., KUMAR, D., SONAL, REKHA, V., ARUN, T. R.
1025 & JENA, D. 2018. Development of real-time PCR assay for the detection of
1026 Mycoplasma bovis. *Trop Anim Health Prod*.
1027 BIDDLE, M. K., FOX, L. K. & HANCOCK, D. D. 2003. Patterns of mycoplasma shedding in
1028 the milk of dairy cows with intramammary mycoplasma infection. *J Am Vet Med Assoc,*
1029 223**,** 1163-6.
1030 BYRNE, W. J., BALL, H. J., BRICE, N., MCCORMACK, R., BAKER, S. E., AYLING, R. D.
1031 & NICHOLAS, R. A. 2000. Application of an indirect ELISA to milk samples to
1032 identify cows with Mycoplasma bovis mastitis. *Vet Rec,* 146**,** 368-9.
1033 HAZELTON, M., SHEEHY, P., BOSWARD, K., PARKER, A., MORTON, J., DWYER, C.,
1034 NIVEN, P. & HOUSE, J. 2017. Shedding of Mycoplasma bovis and antibody responses
1035 in cows recently diagnosed with clinical infection. *Journal of Dairy Science*.

1036 MARKEY, B., LEONARD, F., ARCHAMBAULT, M., CULLINANE, A. & MAGUIRE, D.
1037 2013. *Clinical veterinary microbiology*, Elsevier Health Sciences.
1038 MAUNSELL, F. P., WOOLUMS, A. R., FRANCOZ, D., ROSENBUSCH, R. F., STEP, D. L.,
1039 WILSON, D. J. & JANZEN, E. D. 2011. Mycoplasma bovis infections in cattle. *J Vet*
1040 *Intern Med,* 25**,** 772-83.
1041 NICHOLAS, R. A., FOX, L. K. & LYSNYANSKY, I. 2016. Mycoplasma mastitis in cattle:
1042 To cull or not to cull. *The Veterinary Journal,* 216**,** 142-147.
1043 NIELSEN, P. K., PETERSEN, M. B., NIELSEN, L. R., HALASA, T. & TOFT, N. 2015. Latent
1044 class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of
1045 Mycoplasma bovis. *Prev Vet Med,* 121**,** 338-42
1046 PARKER, A., HOUSE, J., HAZELTON, M., BOSWARD, K., MORTON, J. & SHEEHY, P.
1047 2017a. Bulk tank milk antibody ELISA as a biosecurity tool for detecting dairy herds
1048 with past exposure to Mycoplasma bovis. *Journal of Dairy Science,* 100**,** 8296-8309.
1049 PARKER, A. M., HOUSE, J. K., HAZELTON, M. S., BOSWARD, K. L. & SHEEHY, P. A.
1050 2017b. Comparison of culture and a multiplex probe PCR for identifying Mycoplasma
1051 species in bovine milk, semen and swab samples. *PloS one,* 12**,** e0173422.
1052 SACHSE, K. & FREY, J. 2003. *PCR detection of microbial pathogens*, Springer Science &
1053 Business Media.
1054 STELWAGEN, K. & SINGH, K. 2014. The role of tight junctions in mammary gland function.
1055 Journal of mammary gland biology and neoplasia, 19, 131-138.
1056 SUN, Z., FU, P., WEI, K., ZHANG, H., ZHANG, Y., XU, J., JIANG, F., LIU, X., XU, W. &
1057 WU, W. 2014. Identification of novel immunogenic proteins from Mycoplasma bovis
1058 and establishment of an indirect ELISA based on recombinant E1 beta subunit of the
1059 pyruvate dehydrogenase complex. *PLoS One,* 9**,** e88328.
1060 TIMONEN, A. A., KATHOLM, J., PETERSEN, A., MÕTUS, K. & KALMUS, P. 2017.
1061 Within-herd prevalence of intramammary infection caused by Mycoplasma bovis and
1062 associations between cow udder health, milk yield, and composition. *Journal of Dairy*
1063 *Science*.
1064 WAWEGAMA, N. K., BROWNING, G. F., KANCI, A., MARENDA, M. S. & MARKHAM,
1065 P. F. 2014. Development of a recombinant protein-based enzyme-linked
1066 immunosorbent assay for diagnosis of Mycoplasma bovis infection in cattle. *Clin*
1067 *Vaccine Immunol,* 21**,** 196-202.
1068 WAWEGAMA, N. K., MARKHAM, P. F., KANCI, A., SCHIBROWSKI, M., OSWIN, S.,
1069 BARNES, T. S., FIRESTONE, S. M., MAHONY, T. J. & BROWNING, G. F. 2016.
1070 Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for
1071 detection of Mycoplasma bovis infection in feedlot cattle. *Journal of clinical*
1072 *microbiology,* 54**,** 1269-1275.

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1074 *Table 5-1. Count of Mycoplasma bovis samples per outcome of indirect ELISA (□140AU) versus both culture and qPCR results of 291 samples*
1075 *collected aseptically from two farms in South Australia.*

ELISA results (no.)	**Culture results (no.)**							**PCR results (no.)**						
	Positive			**Negative**			**Total**	**Positive**			**Negative**			**Total**
	Farm 1	**Farm 2**	**Total**	**Farm 1**	**Farm 2**	**Total**		**Farm** 1	**Farm 2**	**Total**	**Farm 1**	**Farm 2**	**Total**	
Positive	43	1	**44**	20	4	**24**	**68**	45	0	**45**	18	5	**23**	**68**
Negative	101	21	**122**	87	14	**101**	**223**	105	0	**105**	83	35	**118**	**232**
Total	144	22	**166**	107	18	**125**	**291**	150	0	**150**	101	40	**141**	**291**

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1079 **Chapter 6 Evaluation of effects of *Mycoplasma* mastitis on milk composition in dairy cattle**
1080 **from South Australia**

1081 **Aims:** to determine the effects of different *Mycoplasma* spp. and *A. laidlawii* compared to
1082 conventional mastitis pathogens on milk yield and other milk components in cattle with high
1083 SCC (subclinical mastitis) from a single dairy herd in South Australia with low response rate
1084 to conventional antimicrobial therapy, and to identify *Mycoplasma* spp. and *A. laidlawii* using
1085 novel PCR applied directly to milk samples.

1086 **Null Hypothesis:** *M. bovis* is the only pathogenic Mycoplasma which has effect on milk
1087 composition.

1088 **Note:** Raw data available in Appendix 1.

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Contribution to the Paper	Assisted in the conceptualisation of the study and the study design, performed literature search, prepared the samples, performed culture and PCR for all samples, wrote the manuscript and acted as a corresponding author.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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Contribution to the Paper	Assisted in the conceptualisation of the study and the study design, principle supervisor, assisted in sampling, performed statistics, edited and approved the manuscript.		
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RESEARCH ARTICLE Open Access

Evaluation of effects of *Mycoplasma* mastitis on milk composition in dairy cattle from South Australia

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Abstract

Background: *Mycoplasma* mastitis is increasingly posing significant impact on dairy industry. Although the effects of major conventional mastitis pathogens on milk components has been widely addressed in the literature, limited data on the effects of different *Mycoplasma* and *Acholeplasma* spp. on milk quality and quantity is available. The aim of this study was to determine the casual relationship of *Mycoplasma* spp. and *A. laidlawii* to mastitis and compare them to subclinical mastitis caused by conventional mastitis pathogens from a single dairy herd in South Australia; *Mycoplasma* spp. and *A. laidlawii* were detected using PCR applied directly to milk samples. The herd had mastitis problem with high somatic cell count and low response rate to conventional antimicrobial therapy. A total of 288 cow-level milk samples were collected aseptically and used in this study.

Results: Conventional culture showed a predominance of coagulase-negative staphylococci, followed by coagulase-positive staphylococci, *Streptococcus* spp., *Enterococcus* spp., *E. coli*, and *Klebsiella* spp. PCR results showed a high prevalence of mycoplasmas (76.7%), including *A. laidlawii* (10.8%), *M. bovis* (6.2%), *M. bovirhinis* (5.6%), *M. arginini* (2%), and (52.1%) of cows were co-infected with two or more *Mycoplasma* and *Acholeplasma* species. *Mycoplasma* co-infection significantly increased somatic cell counts (SCC) similar to conventional mastitis pathogens and compared to non-infected cows with 389.3, 550.3 and 67.3 respectively; and decreased the milk yield with 29.0, 29.9 and 34.4 l, respectively. *Mycoplasma* co-infection caused significant increase in protein percentage, and significant decrease in fat percentage and total milk solids, similar to other conventional mastitis pathogens. In contrast, changes in milk composition and yield caused by various individual *Mycoplasma* species were non-significant.

Conclusions: *Mycoplasma* mastitis had on-farm economic consequences similar to common conventional mastitis pathogens. Results of our study indicate that co-infection *Mycoplasma* mastitis caused similar effect on milk composition to other mastitis pathogens and we hope these findings raise the awareness of the importance of their detection on routine diagnostic panels.

Keywords: *Mycoplasma*, Mastitis, Dairy cattle, Milk composition, Somatic cell count (SCC)

Background

The genus *Mycoplasma* belongs to the class *Mollicutes* and is responsible for many diseases in cattle, including respiratory disorders, arthritis, otitis media, and mastitis [12, 23, 41]. *Mycoplasma* mastitis is highly resilient to antimicrobial therapy and can be easily missed during laboratory culture and susceptibility testing diagnostic panels [25]. Among the 200 species of *Mycoplasma* discovered to date, several have been reported to be involved in bovine mastitis such as *M. bovis, M. bovigenitalium, M. californium, M. bovirhinis, M. arginini, M. dispar, M. canadense, M. bovoculi*, and *Mycoplasma* spp. bovine groups 7 and F-38 [13]. Some studies have also indicated that *Acholeplasma* spp. can be a milk contaminant or non-pathogenic saprophyte [5, 27]. However, others have reported isolation of *A. laidlawii* from clinical and subclinical cases of bovine mastitis, suggesting a causal relationship [31, 61, 63].

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In dairy herds, mycoplasmas can cause clinical, subclinical or chronic mastitis [16]. *M. bovis* is considered the most common pathogen among mycoplasmas [14]. The possibility of isolating *Acholeplasma* spp. from mastitis cases is not excluded [8, 31, 61, 63]. The incubation period of *Mycoplasma* mastitis is 10–14 days [48], and shedding of the causative pathogen may occur during this period contributing to the spread of bacteria. Economic consequences of *Mycoplasma* mastitis in cattle are due to decreased milk production, cost of implementing control procedures, and cost of diagnosis and treatment [39]. For instance, the cost of *M. bovis* infection in cattle is more than US$140 million annually in the United States, and even higher losses have been reported in Europe [2].

Bacteriological culture of mycoplasmas from milk samples was once the most common method of detection. However, this method is relatively slow often taking one to 2 weeks with potential non-growth of these bacteria due to their fastidious culture requirements [45, 51], *Mycoplasma* mastitis is usually excluded from general mastitis screening tests due to its special growth requirements and time delay [30]. Similarly, serological detection method is time-consuming as antibody formation requires approximately 2 weeks [26]. Furthermore, there is a variation in the growth requirements of different species of *Mycoplasma* [20] which consequently affects disease detection, particularly when *Mycoplasma* co-infection occurs. However, most mastitis diagnostic investigations focus on the predominant species of mycoplasma associated with infection, *M. bovis,* and disregard other causative *Mollicutes* [52]. Studies of clinical *Mycoplasma* co-infection also deserve more attention, especially for epidemiological and treatment investigations. Therefore, development of a rapid and reliable screening diagnostic method is required which will be capable in distinguishing between different mycoplasma genera and species.

The association between *Mycoplasma* mastitis, individual somatic cell count (SCC) and milk yield also requires clarification. The association between conventional pathogens causing mastitis, such as *Streptococcus* and *Staphylococcus* spp., and elevated SCC has been previously reported [10, 19]. *Mycoplasma* mastitis can also affect SCC patterns [32, 47]. A marked decrease in milk production has been estimated particularly from mastitis caused by *S. agalactiae*, *Mycoplasma* spp. and *Pasteurella* spp. [62]. However, the effect of *Mycoplasma* mastitis compared to conventional bacterial pathogens on other milk composition has yet to be evaluated. Furthermore, the pathogenicity of each individual *Mycoplasma* spp., either as a single or co-infection, needs to be explored.

This study had two aims. The first aim of this study was to determine the effects of different *Mycoplasma* spp. and *A. laidlawii* compared to conventional mastitis pathogens on milk yield and other milk components in cattle with high SCC (subclinical mastitis) from a single dairy herd in South Australia with low response rate to conventional antimicrobial therapy. The second aim was to identify *Mycoplasma* spp. and *A. laidlawii* using novel PCR applied directly to milk samples.

Methods

Sample collection

Milk samples were collected aseptically once from each individual cow from a single commercial dairy farm near Mount Gambier, South Australia. This farm had a history of repeated failure of mastitis treatment with high SCC. Samples originated from cows aged 2–10 years in the hospital mob or main milking mob if they had a high SCC. Composite milk samples were collected aseptically in sterile 50 mL tubes from each functional quarter of individual cows ($n = 288$). Samples were kept on ice and were sent immediately to the PC2 laboratory at The University of Adelaide, Roseworthy Campus. In the laboratory, milk samples were subjected to conventional microbial culture using 10 μL aliquots according to National Mastitis Council guidelines [24]. All samples were then frozen at −20 °C for further analysis.

Milk analysis data

A database of individual cow yield production parameters (yield, total milk solids, fat and protein percentage) and SCC for sampled and non-sampled cows (the rest of the herd's cows that have also high and low SCC) was obtained from herd testing information for the 4 herd tests closest to the sampling points ($n = 7609$ cow data points). SCC was performed for each milk sample using a FOSS Fossomatic 5000 (Hillerød, Denmark), and instrumental milk components assay by a commercial laboratory (NHIS, Cohuna, VIC, Australia).

DNA extraction

After thawing milk at ambient temperature, 2 mL of each sample was centrifuged at 8000 X g for 20 min to remove supernatant fat and excess liquid. DNA extraction was performed directly from milk using the QIAmp DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions with minor modifications. In order to increase the DNA yield, a much larger milk sample (1 mL) was centrifuged at 5000 X g for 30 min and the lipid layer removed. Genomic DNA concentration was measured using Nanodrop 1000c (Thermofisher Scientific Inc., Waltham, MA, USA) and stored at −20 °C until further use.

PCR probes and protocol

A modified PCR protocol using four pairs of species-specific primers previously published elsewhere was developed (Table 1). In-vitro amplification of DNA to

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Table 1 Oligonucleotides primers for detection of mycoplasmas and *A. laidlawii* identified by conventional PCR

Primers	Sequencing 5′-3′	Species	Targets	Amplicon size (bp)	Ref
Mb-F	CTTGGATCAGTGGCTTCATTAGC	*M.bovis*	*VspA* gene	400	[1]
Mb-R	GTCATCATGCGGAATTCTTGGGT				
Arg-F	AGAGTTTGATCCTGGCTCAGGA	*M. arginini*	16S rRNA gene	449	[11]
Arg-R	TCAACCAGGTGTTCTTTCCC				
Mbv-F	GCTGATAGAGAGGTCTATCG	*M. bovirhinis*	16S rRNA gene	316	[34]
Mbv-R	ATTACTCGGGCAGTCTCC				
Acho-F	AGCCGGACTGAGAGGTCTAC	*A. lailawii*	16S rRNA gene	505	[11]
Acho-R	TAATCCTGTTTGCTCCCCAC				

detect *Mycoplasma* and *Acholeplasma* spp. was carried out in 25 μL volumes consisting of 0.25 μL Taq DNA polymerase, 5 μL of 5× reaction buffer (Biolab, UK), 1 μL forward primer, 1 μL reverse primer, 1 μL of template DNA and 16.75 μL of DEPC-treated water. Amplifications were performed for 35 PCR cycles using the T100™ Thermal Cycler (Biorad Australia) and consisted of preheating activation for 5 min at 95 °C, denaturation at 95 °C for 30 s, and annealing at 60 °C for *M. bovis* and A. *laidlawii,* 55 °C for *M. arginini* and 64 °C for *M. bovirhinis.* The final extension step was performed at 72 °C for 10 min. The PCR products were subjected to electrophoresis in 1.5% agarose gels and visualised by staining with Gel Red.

Statistical analysis

For statistical analysis individual cows were grouped as follows:

a. Bacteriologically analysed milk samples (all with SCC of >250,000 cells/mL and assumed to have subclinical mastitis, *n* = 288): which were positive for pure *M. bovis* detection (GROUP 1, *n* = 11); positive for pure *A. laidlawi* detection (GROUP 2, n = 28); positive for pure *M. bovirhinis* detection (GROUP 3, *n* = 13); positive for pure *M. arginini* detection (*n* = 6); positive for *Mycoplasma* co-infection by two or more detected species (GROUP 4, *n* = 119); positive for conventional mastitis pathogens and *Mycoplasma* negative (GROUP 5, *n* = 58), and cows with positive *Mycoplasma/Acholeplasma* detection and positive on conventional mastitis culture (GROUP 6, *n* = 53).
b. Milk samples not bacteriologically analysed: cows with SCC of >250,000 cells/mL – assumed to have subclinical mastitis but not sampled (GROUP 7, *n* = 1146); and cows with SCC of ≤250,000 cells/mL assumed to be non-affected by subclinical mastitis 'healthy cows', (GROUP 8, *n* = 6181).

Statistical analysis software (SAS) version 9.4 (Cary Inc., USA) was used to analyse data.

For assessment of the effect of subclinical mastitis (1) or no subclinical mastitis (0) on SCC, herd test data were log transformed (somatic cell score; SCS). Due to the small number of positive detections (n = 6), the effect of *M. arginini* on milk composition and yield was not estimated.

The effect of subclinical mastitis on the test-day geometric mean of SCC was estimated using LOGISTIC REGRESSION in PROC GLIMMIX of SAS. The values obtained by modelling for the SCS were back-transformed to obtain the geometric mean of SCC. The effect of subclinical mastitis on test-day milk production parameters (yield, fat, protein and total milk solids) with pathogen as fixed effect and a cow as a random effect, was estimated using ANOVA in PROC MIXED of SAS. The effect of age and stage of production (i.e. days in milk) were not included in the final analysis as the preliminary model demonstrated no significant effect. A statistical difference of $P < 0.05$ was set as significant and $P < 0.001$ was highly significant.

Results

Results showed that 221 of the 288 milk samples (76.7%) collected from the dairy farm were positive for *Mycoplasma* spp. and *A. laidlawii*. Among these, *Mycoplasma* co-infection with two or more genera/species predominated (52.1%), followed by single infections with *A. laidlawii* (10.8%), *M. bovis* (6.2%), *M. bovirhinis* (5.6%), and *M. arginini* (2%) (Table 2). Agarose gel electrophoresis for PCR products revealed amplicon sizes stated in (Table 1). Conventional culture for all milk samples, independently of *Mollucites* isolation, showed a predominance of coagulase-negative staphylococci (CoNS) (12.2%), and relatively low occurrence of coagulase-positive staphylococci (CoPS) (2.4%), *Streptococcus* spp. (2.1%), *Enterococcus* spp. (1.7%), *E. coli* (1.4%), and *Klebsiella* spp. (0.4%) (Table 3).

Significant difference in test-day SCC was detected between groups (*Mycoplasma* co-infection; GROUP 4, conventional mastitis pathogens; GROUP 5, mycoplasmas and other pathogens; GROUP 6, and not tested high SCC cows; GROUP 7) and assumed healthy cows (low SCC;

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Table 2 Distribution of *Mycoplasma* spp. detected by PCR for various conventional mastitis culture as detected directly from milk samples

Conventional mastitis culture	*Mycoplasma* detected spp.						Total
	A. laidlawii	*M. bovis*	*M. bovirhinis*	*M. arginini*	Mixed	Negative	
Negative (%)	28 (9.7)	11 (3.8)	13 (4.5)	3 (1)	119 (41.3)	56 (19.4)	230 (79.9)
Positive (%)	3 (1)	7 (2.4)	3 (1)	3 (1)	31 (10.76)	11 (3.8)	58 (20.1)
Total (%)	31 (10.8)	18 (6.2)	16 (5.6)	6 (2)	150 (52.1)	67 (23.2)	288 (100)

GROUP 8; $P < 0.001$) with 389.32, 550.26, 611, 960.7 and 67.33 SCCx10^3 cell/mL respectively. The *Mycoplasma* co-infected cows (GROUP 4) showed significant difference of SCC from assumed healthy cows (GROUP 8) at 376.15 SCCx10^3 cell/mL ($P \leq 0.05$). However, no significant difference was observed between cows infected with *M. bovis* (GROUP 1) and *A. laidlawii* (GROUP 2) in comparison with assumed healthy cows (GROUP 8) with 255.09 and 216.14, SCCx10^3 cell/mL respectively ($P > 0.05$). Correspondingly, test day milk yield was similarly affected significantly in *Mycoplasma* co-infection (GROUP 4), conventional mastitis pathogens (GROUP 5) and mycoplasmas and conventional pathogens (GROUP 6) in comparison with assumed healthy cows (GROUP 8) with 29.0, 29.9, 27.9 and 34.4 l, respectively ($P < 0.001$). In contrast, milk yield did not decline significantly in cows infected with *M. bovis* (GROUP 1), *A. laidlawii* (GROUP 2), or *M. bovirhinis* (GROUP 3) with 32.4, 32.4 and 30.0 l/day, respectively ($P > 0.05$).

Fat percentage was significantly decreased in cows co-infected with *Mycoplasma* (GROUP 4) with 3.1% ($P < 0.01$), while no differences were observed in all other tested groups with 3.4, 3.3, 3.3, 3.2 and 3.3% for groups *M. bovis* (GROUP 1), *A. laidlawii* (GROUP 2), *M. bovirhinis* (GROUP 3), conventional mastitis pathogens (GROUP 5) and mixed *Mycoplasma/Acholeplasma* and conventional mastitis culture (GROUP 6) respectively ($P > 0.05$). Protein percentage increased significantly in cows with *Mycoplasma* co-infection (GROUP 4), conventional mastitis pathogens (GROUP 5), mixed *Mycoplasma/Acholeplasma* and conventional mastitis culture (GROUP 6) and non-tested high SCC (GROUP 7) in comparison with cows assumed to be healthy (GROUP 8) with 3.4, 3.3, 3.4, 3.4 and 3.3%, respectively ($P < 0.05$), while other *Mycoplasma* groups showed no differences being 3.4, 3.4 and 3.3% for GROUPs s 1, 2 and 3 respectively ($P > 0.05$). In comparison, total milk solids decreased significantly in GROUPs 4, 5, 6 and 7 in comparison to GROUP 8 with 1.8, 1.9, 1.8, 2.0 and 2.2%, respectively ($P < 0.001$). However, there were no differences in total milk solids for GROUPs 1, 2 and 3 with 2.1, 2.1 and 2.0%, respectively (P > 0.05) (Table 4).

Table 3 Prevalence of non-*Mycoplasma* bacteria isolated by conventional mastitis culture from 288 milk samples collected purposively to identify *Mycoplasma* infections

Species.	Frequency (%)
CoNS	35 (12.2)
CoPS	7 (2.4)
Streptococcus	6 (2.1)
Enterococcus	5 (1.7)
E.coli	4 (1.4)
Klebsiella	1 (0.4)
ND	230 (79.86)
Total	288 (100%)

Discussion

Results showed a high prevalence of *Mycoplasma* and *Acoleplasma* species isolated from the purposive sampled cows. In addition, *Mycoplasma* co-infection significantly changed milk quality and quantity, similarly to other mastitis pathogens. Details on the results of culture and PCR, including test characteristics, were not subject of this study and are not further detailed.

The study farm had a history of mastitis treatment failure and high SCC. The long persistence of this problem may be due to the variety of transmission methods, such as via direct contact, milking machines and other fomites [29]. Intermittent shedding of the pathogen from cows suffering chronic mastitis may be another important reason for the relatively high prevalence of *Mycoplasma* mastitis in the studied herd. Chronic mastitis results from the capability of *Mycoplasma* spp. to form multiple micro-abscesses within the infected mammary gland [28]. Due to the high occurrence of mastitis, the studied farm is not representative of the South Australian dairy cattle population. As the primary objective of this study was not to carry out an epidemiological investigation, but to purposively sample and collect a significant number of isolates for research, the validity of the study is not decreased. In addition, study results may raise awareness of the importance of *Mycoplasma* mastitis to the dairy industry. In order to establish the prevalence of this disease in Australia, further attempts in affected herds using methodology similar to that described in this study are required. It is possible that the PCR technique used in

Table 4 The mean values (± SE) of SCC, milk yield, milk fat, protein and total solids for dairy cattle infected with *Mycoplasma* and other mastitis pathogens (in individual cow-testing points)

		Group	Frequency	Milk yield (litre/day)	Fat percentage	Protein percentage	Total milk solids	SCCx10^3 cell/mL
Tested	1	*Mycoplasma bovis*	11	32.4 ± 3.73	3.4 ± 0.26	3.4 ± 0.09	2.1 ± 0.20	255.1 ± 140.07
	2	*Acholeplasma laidlawii*	28	32.4 ± 2.33	3.3 ± 0.16	3.4 ± 0.06	2.1 ± 0.13	216.1 ± 87.79
	3	*M. bovirhinis*	13	30.0 ± 3.43^a	3.3 ± 0.24	3.3 ± 0.09	2.0 ± 0.19	376.2 ± 128.85^a
	4	*Mycoplasma* co-infection	119	29.0 ± 1.13^A	3.1 ± 0.08^a	3.4 ± 0.03^A	1.9 ± 0.06^A	389.3 ± 42.58^A
	5	Conventional mastitis pathogens	58	29.9 ± 1.57^a	3.2 ± 0.11	3.3 ± 0.04^a	1.9 ± 0.09^A	550.3 ± 59.00^A
	6	Mycoplasmas and conventional pathogens	53	27.9 ± 1.79^A	3.3 ± 0.12	3.4 ± 0.04^A	1.8 ± 0.11^A	611 ± 12.08^A
Total			282 (3.7%)					
Non tested	7	High SCC	1146	29.2 ± 0.36^A	3.6 ± 0.02^A	3.4 ± 0.01^A	2.0 ± 0.02^A	960.7 ± 13.72^A
	8	Low SCC	6181	34.4 ± 0.15	3.4 ± 0.01	3.3 ± 0.00	2.2 ± 0.01	67.3 ± 5.90
Total			7327 (96.3%)					

Lower case superscripts indicate statistical difference within column of $P < 0.05$ or capital superscripts indicate statistical difference within column of $P < 0.001$

this study detects foreign pieces of DNA. To ensure validity of our results we also carried out conventional culture for *Mycoplasma* (data not shown). Indeed, some culture-negative samples yielded positive PCR. To ensure that we were detecting *Mycoplasma* spp. and *A. laidlawii,* 16S rRNA sequencing was also carried out (data not shown).

Results of this study highlight the tendency of *Mycoplasma* co-infection to cause a significant alteration in milk composition in comparison to any individual *Mycoplasma* spp. group. This seems to be a result of developing evidence for pathogenicity of *Mycoplasma* co-infection [17], and can be a reflection of the advanced stage of the disease. It is often thought that the mechanism of milk alteration is attributed to the pathogen severity, proportion of involved mammary glands alveoli, interference with blood or hormonal nourishment to these alveoli, epithelial integrity disruption, and milk decomposition due to enzymatic activity.

Differences between the effects of each individual *Mycoplasma* spp. and *A. laidlawii* on milk composition in this study is noteworthy. It has been reported worldwide that *M. bovis* is the primary contagious pathogen in bovine *Mycoplasma* mastitis [14, 35, 39, 43]. In our study, cows infected only with *M. bovis* (GROUP 1) showed no significant impacts on milk composition apart from the SCC. Cows in this group may have been at an early stage of the disease. In addition, the limited sample size of this group ($n = 11$) needs to be considered. However, the effect of *M. bovis* was significant when it contributed to the *Mycoplasma* co-infection group. Multiple *Mycoplasma* spp. infection tends to be more common in *Mycoplasma*-associated diseases [59]. Although, some studies indicate the possibility of *A. laidlawii* being involves in mastitis cases [8, 31, 63], our data showed no effect on milk composition as an individual pathogen, being similar to common veterinary literature that establishes these bacteria as a milk contaminant [4, 27, 40]. We considered increased SCC and decreased milk production in affected cows as a crucial factor confirming the contribution of *Mycoplasma* in bovine mastitis.

M. arginini was another species detected in this study. However, we excluded it from the comparison as an individual group due to the limited number of detection in milk samples ($n = 6$). *M. arginini* has been reported elsewhere with no significant impact on milk composition, but can be considered a predisposing agent to *Str. dysagalactiae* leading to severe mammary gland inflammation [58]. It is important to consider that *M. arginini* is associated with some diseases in human [56, 64]. Evidence of transmission of *M. arginini* to human through bovine milk is available as it has been isolated from blood of a man with a history of milk product consumption who had eosinophilic ascites for 2 years [57]. *M. bovirhinis* (GROUP 3) showed remarkable effect on SCC and milk yield in our study. *M. bovirhinis* was first isolated in mastitis cows in England [21] and named by Leach [37]. These bacteria showed predominance to other mycoplasmas in some mastitis studies [22, 50]. SCC can be elevated in cows inoculated experimentally with four different strains of *M. bovirhinis* causing subclinical mastitis [6]. However, the study of Brownlie et al. [6] lacks information on other milk composition changes.

Somatic cells mainly include macrophages, lymphocytes, polymorphonuclear and epithelial cells [44]. The elevation of these cells in bovine quarters reflects the possibility of infection and is the standard method to discriminate between healthy and mastitic cows [7, 42, 46, 54]. The acceptable limit of individual cow SCC in raw milk in Australia has been established at 250,000 cell/mL [9]. Elevated SCC appears to be an immune response to mastitis caused by *Mycoplasma*, often followed by spontaneous recovery [15]. The negative correlation between high SCC and low milk production has been widely accepted for mastitis cases. In this study, low milk yield

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also appeared to be associated with positive *Mycoplasma* infection which in turn was consistently associated with higher SCC, confirming the economic impact of mastitis caused by *Mycoplasma* mastitis. This decline in milk yield can be attributed to reduction of synthesis ability of the secretory mammary tissue [53].

The role of *Mycoplasma* mastitis in declining milk fat percentage during mastitis has not been established as yet. For other mastitis causing pathogens, contradictory results have been stated for both elevation [46, 55] and depression of milk fat percentage [3, 33, 53]. The decline in fat percentage seems to be a result of leukocyte ingestion to fat globule [49] or due to a decrease in the synthesis and secretion activities of mammary glands [36]. It is also important to note that variation of fat percentage can also affected by stage of lactation, genetics, management, nutrition and hormonal changes [38]. This most likely explains the elevation of fat percentage in the non-tested high SCC (GROUP 7) in this study. Our results show that milk protein percentage can be elevated significantly during mastitis caused by *Mycoplasma* co-infection (GROUP 5) or other non-*Mycoplasma* pathogens (GROUP 6). It is generally thought that milk proteins increase during mastitis due to increases in blood albumins and immunoglobulins influx as an immune response [3, 18]. Protein percentage increases have been reported during *M. agalactiae* experimental mastitis in small ruminant [60].

Decreases in total milk solids were tested to allow farmers, practitioners and scientists who deal with mastitis to directly compare effects of *Mycoplasma* infection on milk yield to each other and to other mastitis pathogens. SCC are usually available only at herd testing level, while total milk solids can be detected daily by in-line milk meters on some farms. Hence, the effect of different mastitis pathogens on total milk solids tends to be more important than SCC records to the current dairy industry. We are aware that SCC are more sensitive to detecting early intramammary infections. With time, in-line SCC measures may be available on many farms, and the importance of total milk solids may not be as high as at this stage. Data on decreases of total milk solids will also allow for further research into economic effects of *Mycoplasma* mastitis to a particular dairy herd or to the entire dairy industry.

Effects on milk production and SCC detected in our study may vary between farms and this should be an area of future research.

Conclusions

In summary, we report bovine milk composition alteration during *Mycoplasma* mastitis. In addition, effects of *Mycoplasma* mastitis were compared with conventional mastitis pathogens. Results of our study indicate that co-infection *Mycoplasma* mastitis (GROUP 4) caused similar effect on milk composition to other conventional mastitis pathogens (GROUP 5), and we hope these findings raise the awareness of the importance of their detection on routine diagnostic panels. Ignoring timely detection may lead to developing *Mycoplasma* co-infection which may result in severe alterations in milk composition. However, roles of each individual *Mycoplasma* spp. in mastitis economics, and produced milk quantity and quality, needs further investigations, particularly when present as co-infections.

Abbreviations

CoNS: Coagulase-negative staphylococci; CoPS: Coagulase-positive staphylococci; PCR: Polymerase chain reaction; SAS: Statistical analysis software; SCC: Somatic cell counts; SCS: Somatic cell scour

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Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AA, FH and KP participated in the study design and coordination. AA, KP, MK & AH contributed to sample collection. AH contributed in herd test data collection and coordination with the farm. AA, MK and FH contributed in sample processing. AA and FH contributed in bacterial culture and PCR in the PC2 laboratory. KP performed the statistical analyses. All authors were involved in drafting the manuscript, read, corrected and approved the final manuscript.

Ethics approval and consent to participate

Not applicable. Samples were collected by field veterinary support that was dealing with the case and no Animal Ethics application was required (Australian code for the care and use of animals for scientific purposes, 8th edition, 2013).

Consent for publication

Not applicable as farm identity is not disclosed.

Competing interests

The authors declare that they have no competing interests.

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References

1. Alberti A, Addis MF, Chessa B, Cubeddu T, Profiti M, Rosati S, Ruiu A, Pittau M. Molecular and antigenic characterization of a Mycoplasma bovis strain causing an outbreak of infectious keratoconjunctivitis. J Vet Diagn Investig. 2006;18:41–51.
2. Amram E, Freed M, Khateb N, Mikula I, Blum S, Spergser J, Sharir B, Ozeri R, Harrus S, Lysnyansky I. Multiple locus variable number tandem repeat analysis of Mycoplasma bovis isolated from local and imported cattle. Vet J. 2013;197:286–90.
3. Auldist M, Hubble I. Effects of mastitis on raw milk and dairy products. Aust J Dairy Technol. 1998;53:28.
4. Boonyayatra S. Diagnosis of mycoplasma mastitis: validation and development. Washington: Washington State University; 2010.
5. Boonyayatra S, Fox LK, Gay JM, Sawant A, Besser TE. Discrimination between Mycoplasma and Acholeplasma species of bovine origin using digitonin disc diffusion assay, nisin disc diffusion assay, and conventional polymerase chain reaction. J Vet Diagn Investig. 2012;24:7–13.
6. Brownlie J, Howard C, Gourlay R. Pathogenicity of certain mycoplasma species in the bovine mammary gland. Res Vet Sci. 1976;20:261–6.
7. Carrillo-Casas EM, Miranda-Morales RE. Bovine mastitis pathogens: prevalence and effects on somatic cell count. Croatia: INTECH Open Access Publisher; 2012.
8. Counter D. A severe outbreak of bovine mastitis assoicated with Mycoplasma bovigenitalium and Acholeplasma laidlawii. Vet Rec. 1978;103:130–1.
9. DairyAustralia. Mastitis. Australia: Dairy Australia; 2014.
10. De Haas Y, Veerkamp R, Barkema H, Gröhn Y, Schukken Y. Associations between pathogen-specific cases of clinical mastitis and somatic cell count patterns. J Dairy Sci. 2004;87:95–105.
11. Dussurget O, Roulland-Dussoix D. Rapid, sensitive PCR-based detection of mycoplasmas in simulated samples of animal sera. Appl Environ Microbiol. 1994;60:953–9.
12. Foster AP, Naylor RD, Howie NM, Nicholas RA, Ayling RD. Mycoplasma bovis and otitis in dairy calves in the United Kingdom. Vet J. 2009;179:455–7.
13. Fox LK. Mycoplasma mastitis: causes, transmission, and control. Vet Clin North Am Food Anim Pract. 2012;28:225–37.
14. George LW, Divers TJ, Ducharme N, Welcome FL. Diseases of the Teats and Udder, Rebhun's diseases of dairy cattle, Second Edition ed. Missouri: Elsevier Health Sciences; 2007. p. 327–93.
15. Ghadersohi A, Hirst RG, Forbes-Faulkener J, Coelen RJ. Preliminary studies on the prevalence of Mycoplasma bovis mastitis in dairy in cattle in Australia. Vet Microbiol. 1999;65:185–94.
16. González RN, Wilson DJ. Mycoplasmal mastitis in dairy herds. Vet Clin N Am Food Anim Pract. 2003;19:199–221.
17. Gourlay RN, Thomas LH, Wyld SG. Increased severity of calf pneumonia associated with the appearance of Mycoplasma bovis in a rearing herd. Vet Rec. 1989;124:420–2.
18. Gráff M, Miko E. Analysis of mastitis in Holstein-Fresian cows and economic effects of mastitis. Agricult Manage/Lucrari Stiintifice Ser I Manage Agricol. 2015;17
19. Green M, Green L, Schukken Y, Bradley A, Peeler E, Barkema H, De Haas Y, Collis V, Medley G. Somatic cell count distributions during lactation predict clinical mastitis. J Dairy Sci. 2004;87:1256–64.
20. Hahn RG, Kenny GE. Differences in arginine requirement for growth among arginine-utilizing Mycoplasma species. J Bacteriol. 1974;117:611–8.
21. Harbourne J, Hunter D, Leach R. The isolation of Mycoplasma from bovine lungs and nasal swabs. Res Vet Sci. 1965;6:178–88.
22. Hirose K, Kawasaki Y, Kotani K, Tanaka A, Abiko K, Ogawa H. Detection of mycoplasma in mastitic milk by PCR analysis and culture method. J Vet Med Sci. 2001;63:691–3.
23. Hirose K, Kobayashi H, Ito N, Kawasaki Y, Zako M, Kotani K, Ogawa H, Sato H. Isolation of Mycoplasmas from nasal swabs of calves affected with respiratory diseases and antimicrobial susceptibility of their isolates. J Veterinary Med Ser B. 2003;50:347–51.
24. Hogan J, Gonzalez R, Harmon R, Nickerson S, Oliver S, Pankey J, Smith KL. Laboratory handbook on bovine mastitis. Madison: National Mastitis Council; 1999. p. 6–10.
25. Horwood PF, Schibrowski MI, Fowler EV, Gibson JS, Barnes TS, Mahony TJ. Is Mycoplasma bovis a missing component of the bovine respiratory disease complex in Australia? Aust Vet J. 2014;92:185–91.
26. Hotzel H, Frey J, Bashiruddin J, Sachse K. Detection and differentiation of ruminant mycoplasmas. PCR Detect Microbial Pathog. 2003;216:231–45.
27. Jasper D, Dellinger J, Rollins M, Hakanson H. Prevalence of mycoplasmal bovine mastitis in California. Am J Vet Res. 1979;40:1043–7.
28. Jasper DE. The role of Mycoplasma in bovine mastitis. J Am Vet Med Assoc. 1982;181:158–62.
29. Justice-Allen A, Trujillo J, Corbett R, Harding R, Goodell G, Wilson D. Survival and replication of Mycoplasma species in recycled bedding sand and association with mastitis on dairy farms in Utah. J Dairy Sci. 2010;93:192–202.
30. Kalogridou-Vassiliadou D. Mastitis-related pathogens in goat milk. Small Rumin Res. 1991;4:203–12.
31. Kirk JH, Glenn K, Ruiz L, Smith E. Epidemiologic analysis of Mycoplasma spp isolated from bulk-tank milk samples obtained from dairy herds that were members of a milk cooperative. J Am Vet Med Assoc. 1997;211:1036–8.
32. Kirk JH, Lauerman LH, Roberts C. Mycoplasma mastitis in dairy cows. Compend Contin Educ Pract Vet. 1994;16:541–52.
33. Kitchen BJ. Bovine mastitis: milk compositional changes and related diagnostic tests. J Dairy Res. 1981;48:167–88.
34. Kobayashi H, Hirose K, Worarach A, Paugtes P, Ito N, Morozumi T, Yamamoto K. In vitro amplification of the 16S rRNA genes from Mycoplasma bovirhinis, Mycoplasma alkalescens and Mycoplasma bovigenitalium by PCR. J Vet Med Sci. 1998;60:1299–303.
35. Kunkel JR. Isolation of Mycoplasma bovis from bulk milk. Cornell Vet. 1985; 75:398–400.
36. Le Maréchal C, Thiéry R, Vautor E, Le Loir Y. Mastitis impact on technological properties of milk and quality of milk products-a review. Dairy Sci Technol. 2011;91:247–82.
37. Leach R. Comparative studies of mycoplasma of bovine origin. Ann N Y Acad Sci. 1967;143:305–16.
38. Linn J. Factors affecting the composition of milk from dairy cows. Designing foods: animal product options in the marketplace. National Research Council (US) committee on technological options to improve the nutritional attributes of animal products, ed. Washington, DC: Natl. Acad. Press; 1988. p. 224.
39. Maunsell FP, Woolums AR, Francoz D, Rosenbusch RF, Step DL, Wilson DJ, Janzen ED. Mycoplasma bovis infections in cattle. J Vet Intern Med. 2011;25: 772–83.
40. Nicholas, R., Ayling, R., McAuliffe, L., 2008. Mycoplasma diseases of ruminants. CABI.
41. Nicholas RA, Ayling RD. Mycoplasma bovis: disease, diagnosis, and control. Res Vet Sci. 2003;74:105–12.
42. Peters M, Silveira I, Fischer V. Impact of subclinical and clinical mastitis on sensitivity to pain of dairy cows. Animal. 2015;9:2024–8.
43. Pfutzner H, Busch S, Hauke H, Landsiedel U, Schimmel D, Templin G, Wehnert C. Studies of Mycoplasma mastitis in cattle. 7. Mycoplasma mastitis in 3 dairy cattle herds. Arch Exp Vet. 1979;33:685–97.
44. Pillai S, Kunze E, Sordillo L, Jayarao B. Application of differential inflammatory cell count as a tool to monitor udder health. J Dairy Sci. 2001; 84:1413–20.
45. Pinnow CC, Butler JA, Sachse K, Hotzel H, Timms LL, Rosenbusch RF. Detection of Mycoplasma bovis in preservative-treated field milk samples. J Dairy Sci. 2001;84:1640–5.
46. Pyörälä S. Indicators of inflammation in the diagnosis of mastitis. Vet Res. 2003;34:565–78.
47. Radaelli E, Castiglioni V, Losa M, Benedetti V, Piccinini R, Nicholas RA, Scanziani E, Luini M. Outbreak of bovine clinical mastitis caused by Mycoplasma bovis in a north Italian herd. Res Vet Sci. 2011;91:251–3.
48. Razin S. Methods in Mycoplasmology V1: Mycoplasma characterization. New York: Elsevier; 2012.
49. Reiter B, Bramley A. Defence mechanisms of the udder and their relevance to mastitis control. Doc Int Dairy Fed. 1975;85:210–22.
50. Saad N, Hameed K. Detection of Mycoplasma species in raw milk of lactating animals in Assiut and Qena city of Egypt. Vet World. 2012;5:80-85.
51. Sachse K, Pfutzner H, Hotzel H, Demuth B, Heller M, Berthold E. Comparison of various diagnostic methods for the detection of Mycoplasma bovis. Rev Sci Tech. 1993;12:571–80.
52. Schnee C, Schulsse S, Hotzel H, Ayling RD, Nicholas RA, Schubert E, Heller M, Ehricht R, Sachse K. A novel rapid DNA microarray assay enables identification of 37 Mycoplasma species and highlights multiple Mycoplasma infections. PLoS One. 2012;7:e33237.
53. Schultz L. Somatic cells in milk-physiological aspects and relationship to amount and composition of milk. J Food Prot®. 1977;40:125–31.

1119

1120

1121

54. Schwarz D, Diesterbeck U, König S, Brügemann K, Schlez K, Zschöck M, Wolter W, Czerny C-P. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. J Dairy Sci. 2011;94:5033–44.
55. Shuster D, Harmon R, Jackson J, Hemken R. Suppression of milk production during endotoxin-induced mastitis. J Dairy Sci. 1991;74:3763–74.
56. Sillis M. Mycoplasma arginini–a new human zoonosis? Clin Infect Dis. 1994; 18:488.
57. Sillo P, Pinter D, Ostorhazi E, Mazan M, Wikonkal N, Ponyai K, Volokhov DV, Chizhikov VE, Szathmary S, Stipkovits L, Karpati S. Eosinophilic fasciitis associated with Mycoplasma arginini infection. J Clin Microbiol. 2012;50:1113–7.
58. Stipkovits L, Somogyi M, Asvanyi B, Toth A, Szathmary S. Short communication: role of Mycoplasma arginini in mastitis caused by streptococcus dysgalactiae. J Dairy Sci. 2013;96:1661–7.
59. Szacawa E, Niemczuk K, Dudek K, Bednarek D, Rosales R, Ayling R. Mycoplasma bovis infections and co-infections with other Mycoplasma spp. with different clinical manifestations in affected cattle herds in eastern region of Poland. Bull Vet Inst Pulawy. 2015;59:331–8.
60. Todaro M, Puleio R, Sabelli C, Scatassa M, Console A, Loria G. Determination of milk production losses in Valle del Belice sheep following experimental infection of Mycoplasma agalactiae. Small Rumin Res. 2015;123:167–72.
61. Watts JL. Etiological agents of bovine mastitis. Vet Microbiol. 1988;16:41–66.
62. Wilson DJ, Gonzalez RN, Das HH. Bovine mastitis pathogens in New York and Pennsylvania: prevalence and effects on somatic cell count and milk production. J Dairy Sci. 1997;80:2592–8.
63. Windsor HM, Windsor GD, Noordergraaf J. The growth and long term survival of Acholeplasma laidlawii in media products used in biopharmaceutical manufacturing. Biologicals. 2010;38:204–10.
64. Yechouron A, Lefebvre J, Robson HG, Rose DL, Tully JG. Fatal septicemia due to Mycoplasma arginini: a new human zoonosis. Clin Infect Dis. 1992; 15:434–8.

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Chapter 7 Evaluation of three cryoprotectants used with bovine milk affected with *Mycoplasma bovis* in different freezing conditions

Aim: This study aimed to compare different cryopreservation compounds and storage temperatures for *M. bovis*.

Null Hypotheses: cryoprotectants additives to milk have no effects on *Mycoplasma bovis* survival during storage at different temperatures.

Notes:

1- Raw data available in Appendix 4.
2- Identification of *Mycoplasma bovis* was carried out by axenization as stated in Chapter 2

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Signature		Date	2/05/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Contributed in sampling, herd test data collection and coordination with the farm. Edited and approved the manuscript.	
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Contribution to the Paper	Assisted in the conceptualisation of the study and the study design, principle supervisor, assisted in sampling, performed statistics, edited and approved the manuscript.	
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BMC Research Notes

RESEARCH NOTE

Open Access

Evaluation of three cryoprotectants used with bovine milk affected with *Mycoplasma bovis* in different freezing conditions

Abd Al-Bar Al-Farha[1,2*], Manouchehr Khazandi[1,3], Farhid Hemmatzadeh[1,3], Razi Jozani[5], Rick Tearle[1,4], Andrew Hoare[6] and Kiro Petrovski[1,3,4]

Abstract

Objectives: Currently, there is no consensus protocols regarding the combination of glycerol (GLY), gelatin or foetal bovine serum (FBS) with dimethyl sulphoxide (DMSO) as cryoprotectants for *Mycoplasma bovis* in bovine milk samples. This study aimed to compare different cryopreservation compounds and storage temperatures for *M. bovis*.

Results: There were significant differences in the survival of *M. bovis* on different media. Differences were also observed between different storage conditions. All additives improved the survival of *M. bovis* in comparison to control (CON). The combination of GLY and DMSO was shown to be significantly different to CON with 57.1% (95% CI = 21.43–133.34) and 19.1% (95% CI = 11.73–60.27), respectively at week 16, and its use should be encouraged as a cryoprotectant for *M. bovis* at − 20 and − 80 °C. GEL/DMSO showed the highest survival rate for *M. bovis* with 57.14% (95% CI = 21.43–133.34) at 4 °C in comparison with CON 14.29% (95% CI = 9.60–50.39). FBS/DMSO showed the highest survival rate for the short-term preservation similarly to other additives. The evaluated cryopreservative compounds would improve survivability of *M. bovis* in milk for both transport and long-term storage. Hence, it is recommended to use the mentioned methods for routine transportation or storage purposes for suspicious *M. bovis* milk samples.

Keywords: *Mycoplasma bovis*, Mastitis, Cryopreservation, Glycerol, DMSO, Gelatin

Introduction

Mycoplasma bovis mastitis is increasingly generating considerable interest in the bovine dairy industry. The current method for isolation of mycoplasmas is using specific mycoplasma culture media. For this purpose, milk samples are sent as frozen or fresh to diagnostic laboratories. An important factor for successful bacterial isolation is to keep *Mycoplasma* organisms viable for growth. Consequently, appropriate sample handling and storage is the main key for diagnosis or research purposes. Due to the lack of cell wall, viability of *M. bovis* under freeze-thaw conditions is a significant challenge in short and long-term preservation. Previous studies have focused on different animal sources of mycoplasmas rather than bovine milk [1, 2]. For bovine milk, standard protocols for prolonged storage of non-*Mycoplasma* mastitis pathogens have been proposed [3–5]. However, these protocols are not applicable to *M. bovis* due to the structural variation between these bacteria and conventional mastitis pathogens. Furthermore, farmers commonly freeze collected milk samples for submission to the diagnostic labs as part of their mastitis management program or if mastitis is a perceived problem on their farm (e.g. increase in incidence of mastitis or treatment failure). Preserving the collected milk is the most important step for bacterial isolation/detection in both bacteriological and molecular methods. A cornerstone for prospective microbiological studies is successful culture of *Mycoplasma*. For routine culture of *Mycoplasma* mastitis, the use of fresh milk samples has

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been recommended [6]. However, preserved milk samples should be considered. Thus, appropriate storage conditions are required to obtain maximal survival of *M. bovis*. A dearth of knowledge regarding appropriate storage of milk samples, both for farmers and researchers, is evident. This study evaluated survival of *M. bovis* in bovine milk following various storage times under three different temperature storage conditions (4 , −20 and −80 °C) using milk only as a control (CON) or three different storage media [milk supplemented with dimethyl sulphoxide (DMSO) and foetal bovine serum (FBS), gelatin (GEL) or glycerol (GLY)].

Main text

Methods

Milk samples

As part of a previous study, milk samples were collected aseptically from 288 cows at individual cow-level from a single commercial dairy farm near Mount Gambier in South Australia [7]. All samples were subjected to cryopreservation, culture and PCR. In this study, twenty-one positive samples for *M. bovis* were selected based on positive culture and PCR results.

Bovis *culture*

Milk samples were subjected to *Mycoplasma* culture using *Mycoplasma* selective media (Oxoid, Sydney, Australia) according to the manufacturer's instructions. *Mycoplasma* colonies were counted using a stereomicroscope at 10× magnification after 7–14 days. Cultures were considered positive when a minimum of one *M. bovis* colony was recorded [8]. At the moment of counting the person who carried out the procedure was not aware of the group allocation. The initial concentration of the organisms in each milk sample was calculated at week 0 for all samples.

Identification of M. bovis *by PCR*

DNA was extracted directly from milk using QIAmp DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Specific 16S rRNA primers designed for *M. bovis* (442 bp), composed of Mbov-F: 5′-CCAGCTCACCCTTATACATGAGCGC-3′ and Mbov-R: 5′-TGACTCACCAATTAGACCGACTATTTCACC-3′. Amplifications were carried out in 25 µL containing 0.25 µL Taq DNA polymerase, 5 µL of 5× reaction buffer (Bioline, UK), 1 µL (0.5 µM) of each forward and reverse primers, 1 µL (approximately 20 ng) of template, and 16.75 µL of DEPC-treated water. Amplifications were performed for 35 PCR cycles conditions using T100™ Thermal Cycler (Biorad thermocycler, Australia), and consisted of pre-heating activation for 5 min at 95 °C, denaturation at 95 °C for 30 s, annealing at 60 °C and primer extension at 72 °C for 45 s. The final extension step was performed at 72 °C for 10 min. The PCR products were analysed by 1.5% agarose gel electrophoresis and visualised by staining with Gel Red.

Evaluating storage-recovery of M. bovis

The following storage media were selected for this study: (a) milk supplemented with 40% FBS and 10% DMSO (treatment group FBS), (b) milk supplemented with 40% GEL (conc. 150 g/L) and 10% DMSO (treatment group GEL), (c) milk supplemented with 40% GLY and 10% DMSO (treatment group GLY), and (d) milk alone (treatment group CON). For each preparation, 8 mL of each *M. bovis* positive milk sample were added into 8 mL of each storage medium, and CON. Each of the diluted samples was aliquoted into 15 Eppendorf tubes (1 mL each) at the same day of collection. For each storage medium, five tubes of aliquots was stored at 4, −20 or −80 °C. There were therefore 21 samples × 4 storage media × 3 storage conditions × 5 time points, totalling 1260 combinations. At each particular time point, one aliquot from each storage media was thawed and cultured onto *Mycoplasma* selective media as described above.

Statistical analysis

The data were binomially distributed (0 = no recovery; 1 = recovery). Hence, a generalised linear model using (R version 3.1.1, R Development Core Team, New Zealand) package was run for the dataset. The predicted survival was estimated accounting for the fixed effect of storage media (FBS, GEL, GLY, or CON), time (weeks), storage condition (4, −20 or −80 °C), and their three-way interaction. Means of survival as rate, 95% confidence intervals, and differences between means were obtained, and are used in the comparison between storage media, time and storage conditions. Survival analysis per treatment group was also carried out and results are presented as figures.

Analysis of variance (ANOVA) using MIXED of the SAS of the number of colony-forming units (CFUs) per plate at each time point was estimated accounting for the fixed effect of storage media (FBS, GEL, GLY, or CON), time (weeks), storage condition (4, −20 or −80 °C), and their three-way interaction. Means of CFU, standard errors and were obtained, and are used in the comparison between storage media, time and storage conditions.

Results

The viability of *M. bovis* for the 21 milk samples after storage at different temperature conditions and milk alone or in combination with three different cryoprotectants (treatment groups FBS, GEL, GLY or CON) are shown in Fig. 1. A significant differences in survival

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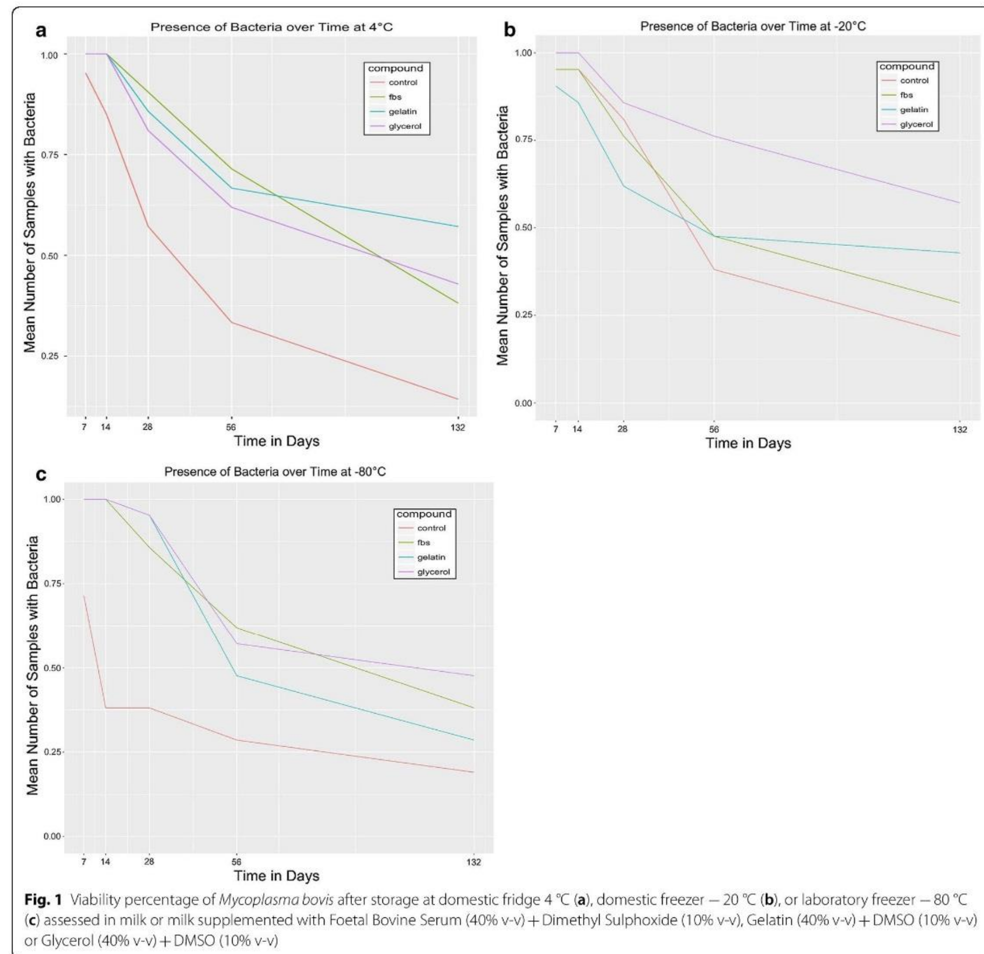

Fig. 1 Viability percentage of *Mycoplasma bovis* after storage at domestic fridge 4 °C (**a**), domestic freezer −20 °C (**b**), or laboratory freezer −80 °C (**c**) assessed in milk or milk supplemented with Foetal Bovine Serum (40% v-v) + Dimethyl Sulphoxide (10% v-v), Gelatin (40% v-v) + DMSO (10% v-v) or Glycerol (40% v-v) + DMSO (10% v-v)

rate of *M. bovis* were detected between different cryoprotectants and temperature conditions. In general, all additives improved the survival rate of *M. bovis* in comparison with CON. The highest survival rate for *M. bovis* isolates was observed at −80 °C followed by −20 and 4 °C. For the long term preservation, GLY was the most effective cryoprotectant; at −20 and −80 °C the survival rate was 57.1% (95% CI = 21.43–133.34) and 47.6% (95% CI = 20–116.04), respectively, in comparison with CON 19.1% (95% CI = 11.73–60.27) in week 16. GEL showed highest *M. bovis* survival rate at 4 °C with 57.1% (95% CI = 21.4–133.34) in comparison with CON 14.3% (95% CI = 9.6–50.39) in week 16. FBS showed the highest survival rate for the short-term preservation, similarly to other additives. However, contrary to survivability rates, no significant differences were observed in the CFUs among the survived isolates Table 1. Agarose gel electrophoresis for specific *M. bovis* PCR of all 21 samples tested revealed amplicon size of 442 bp.

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Table 1 The mean values (± SE) of viable colonies of *Mycoplasma bovis* for 21 milk samples after storage at 4, − 20, or − 80 °C in milk or milk supplemented with Foetal Bovine Serum (40% v-v) + Dimethyl Sulphoxide (10% v-v), Gelatin (40% v-v) + DMSO (10% v-v) or Glycerol (40% v-v) + DMSO (10% v-v)

Storage time (weeks)	The number of *M. bovis* (CFU/mL)											
	4 °C				− 20 °C				− 80 °C			
	CON[a]	FBS[b]	GEL[c]	GLY[d]	CON	FBS	GEL	GLY	CON	FBS	GEL	GLY
1	301.7 ± 94.5	327.6 ± 97.4	361.9 ± 100	312 ± 101	304.7 ± 93.7	342.8 ± 99.5	323.8 ± 96.3	320 ± 100.7	316.1 ± 106.9	327.6 ± 58.1	342.8 ± 100.5	320 ± 100.9
2	285.3 ± 95.8	300.9 ± 92	369.5 ± 99	281 ± 91	281.9 ± 90.6	335.2 ± 94.9	327.6 ± 91.0	308.5 ± 96.1	204.2 ± 90.8	266.6 ± 58.5	300.9 ± 100.1	350.4 ± 105.9
4	154.6 ± 23.3	194 ± 64.3	388 ± 102	300 ± 96	262.8 ± 85.9	274.2 ± 79.7	300.9 ± 110.3	297.1 ± 93.8	117.1 ± 43.8	240 ± 63.8	281.9 ± 89.1	380.9 ± 98.7
8	56.5 ± 23.8	125.7 ± 46	201.9 ± 75.3	289.5 ± 95	137.1 ± 74.9	148.5 ± 52.4	262.8 ± 110.0	335.2 ± 103.3	38 ± 20.9	91.4 ± 20.9	224.7 ± 96.8	384.7 ± 102.3
16	16.6 ± 11.4	72.3 ± 28.9	160 ± 66.6	186.6 ± 73.7	57.1 ± 34	83.8 ± 42.2	110.4 ± 52.8	251.4 ± 78.1	11.4 ± 6.1	38.1 ± 19.6	144.7 ± 83.6	266.6 ± 94.5

[a] Control (milk only); [b] Foetal Bovine Serum (40% v-v) + Dimethyl Sulphoxide (10% v-v); [c] Gelatin (40% v-v) + DMSO (10% v-v); [d] Glycerol (40% v-v) + DMSO (10% v-v)

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Discussion

The effect of *M. bovis* viability under freeze-thaw conditions is a significant challenge in preservation of these bacteria. Lacking of peptidoglycan cell wall in mycoplasmas makes them sensitive to formation of ice crystal during freezing/thawing processes. Sensitivity of *Mycoplasma* spp. to freezing injuries due to phospholipid membrane lipids leakage has been reported in previous studies [9]. *M. bovis* is a fastidious pathogen, and its survival during storage is often affected by both bacterial overgrowth and pH alteration of milk [10]. Intracellular and extracellular ice crystallisation play an important role in cell damage during freezing processes [9, 11]. Our results indicated maximal survival rate of isolates after short- and long-term storage in the treatment group GLY. We hypothesise that optimum survival in freezing conditions containing DMSO was a result of prevention of formation of intracellular ice crystals [12]. GLY has a similar role in cryopreservation that likely results from binding the hydrogen–hydrogen bonds of water intracellularly [13]. The bacteriostatic activity of GLY contributes to inhibition of other bacterial growth leading to improved survival of *Mycoplasma* [14]. The effect of DMSO and GLY as cryoprotectants for various kinds of microorganisms has been previously reported [15]. A lower survival was detected when a combination of FBS and DMSO were used. FBS has been used as a preservation for many types of cells either alone or in combination with DMSO. It is hypothesised that FBS protects cells from osmotic shock, in addition to the neutralising activity against toxic materials released from the damaged cell during the freezing process [15–17]. GEL has been previously used as a preservative for various bacterial species [18], and can act as coating factor to cells similar to GLY. Together, these findings demonstrate that the combination of DMSO + GLY significantly preserves the viability of *M. bovis* under different storage conditions. Our results indicated the legitimacy of using a combination of GEL + DMSO solution as well as FBS + DMSO as additives to milk samples stored at different temperatures.

In conclusion, this study revealed that milk samples supplemented with DMSO and GEL or GLY improved the survival of *M. bovis* associated with mastitis. Our cryoprotectants need to be studied for conventional mastitis pathogens. If the results are similar, addition of preservation additives used in this study can be recommended as a routine procedure for transforming or storing milk samples for different purpose processing.

Limitations

Although there was a dramatic decrease in CFU over the period of preservation points, no significant differences were found. This may be a true effect but may also be due to small sample size. Samples used in this study were not randomly selected. They were selected by chance from the original pool of samples. Hence, one may suspect that the results may not be applicable to the external population. However, in this study each sample had similar chance of being selected for the study, as cows were sampled in non-particular order, and selection of milk samples was in non-particular order as well.

Abbreviations

CON: control; GLY: glycerol; GEL: gelatin; FBS: foetal bovine serum; DMSO: dimethyl sulphoxide; CFU: colony forming unit.

Authors' contributions

AA, MK, FH and KP participated in the study design and sample processing. AA, FH and RJ contributed to the bacterial culture and molecular detection. AA, AH, MK, and KP contributed to samples collection. KP and RT carried out the statistical analysis. All authors were involved in drafting the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent to publish

Not applicable.

Ethics approval and consent to participate

Not applicable. Samples were collected by field veterinary support as a part of the mastitis investigation as per farmer request and no Animal Ethics application was required (Australian code for the care and use of animals for scientific purposes, 8th edition, 2013).

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References

1. Addey JP, Taylor-Robinson D, Dimic M. Viability of mycoplasmas after storage in frozen or lyophilised states. J Med Microbiol. 1970;3(1):137–45.
2. Christensen NH, Yavari CA, McBain AJ, Bradbury JM. Investigations into the survival of Mycoplasma gallisepticum, *Mycoplasma synoviae* and *Mycoplasma iowae* on materials found in the poultry house environment. Avian Pathol. 1994;23(1):127–43.
3. Petzer I-M, Karzis J, Van der Schans TJ, Watermeyer JC, Mitchell-Innes N, Eloff S, Fosgate GT. Comparing effects of freezing at −196 °C and −20 °C on the viability of mastitis pathogens. Onderstepoort J Vet Res. 2012;79(1):01–6.
4. Villanueva M, Tyler J, Thurmond M. Recovery of *Streptococcus agalactiae* and *Staphylococcus aureus* from fresh and frozen bovine milk. J Am Vet Med Assoc. 1991;198(8):1398–400.
5. Schukken Y, Smit J, Grommers F, Vandegeer D, Brand A. Effect of freezing on bacteriologic culturing of mastitis milk samples. J Dairy Sci. 1989;72(7):1900–6.
6. Biddle MK, Fox LK, Hancock DD, Gaskins CT, Evans MA. Effects of storage time and thawing methods on the recovery of Mycoplasma species in milk samples from cows with intramammary infections. J Dairy Sci. 2004;87(4):933–6.
7. Al-Farha AA-B, Hemmatzadeh F, Khazandi M, Hoare A, Petrovski K. Evaluation of effects of *Mycoplasma mastitis* on milk composition in dairy cattle from South Australia. BMC Vet Res. 2017;13(1):351.
8. Markey B, Leonard F, Archambault M, Cullinane A, Maguire D. Clinical veterinary microbiology. Amsterdam: Elsevier Health Sciences; 2013.
9. Raccach M, Rottem S, Razin S. Survival of frozen mycoplasmas. Appl Microbiol. 1975;30(2):167–71.
10. Boonyayatra S, Fox LK, Besser TE, Sawant A, Gay JM. Effects of storage methods on the recovery of Mycoplasma species from milk samples. Vet Microbiol. 2010;144(1–2):210–3.
11. Mazur P. Physical and chemical basis of injury in single-celled microorganisms subjected to freezing and thawing. In: Meryman HT, editor. Cryobiology. Sapporo: Academic Press; 1966. P. 213–5.
12. Pegg DE. Principles of cryopreservation. In: Day JG, Stacey GN, editors. Cryopreservation and freeze-drying protocols. Berlin: Springer; 2007. p. 39–57.
13. Best B. Cryoprotectant toxicity: facts, issues, and questions. Rejuvenation Res. 2015;18(5):422–36.
14. Roger V, Fonty G, Andre C, Gouet P. Effects of glycerol on the growth, adhesion, and cellulolytic activity of rumen cellulolytic bacteria and anaerobic fungi. Curr Microbiol. 1992;25(4):197–201.
15. Hubalek Z. Protectants used in the cryopreservation of microorganisms. Cryobiology. 2003;46(3):205–29.
16. Castro SV, de Carvalho AA, da Silva CMG, Faustino LR, Campello CC, Lucci CM, Báo SN, de Figueiredo JR, Rodrigues APR. Freezing solution containing dimethylsulfoxide and fetal calf serum maintains survival and ultrastructure of goat preantral follicles after cryopreservation and in vitro culture of ovarian tissue. Cell Tissue Res. 2011;346(2):283–92.
17. Mitchell A, Rivas KA, Smith R III, Watts AE. Cryopreservation of equine mesenchymal stem cells in 95% autologous serum and 5% DMSO does not alter post-thaw growth or morphology in vitro compared to fetal bovine serum or allogeneic serum at 20 or 95% and DMSO at 10 or 5%. Stem Cell Res Ther. 2015;6(1):1–12.
18. Obara Y, Yamai S, Nikkawa T, Shimoda Y, Miyamoto Y. Preservation and transportation of bacteria by a simple gelatin disk method. J Clin Microbiol. 1981;14(1):61–6.

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Chapter 8: General discussion

The common themes of this project were to identify a reliable diagnostic strategy for the detection of *Mycoplasma* spp and *A. laidlawii*, investigate the effects of each of these mollicutes on milk quantity and composition, and compare different cryopreservation compounds and storage temperatures for *M. bovis* survivability. Remarkably, a high prevalence of mollicutes was detected in the 368 purposively sampled cows using 16S rRNA universal PCR. Culture was inferior in detecting infected milk samples. The novel universal PCR demonstrated best concordance with the species-specific PCR. The novel real time PCR-HRM analysis was able to discriminate between four of the field isolates of *Mycoplasma* spp. and *Acholelasma laidlawii*. An indirect ELISA used to identify *M. bovis* antibodies in milk detected antibodies in 79/291 samples (27.1%). The co-infection with two or more mollicutes had a similar effect on milk composition to other major mastitis pathogens. All cryopreservatives improved the survivability of mollicutes in milk samples stored under freezing conditions. The combination of GLY and DMSO was shown to be significantly different to CON.

All aims of the research have been discussed in the relevant papers. However, a brief summary of the key findings, principal significance, implementations of this study in the dairy industry, limitations, and recommendations for further research will be discussed here.

For the majority of the work, milk samples originated from a single commercial dairy farm. The studied farm had a high bulk milk SCC with low milk production, and poor response to a wide range of antimicrobial treatment. The routine mastitis culture showed some conventional mastitis pathogens involved (Table 3, Chapter 6). The limited number of major mastitis pathogens isolated from this farm did not reflect the significant number of repeated mastitis cases and treatment failures with conventional antimicrobial therapy, which raised the concern of the potential involvement of minor mastitis bacteria, mainly *M. bovis*.

1185 The relatively high prevelance of *Mycoplasma* mastitis detected on this farm could be attributed
1186 to a variety of transmission methods exhibited by mollicutes including direct contact through
1187 milking machines and other fomites, shedding of the pathogen from mastitic cows or through
1188 infected semen used for artificial insimination (Haapala et al., 2018, Maunsell et al., 2011,
1189 Radaelli et al., 2011, Justice-Allen et al., 2010).

1190 To date, the traditional gold standard method for identification of mastitis caused by mollicutes
1191 is via conventional culture which is well known to be costly and laborious (Wawegama and
1192 Browning, 2017). Thus, molecular diagnostic methods have been developed aiming to become
1193 the gold standard diagnostics (El-Sayed et al., 2017, Nielsen et al., 2015). However, most PCR
1194 techniques previously used to identify *Mycoplasma* mastitis lack the overarching of different
1195 genera and species of mollicutes involved in bovine mastitis, and discrimination between these
1196 mollicutes is poor or not demonstrated. This project, therefore, aimed to provide rapid
1197 identification and discrimination between milk mollicutes, evaluate the effects of these
1198 mollicutes on milk composition and highlight the role of co-infections of these mollicutes
1199 resulting in increased SCC and decreased milk production, similar to other major mastitis
1200 pathogens.

1201 The series of two studies comprised in this thesis (Chapters 3 and 4) aimed to rapidly and
1202 accurately identify mollicutes directly from milk. Early and accurate detection of *Mycoplasma*
1203 mastitis is essential for any control strategy of the disease (Kirk et al., 1994). Current PCR
1204 primers detect few species of cattle-associated mycoplasmas. With aim to save time and money,
1205 a universal primer, capable of detecting all cattle-associated mollucites would be beneficial.
1206 Hence, I have designed and used the universal primer for cattle-associated mollicutes important
1207 for bovine mastitis. In Chapter 3, a universal PCR was developed that can identify a wide range
1208 of bovine mollicutes including mycoplasmas and *A. laidlawii,* and compared it with the species-
1209 specific PCRs. Clinically, this step could be the key element for further investigations or

1210 speciation using sequencing. The test showed significant improvement over the culture method,
1211 and a good Kappa agreement with the species-specific PCRs.
1212 Based on the findings of Chapter 6, co-infection with two or more of these mollicutes had
1213 significant effects on milk compositions, more than infection with a single individual species.
1214 Therefore, developing a highly sensitive test, targeting the genus-level detection method for
1215 bovine milk mollicutes, provided an initial image of the existing mollicutes regardless of the
1216 species involved, taking into consideration the importance of the sterility of the collected milk
1217 samples in order to avoid contamination with the environmental mollicutes. Although detection
1218 by culture was also non-specific for mollicutes, the 16 rRNA amplification was more sensitive.
1219 A previous attempt to target 16S rRNA of group-specific mollicutes has been performed (van
1220 Kuppeveld et al., 1994b); however, this study was conducted on cell culture contaminants, and
1221 detected mollicutes species included: *M. hyorhinis, M. arginini, M. orale, M. fermentans, A,*
1222 *laidlawii* and *M. pirum.* In addition, false-positive results were detected in their study. Hence,
1223 as it targets bovine milk specific mollicutes, the PCR developed in this project had an advantage
1224 over the former genus-specific PCR. Another screening PCR has been developed and used on
1225 several pathogenic mycoplasmas which cause mastitis in cattle (Higuchi et al., 2011). However,
1226 the PCR could only be performed after incubation of *Mycoplasma* in broth, which needs several
1227 days before the PCR process, in addition to culture costs that may become very important or
1228 limiting when dealing with a large sample size. Extraction of DNA directly from milk as carried
1229 out in this project, tended to be significantly faster and more cost-effective. To save on time
1230 and money, this study used PCR as detection tool for the existing mollicutes. Real-life situations
1231 and application in practice would benefit from these two approaches. PCR was conducted on
1232 broth and milk sample directly. The PCR isolation from broth prevents the crude bacterial
1233 growth and restricts it to mollicutes only that were of interest for this study. The method of
1234 culture was the method of axenization. It may be beneficial to pick up a single colony and deal

1235 with an isolate rather than crude growth. However, this would be impractical, laborious and

1236 costly under real-life situation.

1237 Samples collected for this project were not representing all South Australian dairy farms.

1238 However, the sample size was adequate to fulfil the objectives of the project.

1239 This research revealed a novel and rapid real time PCR-HRM assay for the detection of milk

1240 mollicutes. The advantages of the newly developed PCR for screening purposes established in

1241 Chapter 3 were continued in Chapter 4 through a discrimination method between each

1242 individual isolated mollicute using the HRM profile. Five different field mollicutes were

1243 distinguished by this assay including *A. laidlawii, M. arginini, M bovirhinis, M. bovis,* and

1244 unculturable mollicutes. Moreover, results of this experiment may be linked to results in

1245 Chapter 6 when the significant impact of each of these isolates on milk components was

1246 demonstrated. Due to its fast, accurate detection and distinguishing power between various

1247 pathogenic and environmental mollicutes, the clinical applications of this assay can be

1248 implemented immediately in the dairy industry. Validation of the HRM procedure can be

1249 performed through 16S rRNA sequencing. Findings of the HRM analysis in this thesis has

1250 opened up more opportunities for detection and identification of other common *Mycoplasma*

1251 mastitis referenced isolates such as *M. alkalesence, M. californicum, M. candense, M. dispar*

1252 and *M. leachii*. The HRM analysis in this thesis was developed to identify various mycoplasmas

1253 and acholeplasmas. However, only five distinctive profiles were detected most likely due to the

1254 limited variability in the field isolates of this study. Another limitation of HRM analysis is the

1255 inability for identification of co-infection by mycoplasmas and acholeplasmas. Primers

1256 designed in this study can be recommended for milk mollicute DNA amplification using

1257 conventional and/or real time PCRs.

1258 The phylogenetic tree of field isolates of acholeplasmas and mycoplasmas, based on 16 rRNA

1259 sequencing, demonstrated three distinct groups (Fig 1; Chapter 4). I am aware that the term

1260 'isolate' may be challenged by some, however, I have clearly defined the meaning of 'isolate'
1261 in the context of this thesis (see Chapter 1); this should clear the meaning to the potential
1262 readers. The acholeplasma group has been subdivided into *A. laidlawii* and *A. axanthum*
1263 clusters. *M. alkalescens* was isolated from another commercial dairy farm in the Mid-North
1264 region of South Australia, and showed genetic relatedness with *M. arginini* and *M. bovirhinis*.
1265 However, *M. arginini*, which was previously reported in Chapter 2, has not been included in
1266 this phylogeny due to low sequencing quality. The third group in this phylogeny is of *M. bovis*
1267 which demonstrated two distinct clusters mainly having similarity to other Chinese and
1268 Egyptian sources.
1269 The diagnostic strategy presented in this thesis was completed in Chapter 5, discussing the milk
1270 indirect ELISA test. A quarter of the test samples detected *M. bovis* antibodies. *M. bovis,* the
1271 most common pathogenic *Mycoplasma* among the mastitis-causing mollicutes, is well known
1272 to have a characteristic of intermittent shedding in milk (Hazelton et al., 2017). Therefore,
1273 misdiagnosis via methods stated in Chapters 2, 3 and 4 could not be excluded. Hence, the
1274 identification of *M. bovis* antibodies in milk samples was essential. In addition, *M. bovis*
1275 antibody status reflects previous exposure to the pathogen. Consequently, detection of
1276 antibodies may have a high importance in any eradication strategy of the disease. The detection
1277 methods stated in Chapter 5 could serve as a basis for future studies on epidemiological
1278 screening, disease surveillance and biosecurity assessment of *Mycoplasma* mastitis. To ensure
1279 that the signals in MilA ELISA were due to the *M. bovis* antigen and not the result of the GST
1280 fusion antigen to validate the MilA ELISA, GST protein as coating antigen was also carried
1281 out. All samples tested in the MilA ELISA were negative to GST protein. These results were
1282 enough to reject the H0 hypothesis of this chapter. Most studies on milk *M. bovis* antibodies
1283 ELISA have conducted at bulk tank milk level (Parker et. al. 2017, Petersen et. al 2016). The
1284 sensitivity and specificity of bulk milk often differ to the sensitivity and specificity of individual

1285 cow samples. This is partially results of the concentrations of immunoglobulins that can be
1286 detected. The amount of immunoglobulins in bulk milk is diluted by milk harvested from cows
1287 that do not have antibody response yet.

1288 Further assessment of the test sensitivity through performing comparisons between the studied
1289 indirect ELISA and the available commercial kits is required. A reliable method for testing
1290 ELISA targeting the genus-level of mollicutes is yet to be developed. Based on results from this
1291 thesis, it can be concluded that there is limited correlation between *M. bovis* antibodies in milk
1292 and serum (Petersen et al., 2016). This can open an area for further investigations into the
1293 dissemination of *M. bovis* in the body and the characteristics of the immune response by the
1294 host. As there was no correlation between the tested indirect ELISA with the real time PCR,
1295 the tested hypothesis was rejected.

1296 The controversial argument raised in the Chapters 2 to 4 regarding the involvement of different
1297 mollicutes in mastitis has been answered in Chapter 6. Effects of the aforementioned mollicutes
1298 on cow-level milk composition were evaluated in this chapter. Notably, co-infection with any
1299 two or more of these mollicutes had more evident effects on milk quality and quantity than any
1300 individual invasion by these organisms. Moreover, co-infection with mollicutes had similar
1301 effects on milk composition to those of the major conventional mastitis pathogens. Several
1302 studies highlight the synergistic contribution of *M. bovis* with other organisms resulting in
1303 exacerbation of bovine diseases including mastitis (Bürgi et al., 2018, Szacawa et al., 2015,
1304 Shahriar et al., 2002). The likelihood of co-infection with two or more mycoplasmas has been
1305 also reported in cattle with otitis, pneumonia and pink eye (Levisohn et al., 2004, Thomas et
1306 al., 2002). Among 95 dairy herds tested for mycoplasma mastitis in New York State, co-
1307 infection of two or more mycoplasmas has been identified in 14% of herds (Gioia et al., 2016).
1308 Previous studies just re-iterate the validity of the diagnostic procedure described in Chapter 4

1309 of this study. I hope that my findings will further attract attention to the importance of screening
1310 for *Mycoplasma* co-infection.

1311 It is plausible that a number of limitations may have influenced the results obtained from
1312 Chapter 6. To begin with, given that the focus of this analysis is to compare the effects of
1313 infection with mollicutes or other major mastitis pathogens on milk composition, confounding
1314 effects of age, season, stage of lactation, genetics, management, nutrition and hormonal
1315 changes, were not addressed. However, I assumed that cows on this farm were kept under
1316 similar conditions. Compared to the results obtained in Chapter 4, unculturable mollicutes
1317 (n=26) were excluded from the analysis in the Chapter 6. I assumed that they are milk
1318 contaminants and have no effect on the milk composition. Although the study reported in
1319 Chapter 6 analysed the effect of mollicutes on total milk protein, the effect on casein and
1320 immunoglobulins concentrations are yet to be analysed. This may be important as it has been
1321 reported that immune suppression can be induced by *M. bovis* through peripheral-blood
1322 mononuclear cells proliferation inhibition (Suleman et al., 2018b).

1323 The study reported in Chapter 6 initiated numerous queries and the need for further
1324 investigation into the pathogenicity of each of the detected mollicutes, and their role in causing
1325 mastitis either individually or as a co-pathogen with other mollicutes or the major mastitis
1326 pathogens. Indeed, this thesis does not address all diagnostic and pathogenic aspects of
1327 mollicute-associated mastitis. Experimental infection with each individual isolate can answer
1328 some of these questions. The pathogenicity of *M. bovis* is extensively studied in the literature,
1329 including cytoadherance, release of secondary metabolites, invasion of host cells, immune
1330 system suppression and biofilm formation (Suleman et al., 2018a, Burki et al., 2015, Song et
1331 al., 2012, Rottem, 2003). Cytoadherance of *M. bovis* and the role of fibrinoactin (Fn)-binding
1332 of methylenetetrahydrofolate-tRNA-(uracil-5-)-methyltransferase (TrmFO) has been
1333 previously reported (Guo et al., 2017, Rottem, 2003) suggesting the potential implementation

1334 of these study findings in co-infection mollicute mastitis. In this chapter, the H0 hypothesis that
1335 mycoplasmas and *A. laidlawii* have no effect on milk composition as major pathogens has been
1336 rejected.
1337 Finally, comparison of the efficacy and accuracy of the detection methods used in this project
1338 with flow cytometric analysis can be another area of interest. Considerable efforts have been
1339 put into *M. bovis* mastitis antimicrobial susceptibility (Sulyok et al., 2018, Anholt et al., 2017,
1340 Sulyok et al., 2014). However, further work needs to be carried out on antimicrobial
1341 susceptibility of co-infection mycoplasmal mastitis.

1342 Another key contribution of this project was to identify effective storage conditions for milk
1343 contaminated with *M. bovis* for diagnosticians, lab staff and farmers (Chapter 7). Compounds
1344 such as GEL+DMSO, GLY+DMSO and FBS+DMSO improved the survival of *M. bovis* in
1345 comparison to the control (milk alone). GEL+DMSO showed the highest survival rate for *M.*
1346 *bovis* at 4 ℃ in comparison with CON. Therefore, GEL+DMSO was recommended for
1347 transport of milk samples suspected to contain *M.bovis*. The GLY+DMSO additive improved
1348 the survivability at -80 ℃ and it could be used for long-term storage. Therefore, the null
1349 hypothesis that these milk additives would not improve *M. bovis* survivability was rejected.
1350 Unlike other research carried out in this area (Gille et al., 2018), changes in *M. bovis* CFU/mL
1351 between different time points were not significant. Further data collection would be needed to
1352 determine if there is a significant difference in CFU over variety of storage periods. Results of
1353 cryoprotectants used in this study were promising and should be validated by a larger sample
1354 size of milk samples with *M. bovis* mastitis, and with other mastitis pathogens.

1355 In conclusion, this project improved further diagnostics of *Mycoplasma* mastitis through:

1356 Firstly, devising a rapid screening strategy for identification of mollicutes in milk using a novel
1357 conventional PCR which can identify co-infection of different mollicutes genera/species in
1358 clinical samples.

1359 Secondly, demonstrating an innovative discriminative assay between these mollicutes using
1360 real time the PCR-HRM analysis.

1361 Thirdly, evaluating the usefulness of an indirect ELISA for identification of *M. bovis* antibodies
1362 in milk which can be utilised for improving biosecurity.

1363 Above all, the project underlined the importance of the effects of co-infection of mollicutes
1364 over any individual invasion of these bacteria on milk composition which may improve
1365 treatment and control strategies of *Mycoplasma* mastitis.

1366 Finally, considerable progress has been made to improve microbiology of *M. bovis* after storage
1367 for long period under different storage conditions.

1368

1369 **Chapter 9: Collated References**

1370 ADDEY, J. P., TAYLOR-ROBINSON, D. & DIMIC, M. 1970. Viability of mycoplasmas after
1371 storage in frozen or lyophilised states. *J Med Microbiol,* 3**,** 137-145.
1372 AEBI, M., BODMER, M., FREY, J. & PILO, P. 2012. Herd-specific strains of Mycoplasma
1373 bovis in outbreaks of mycoplasmal mastitis and pneumonia. *Vet Microbiol,* 157**,** 363-8.
1374 AEBI, M., VAN DEN BORNE, B. H., RAEMY, A., STEINER, A., PILO, P. & BODMER, M.
1375 2015. Mycoplasma bovis infections in Swiss dairy cattle: a clinical investigation. *Acta*
1376 *Vet Scand,* 57**,** 10.
1377 ALBERTI, A., ADDIS, M. F., CHESSA, B., CUBEDDU, T., PROFITI, M., ROSATI, S.,
1378 RUIU, A. & PITTAU, M. 2006. Molecular and antigenic characterization of a
1379 Mycoplasma bovis strain causing an outbreak of infectious keratoconjunctivitis.
1380 *Journal of Veterinary Diagnostic Investigation,* 18**,** 41-51.
1381 AL-FARHA, A. A., HEMMATZADEH, F., KHAZANDI, M., HOARE, A. & PETROVSKI,
1382 K. 2017b. Evaluation of effects of Mycoplasma mastitis on milk composition in dairy
1383 cattle from South Australia. *BMC Vet Res,* 13**,** 351.
1384 AL-FARHA, A. A., PETROVSKI, K., JOZANI, R., HOARE, A. & HEMMATZADEH, F.
1385 2018. Discrimination between some Mycoplasma spp. and Acholeplasma laidlawii in
1386 bovine milk using high resolution melting curve analysis. *BMC Res Notes,* 11**,** 107.
1387 AMRAM, E., FREED, M., KHATEB, N., MIKULA, I., BLUM, S., SPERGSER, J., SHARIR,
1388 B., OZERI, R., HARRUS, S. & LYSNYANSKY, I. 2013. Multiple locus variable
1389 number tandem repeat analysis of Mycoplasma bovis isolated from local and imported
1390 cattle. *Vet J,* 197**,** 286-90.
1391 ANHOLT, R. M., KLIMA, C., ALLAN, N., MATHESON-BIRD, H., SCHATZ, C.,
1392 AJITKUMAR, P., OTTO, S., PETERS, D., SCHMID, K. & OLSON, M. 2017.
1393 antimicrobial susceptibility of Bacteria That cause Bovine respiratory Disease complex
1394 in alberta, canada. *Frontiers in veterinary science,* 4**,** 207.
1395 AREDE, M., NIELSEN, P. K., AHMED, S. S., HALASA, T., NIELSEN, L. R. & TOFT, N.
1396 2016. A space-time analysis of Mycoplasma bovis: bulk tank milk antibody screening
1397 results from all Danish dairy herds in 2013-2014. Acta Vet Scand, 58, 16.
1398 AULDIST, M. & HUBBLE, I. 1998. Effects of mastitis on raw milk and dairy products.
1399 *Australian Journal of Dairy Technology,* 53**,** 28.
1400 BASHIRUDDIN, J. B., FREY, J., KONIGSSON, M. H., JOHANSSON, K. E., HOTZEL, H.,
1401 DILLER, R., DE SANTIS, P., BOTELHO, A., AYLING, R. D., NICHOLAS, R. A.,
1402 THIAUCOURT, F. & SACHSE, K. 2005. Evaluation of PCR systems for the
1403 identification and differentiation of Mycoplasma agalactiae and Mycoplasma bovis: a
1404 collaborative trial. *Vet J,* 169**,** 268-75.
1405 BEHERA, S., RANA, R., GUPTA, P. K., KUMAR, D., SONAL, REKHA, V., ARUN, T. R.
1406 & JENA, D. 2018. Development of real-time PCR assay for the detection of
1407 Mycoplasma bovis. *Trop Anim Health Prod.*
1408 BENNETT, R. H. & JASPER, D. E. 1978. Mycoplasma alkalescens-induced arthritis in dairy
1409 calves. *J Am Vet Med Assoc,* 172**,** 484-8.
1410 BEST, B. 2015. Cryoprotectant Toxicity: Facts, Issues, and Questions. *Rejuvenation research,*
1411 18**,** 422-436.
1412 BIDDLE, M. K., FOX, L. K. & HANCOCK, D. D. 2003. Patterns of mycoplasma shedding in
1413 the milk of dairy cows with intramammary mycoplasma infection. *J Am Vet Med Assoc,*
1414 223**,** 1163-6.
1415 BIDDLE, M. K., FOX, L. K., HANCOCK, D. D., GASKINS, C. T. & EVANS, M. A. 2004.
1416 Effects of storage time and thawing methods on the recovery of Mycoplasma species in
1417 milk samples from cows with intramammary infections. *J Dairy Sci,* 87**,** 933-6.

1418 BOONYAYATRA, S. 2010. *Diagnosis of mycoplasma mastitis: Validation and development.*
1419 Washington State University.
1420 BOONYAYATRA, S., FOX, L. K., BESSER, T. E., SAWANT, A. & GAY, J. M. 2010. Effects
1421 of storage methods on the recovery of Mycoplasma species from milk samples. *Vet*
1422 *Microbiol,* 144**,** 210-3.
1423 BOONYAYATRA, S., FOX, L. K., GAY, J. M., SAWANT, A. & BESSER, T. E. 2012.
1424 Discrimination between Mycoplasma and Acholeplasma species of bovine origin using
1425 digitonin disc diffusion assay, nisin disc diffusion assay, and conventional polymerase
1426 chain reaction. *J Vet Diagn Invest,* 24**,** 7-13.
1427 BROWNLIE, J., HOWARD, C. & GOURLAY, R. 1976. Pathogenicity of certain mycoplasma
1428 species in the bovine mammary gland. *Res Vet Sci,* 20**,** 261-266.
1429 BÜRGI, N., JOSI, C., BÜRKI, S., SCHWEIZER, M. & PILO, P. 2018. Mycoplasma bovis co-
1430 infection with bovine viral diarrhea virus in bovine macrophages. *Veterinary research,*
1431 49**,** 2.
1432 BURKI, S., FREY, J. & PILO, P. 2015. Virulence, persistence and dissemination of
1433 Mycoplasma bovis. *Vet Microbiol,* 179**,** 15-22.
1434 BYRNE, W. J., BALL, H. J., BRICE, N., MCCORMACK, R., BAKER, S. E., AYLING, R. D.
1435 & NICHOLAS, R. A. 2000. Application of an indirect ELISA to milk samples to
1436 identify cows with Mycoplasma bovis mastitis. *Vet Rec,* 146**,** 368-9.
1437 BYRNE, W., MARKEY, B., MCCORMACK, R., EGAN, J., BALL, H. & SACHSE, K. 2005.
1438 Persistence of Mycoplasma bovis infection in the mammary glands of lactating cows
1439 inoculated experimentally. Vet Rec, 156, 767-71.
1440 CALCUTT, M. J., LYSNYANSKY, I., SACHSE, K., FOX, L. K., NICHOLAS, R. A. J. &
1441 AYLING, R. D. 2018. Gap analysis of Mycoplasma bovis disease, diagnosis and
1442 control: An aid to identify future development requirements. *Transbound Emerg Dis*.
1443 CARRILLO-CASAS, E. M. & MIRANDA-MORALES, R. E. 2012. *Bovine mastitis*
1444 *pathogens: prevalence and effects on somatic cell count*, INTECH Open Access
1445 Publisher.
1446 CASTRO, S. V., DE CARVALHO, A. A., DA SILVA, C. M. G., FAUSTINO, L. R.,
1447 CAMPELLO, C. C., LUCCI, C. M., BÁO, S. N., DE FIGUEIREDO, J. R. &
1448 RODRIGUES, A. P. R. 2011. Freezing solution containing dimethylsulfoxide and fetal
1449 calf serum maintains survival and ultrastructure of goat preantral follicles after
1450 cryopreservation and in vitro culture of ovarian tissue. *Cell and tissue research,* 346**,**
1451 283-292.
1452 CHEN, S., HAO, H., ZHAO, P., GAO, P., HE, Y., JI, W., WANG, Z., LU, Z., LIU, Y. & CHU,
1453 Y. 2017. Complete Genome Sequence of Mycoplasma bovis Strain 08M. *Genome*
1454 *Announc,* 5.
1455 CHRISTENSEN, N. H., YAVARI, C. A., MCBAIN, A. J. & BRADBURY, J. M. 1994.
1456 Investigations into the survival of Mycoplasma gallisepticum, Mycoplasma synoviae
1457 and Mycoplasma iowae on materials found in the poultry house environment. *Avian*
1458 *Pathol,* 23**,** 127-43.
1459 CONNOLE, M., LAWS, L. & HART, R. 1967. Mastitis in cattle caused by a Mycoplasma sp.
1460 *Australian veterinary journal,* 43**,** 157-162.
1461 COUNTER, D. E. 1978. A severe outbreak of bovine mastitis assoicated with Mycoplasma
1462 bovigenitalium and Acholeplasma laidlawii. *Vet Rec,* 103**,** 130-1.
1463 DAIRYAUSTRALIA. 2014. *Mastitis* [Online]. Australia: Dairy Australia. Available:
1464 http://www.dairyaustralia.com.au/Animal-management/Mastitis.aspx [Accessed
1465 1/3/2016 2016].
1466 DAVIDSON, I. & STUART, P. 1960. Bovine mastitis caused by a mycoplasma. *Veterinary*
1467 *Record,* 72**,** 766.

1468 DE HAAS, Y., VEERKAMP, R., BARKEMA, H., GRÖHN, Y. & SCHUKKEN, Y. 2004.
1469 Associations between pathogen-specific cases of clinical mastitis and somatic cell count
1470 patterns. *Journal of Dairy Science,* 87**,** 95-105.
1471 D'INZEO, T., DE ANGELIS, G., FIORI, B., MENCHINELLI, G., LIOTTI, F. M.,
1472 MORANDOTTI, G. A., DE MAIO, F., NAGEL, D., ANTONACI, M. &
1473 SANGUINETTI, M. 2017. Comparison of Mycoplasma IES, Mycofast Revolution and
1474 Mycoplasma IST2 to detect genital mycoplasmas in clinical samples. *The Journal of*
1475 *Infection in Developing Countries,* 11**,** 98-101.
1476 DUSSURGET, O. & ROULLAND-DUSSOIX, D. 1994. Rapid, sensitive PCR-based detection
1477 of mycoplasmas in simulated samples of animal sera. *Applied and environmental*
1478 *microbiology,* 60**,** 953-959.
1479 EL-SAYED, A., AWAD, W., ABDOU, N.-E. & VÁZQUEZ, H. C. 2017. Molecular biological
1480 tools applied for identification of mastitis causing pathogens. *International Journal of*
1481 *Veterinary Science and Medicine*.
1482 FOSTER, A. P., NAYLOR, R. D., HOWIE, N. M., NICHOLAS, R. A. & AYLING, R. D.
1483 2009. Mycoplasma bovis and otitis in dairy calves in the United Kingdom. *Vet J,* 179**,**
1484 455-7.
1485 FOX, L. K. 2012. Mycoplasma mastitis: causes, transmission, and control. *Vet Clin North Am*
1486 *Food Anim Pract,* 28**,** 225-37.
1487 GAGO, S., ZARAGOZA, Ó., CUESTA, I., RODRÍGUEZ-TUDELA, J. L., CUENCA-
1488 ESTRELLA, M. & BUITRAGO, M. J. 2011. High-resolution melting analysis for
1489 identification of the Cryptococcus neoformans-Cryptococcus gattii complex. *Journal of*
1490 *clinical microbiology,* 49**,** 3663-3666.
1491 GEORGE, L. W., DIVERS, T. J., DUCHARME, N. & WELCOME, F. L. 2007. Diseases of
1492 the Teats and Udder. *Rebhun's diseases of dairy cattle.* Second Edition ed.: Elsevier
1493 Health Sciences.
1494 GHADERSOHI, A., COELEN, R. J. & HIRST, R. G. 1997. Development of a specific DNA
1495 probe and PCR for the detection of Mycoplasma bovis. *Vet Microbiol,* 56**,** 87-98.
1496 GHADERSOHI, A., HIRST, R. G., FORBES-FAULKENER, J. & COELEN, R. J. 1999.
1497 Preliminary studies on the prevalence of Mycoplasma bovis mastitis in dairy in cattle in
1498 Australia. *Vet Microbiol,* 65**,** 185-94.
1499 GHANEM, M. E., HIGUCHI, H., TEZUKA, E., ITO, H., DEVKOTA, B., IZAIKE, Y. &
1500 OSAWA, T. 2013. Mycoplasma infection in the uterus of early postpartum dairy cows
1501 and its relation to dystocia and endometritis. *Theriogenology,* 79**,** 180-5.
1502 GHORASHI, S. A., NOORMOHAMMADI, A. H. & MARKHAM, P. F. 2010. Differentiation
1503 of Mycoplasma gallisepticum strains using PCR and high-resolution melting curve
1504 analysis. *Microbiology,* 156**,** 1019-29.
1505 GIOIA, G., WERNER, B., NYDAM, D. V. & MORONI, P. 2016. Validation of a mycoplasma
1506 molecular diagnostic test and distribution of mycoplasma species in bovine milk among
1507 New York State dairy farms. J Dairy Sci, 99, 4668-4677.
1508 GILLE, L., BOYEN, F., VAN DRIESSCHE, L., VALGAEREN, B., HAESEBROUCK, F.,
1509 DEPREZ, P. & PARDON, B. 2018. Effect of freezer storage time and thawing method
1510 on the recovery of Mycoplasma bovis from bovine colostrum. *Journal of dairy science,*
1511 101**,** 609-613.
1512 GILLE, L., PILO, P., VAN DRIESSCHE, L., VAN LOO, H., BODMER, M., BÜRKI, S.,
1513 BOYEN, F., HAESEBROUCK, F., DEPREZ, P. & PARDON, B. Involvement of
1514 Mycoplasma bovis in postsurgical seromas in Belgian Blue cattle. 49th European
1515 Veterinary Conference: Voorjaarsdagen 2016, 2016.
1516 GONZÁLEZ, R. N. & WILSON, D. J. 2003. Mycoplasmal mastitis in dairy herds. *Veterinary*
1517 *Clinics of North America: Food Animal Practice,* 19**,** 199-221.

1518 GOURLAY, R. N., THOMAS, L. H. & WYLD, S. G. 1989. Increased severity of calf
1519 pneumonia associated with the appearance of Mycoplasma bovis in a rearing herd. *Vet*
1520 *Rec,* 124**,** 420-2.
1521 GRÁFF, M. & MIKO, E. 2015. Analysis Of Mastitis In Holstein-Fresian Cows And Economic
1522 Effects Of Mastitis. *Agricultural Management/Lucrari Stiintifice Seria I, Management*
1523 *Agricol,* 17.
1524 GREEN, M., GREEN, L., SCHUKKEN, Y., BRADLEY, A., PEELER, E., BARKEMA, H.,
1525 DE HAAS, Y., COLLIS, V. & MEDLEY, G. 2004. Somatic cell count distributions
1526 during lactation predict clinical mastitis. *Journal of dairy science,* 87**,** 1256-1264.
1527 GUO, Y., ZHU, H., WANG, J., HUANG, J., KHAN, F. A., ZHANG, J., GUO, A. & CHEN,
1528 X. 2017. TrmFO, a Fibronectin-Binding Adhesin of Mycoplasma bovis. *International*
1529 *journal of molecular sciences,* 18**,** 1732.
1530 HAAPALA, V., POHJANVIRTA, T., VÄHÄNIKKILÄ, N., HALKILAHTI, J., SIMONEN,
1531 H., PELKONEN, S., SOVERI, T., SIMOJOKI, H. & AUTIO, T. 2018. Semen as a
1532 source of Mycoplasma bovis mastitis in dairy herds. *Veterinary Microbiology*.
1533 HAHN, R. G. & KENNY, G. E. 1974. Differences in arginine requirement for growth among
1534 arginine-utilizing Mycoplasma species. *Journal of bacteriology,* 117**,** 611-618.
1535 HARBOURNE, J., HUNTER, D. & LEACH, R. 1965. THE ISOLATION OF
1536 MYCOPLASMA FROM BOVINE LUNGS AND NASAL SWABS. *Research in*
1537 *veterinary science,* 6**,** 178-188.
1538 HATA, E. 2015. Complete Genome Sequence of Mycoplasma arginini Strain HAZ 145_1 from
1539 Bovine Mastitic Milk in Japan. *Genome Announc,* 3.
1540 HATA, E., NAGAI, K. & MURAKAMI, K. 2017. Complete Genome Sequence of
1541 Mycoplasma bovirhinis Strain HAZ141_2 from Bovine Nasal Discharge in Japan.
1542 *Genome Announc,* 5.
1543 HAZELTON, M., SHEEHY, P., BOSWARD, K., PARKER, A., MORTON, J., DWYER, C.,
1544 NIVEN, P. & HOUSE, J. 2017. Shedding of Mycoplasma bovis and antibody responses
1545 in cows recently diagnosed with clinical infection. *Journal of Dairy Science*.
1546 HIGUCHI, H., IWANO, H., KAWAI, K., OHTA, T., OBAYASHI, T., HIROSE, K., ITO, N.,
1547 YOKOTA, H., TAMURA, Y. & NAGAHATA, H. 2011. A simplified PCR assay for
1548 fast and easy mycoplasma mastitis screening in dairy cattle. *J Vet Sci,* 12**,** 191-3.
1549 HIROSE, K., KAWASAKI, Y., KOTANI, K., TANAKA, A., ABIKO, K. & OGAWA, H.
1550 2001. Detection of mycoplasma in mastitic milk by PCR analysis and culture method.
1551 *J Vet Med Sci,* 63**,** 691-3.
1552 HIROSE, K., KOBAYASHI, H., ITO, N., KAWASAKI, Y., ZAKO, M., KOTANI, K.,
1553 OGAWA, H. & SATO, H. 2003. Isolation of Mycoplasmas from nasal swabs of calves
1554 affected with respiratory diseases and antimicrobial susceptibility of their isolates.
1555 *Journal of Veterinary Medicine, Series B,* 50**,** 347-351.
1556 HOGAN, J., GONZALEZ, R., HARMON, R., NICKERSON, S., OLIVER, S., PANKEY, J. &
1557 SMITH, K. L. 1999. Laboratory handbook on bovine mastitis. *National Mastitis*
1558 *Council, Madison, WI***,** 6-10.
1559 HORWOOD, P. F., SCHIBROWSKI, M. I., FOWLER, E. V., GIBSON, J. S., BARNES, T. S.
1560 & MAHONY, T. J. 2014. Is Mycoplasma bovis a missing component of the bovine
1561 respiratory disease complex in Australia? *Aust Vet J,* 92**,** 185-91.
1562 HOTZEL, H., DEMUTH, B., SACHSE, K., PFLITSCH, A. & PFUTZNER, H. 1993. Detection
1563 of Mycoplasma bovis using in vitro deoxyribonucleic acid amplification. *Rev Sci Tech,*
1564 12**,** 581-91.
1565 HOTZEL, H., FREY, J., BASHIRUDDIN, J. & SACHSE, K. 2003. Detection and
1566 differentiation of ruminant mycoplasmas. *PCR Detection of Microbial Pathogens***,** 231-
1567 245.

1568 HUBALEK, Z. 2003. Protectants used in the cryopreservation of microorganisms. *Cryobiology,*
1569 46**,** 205-229.
1570 HUDSON, J. & ETHERIDGE, J. 1963. A NEW TYPE OF PLEUROPNEUMONIA-LIKE
1571 ORGANISM (PPLO) FROM THE NOSE OF CATTLE. *Australian Veterinary Journal,*
1572 39**,** 1-5.
1573 INFANTE-MARTINEZ, F., AGUADO, J. & EDUARD-JASPER, D. 1999. Mastitis outbreak
1574 due to Mycoplasma californicum and Mycoplasma canadense in a commercial dairy
1575 herd in the state of Jalisco, Mexico. *Rev Latinoam Microbiol,* 41**,** 117-20.
1576 JANG, H., KIM, H., KANG, B., KIM, C. & PARK, H. 2009. Oligonucleotide array-based
1577 detection and genotyping of mollicutes (Acholeplasma, Mycoplasma, and Ureaplasma).
1578 *J Microbiol Biotechnol,* 19**,** 265-70.
1579 JASPER, D. E. 1982. The role of Mycoplasma in bovine mastitis. *J Am Vet Med Assoc,* 181**,**
1580 158-62.
1581 JASPER, D., DELLINGER, J., ROLLINS, M. & HAKANSON, H. 1979. Prevalence of
1582 mycoplasmal bovine mastitis in California. *American journal of veterinary research,*
1583 40**,** 1043-1047.
1584 JUSTICE-ALLEN, A., TRUJILLO, J., CORBETT, R., HARDING, R., GOODELL, G. &
1585 WILSON, D. 2010. Survival and replication of Mycoplasma species in recycled
1586 bedding sand and association with mastitis on dairy farms in Utah. *J Dairy Sci,* 93**,** 192-
1587 202.
1588 JUSTICE-ALLEN, A., TRUJILLO, J., GOODELL, G. & WILSON, D. 2011. Detection of
1589 multiple Mycoplasma species in bulk tank milk samples using real-time PCR and
1590 conventional culture and comparison of test sensitivities. *J Dairy Sci,* 94**,** 3411-9.
1591 KALOGRIDOU-VASSILIADOU, D. 1991. Mastitis-related pathogens in goat milk. *Small*
1592 *Ruminant Research,* 4**,** 203-212.
1593 KIRK, J. H., GLENN, K., RUIZ, L. & SMITH, E. 1997. Epidemiologic analysis of
1594 Mycoplasma spp isolated from bulk-tank milk samples obtained from dairy herds that
1595 were members of a milk cooperative. *J Am Vet Med Assoc,* 211**,** 1036-8.
1596 KIRK, J. H., LAUERMAN, L. H. & ROBERTS, C. 1994. Mycoplasma mastitis in dairy cows.
1597 *Compendium on Continuing Education for the Practicing Veterinarian,* 16**,** 541-552.
1598 KITCHEN, B. J. 1981. Bovine mastitis: milk compositional changes and related diagnostic
1599 tests. *Journal of Dairy Research,* 48**,** 167-188.
1600 KOBAYASHI, H., HIROSE, K., WORARACH, A., PAUGTES, P., ITO, N., MOROZUMI, T.
1601 & YAMAMOTO, K. 1998. In vitro amplification of the 16S rRNA genes from
1602 Mycoplasma bovirhinis, Mycoplasma alkalescens and Mycoplasma bovigenitalium by
1603 PCR. *J Vet Med Sci,* 60**,** 1299-303.
1604 KOKOTOVIC, B., FRIIS, N. F. & AHRENS, P. 2007. Mycoplasma alkalescens demonstrated
1605 in bronchoalveolar lavage of cattle in Denmark. *Acta Vet Scand,* 49**,** 2.
1606 KUNKEL, J. R. 1985. Isolation of Mycoplasma bovis from bulk milk. *Cornell Vet,* 75**,** 398-
1607 400.
1608 LARKIN, M. A., BLACKSHIELDS, G., BROWN, N., CHENNA, R., MCGETTIGAN, P. A.,
1609 MCWILLIAM, H., VALENTIN, F., WALLACE, I. M., WILM, A. & LOPEZ, R. 2007.
1610 Clustal W and Clustal X version 2.0. *bioinformatics,* 23**,** 2947-2948.
1611 LE MARÉCHAL, C., THIÉRY, R., VAUTOR, E. & LE LOIR, Y. 2011. Mastitis impact on
1612 technological properties of milk and quality of milk products-A review. Dairy Sci
1613 Technol 91: 247–282.
1614 LEACH, R. 1967. Comparative studies of mycoplasma of bovine origin. *Annals of the New*
1615 *York Academy of Sciences,* 143**,** 305-316.

1616 LEVISOHN, S., GARAZI, S., GERCHMAN, I. & BRENNER, J. 2004. Diagnosis of a mixed
1617 mycoplasma infection associated with a severe outbreak of bovine pinkeye in young
1618 calves. *Journal of veterinary diagnostic investigation,* 16**,** 579-581.
1619
1620 LINN, J. 1988. Factors affecting the composition of milk from dairy cows. *Designing Foods:*
1621 *Animal Product Options in the Marketplace. National Research Council (US)*
1622 *Committee on Technological Options to Improve the Nutritional Attributes of Animal*
1623 *Products, ed. Natl. Acad. Press, Washington, DC***,** 224.
1624 LIU, Y., SINGH, P. & MUSTAPHA, A. 2018. High-resolution melt curve PCR assay for
1625 specific detection of E. coli O157: H7 in beef. *Food Control,* 86**,** 275-282.
1626 LORENZ, T. C. 2012. Polymerase chain reaction: basic protocol plus troubleshooting and
1627 optimization strategies. Journal of visualized experiments: JoVE.
1628 LOWE, J., FOX, L., ENGER, B., PROGAR, A. A. & GAY, J. 2018. Effect of atmospheric
1629 carbon dioxide concentration on the cultivation of bovine Mycoplasma species. *Journal*
1630 *of Dairy Science*.
1631 LYSNYANSKY, I. & AYLING, R. D. 2016. Mycoplasma bovis: Mechanisms of Resistance
1632 and Trends in Antimicrobial Susceptibility. *Front Microbiol,* 7**,** 595.
1633 MANSO-SILVAN, L., TARDY, F., BARANOWSKI, E., BARRE, A., BLANCHARD, A.,
1634 BRETON, M., COUTURE, C., CITTI, C., DORDET-FRISONI, E., DUPUY, V.,
1635 GAURIVAUD, P., JACOB, D., LEMAITRE, C., NIKOLSKI, M., NOUVEL, L. X.,
1636 POUMARAT, F., THEBAULT, P., THEIL, S., THIAUCOURT, F. & SIRAND-
1637 PUGNET, P. 2013. Draft Genome Sequences of Mycoplasma alkalescens, Mycoplasma
1638 arginini, and Mycoplasma bovigenitalium, Three Species with Equivocal Pathogenic
1639 Status for Cattle. *Genome Announc,* 1.
1640 MARKEY, B., LEONARD, F., ARCHAMBAULT, M., CULLINANE, A. & MAGUIRE, D.
1641 2013. *Clinical veterinary microbiology*, Elsevier Health Sciences.
1642 MAUNSELL, F. P., WOOLUMS, A. R., FRANCOZ, D., ROSENBUSCH, R. F., STEP, D. L.,
1643 WILSON, D. J. & JANZEN, E. D. 2011. Mycoplasma bovis infections in cattle. *J Vet*
1644 *Intern Med,* 25**,** 772-83.
1645 MAZUR, P. 1966. Physical and chemical basis of injury in single-celled microorganisms
1646 subjected to freezing and thawing. *Cryobiology,* 213.
1647 MITCHELL, A., RIVAS, K. A., SMITH III, R. & WATTS, A. E. 2015. Cryopreservation of
1648 equine mesenchymal stem cells in 95% autologous serum and 5% DMSO does not alter
1649 post-thaw growth or morphology in vitro compared to fetal bovine serum or allogeneic
1650 serum at 20 or 95% and DMSO at 10 or 5%. *Stem cell research & therapy,* 6**,** 1-12.
1651 MORTON, J., MALMO, J., HOUSE, J., MEIN, G., IZZO, M. & PENRY, J. 2014. Mycoplasma
1652 bovis in Australian dairy herds. *Aust Vet J,* 92**,** 322-3.
1653 NICHOLAS, R. & AYLING, R. 2016. Mycoplasma in cattle. *Vet Rec,* 178**,** 478-9.
1654 NICHOLAS, R. A. & AYLING, R. D. 2003. Mycoplasma bovis: disease, diagnosis, and
1655 control. *Res Vet Sci,* 74**,** 105-12.
1656 NICHOLAS, R. A., AYLING, R. D. & STIPKOVITS, L. P. 2002. An experimental vaccine for
1657 calf pneumonia caused by Mycoplasma bovis: clinical, cultural, serological and
1658 pathological findings. *Vaccine,* 20**,** 3569-75.
1659 NICHOLAS, R. A., FOX, L. K. & LYSNYANSKY, I. 2016. Mycoplasma mastitis in cattle:
1660 To cull or not to cull. *The Veterinary Journal,* 216**,** 142-147.
1661 NICHOLAS, R., AYLING, R. & MCAULIFFE, L. 2007. Mycoplasma mastitis. *Vet Rec,* 160**,**
1662 382; author reply 383.
1663 NICHOLAS, R., AYLING, R. & MCAULIFFE, L. 2008. *Mycoplasma diseases of ruminants*,
1664 CABI.

1665 NIELSEN, P. K., PETERSEN, M. B., NIELSEN, L. R., HALASA, T. & TOFT, N. 2015. Latent
1666 class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of
1667 Mycoplasma bovis. *Prev Vet Med,* 121**,** 338-42.
1668 OBARA, Y., YAMAI, S., NIKKAWA, T., SHIMODA, Y. & MIYAMOTO, Y. 1981.
1669 Preservation and transportation of bacteria by a simple gelatin disk method. *Journal of*
1670 *clinical microbiology,* 14**,** 61-66.
1671 PARKER, A. M., HOUSE, J. K., HAZELTON, M. S., BOSWARD, K. L. & SHEEHY, P. A.
1672 2017b. Comparison of culture and a multiplex probe PCR for identifying Mycoplasma
1673 species in bovine milk, semen and swab samples. *PloS one,* 12**,** e0173422.
1674 PARKER, A. M., SHEEHY, P. A., HAZELTON, M. S., BOSWARD, K. L. & HOUSE, J. K.
1675 A review of mycoplasma diagnostics in cattle. *Journal of Veterinary Internal Medicine,*
1676 0.
1677 PARKER, A. M., SHUKLA, A., HOUSE, J. K., HAZELTON, M. S., BOSWARD, K. L.,
1678 KOKOTOVIC, B. & SHEEHY, P. A. 2016. Genetic characterization of Australian
1679 Mycoplasma bovis isolates through whole genome sequencing analysis. *Vet Microbiol,*
1680 196**,** 118-125.
1681 PARKER, A., HOUSE, J., HAZELTON, M., BOSWARD, K., MORTON, J. & SHEEHY, P.
1682 2017a. Bulk tank milk antibody ELISA as a biosecurity tool for detecting dairy herds
1683 with past exposure to Mycoplasma bovis. *Journal of Dairy Science,* 100**,** 8296-8309.
1684 PEGG, D. E. 2007. Principles of cryopreservation. *Cryopreservation and freeze-drying*
1685 *protocols.* Springer.
1686 PEREDELTCHOUK, M., DAVID, S. A., BHATTACHARYA, B., VOLOKHOV, D. V. &
1687 CHIZHIKOV, V. 2011. Detection of mycoplasma contamination in cell substrates using
1688 reverse transcription-PCR assays. *J Appl Microbiol,* 110**,** 54-60.
1689 PETERS, M., SILVEIRA, I. & FISCHER, V. 2015. Impact of subclinical and clinical mastitis
1690 on sensitivity to pain of dairy cows. *animal,* 9**,** 2024-2028.
1691 PETERSEN, M. B., KROGH, K. & NIELSEN, L. R. 2016. Factors associated with variation
1692 in bulk tank milk Mycoplasma bovis antibody-ELISA results in dairy herds. *Journal of*
1693 *dairy science,* 99**,** 3815-3823.
1694 PETROVSKI, K. 2006. Milk composition changes during mastitis.
1695 PETTERSSON, B., LEITNER, T., RONAGHI, M., BOLSKE, G., UHLEN, M. &
1696 JOHANSSON, K. E. 1996. Phylogeny of the Mycoplasma mycoides cluster as
1697 determined by sequence analysis of the 16S rRNA genes from the two rRNA operons.
1698 *J Bacteriol,* 178**,** 4131-42.
1699 PETZER, I.-M., KARZIS, J., VAN DER SCHANS, T. J., WATERMEYER, J. C.,
1700 MITCHELL-INNES, N., ELOFF, S. & FOSGATE, G. T. 2012. Comparing effects of
1701 freezing at-196 ℃ and-20 ℃ on the viability of mastitis pathogens. *Onderstepoort*
1702 *Journal of Veterinary Research,* 79**,** 01-06.
1703 PFUTZNER, H. & SACHSE, K. 1996. Mycoplasma bovis as an agent of mastitis, pneumonia,
1704 arthritis and genital disorders in cattle. *Rev Sci Tech,* 15**,** 1477-94.
1705 PFUTZNER, H., BUSCH, S., HAUKE, H., LANDSIEDEL, U., SCHIMMEL, D., TEMPLIN,
1706 G. & WEHNERT, C. 1979. [Studies of Mycoplasma mastitis in cattle. 7. Mycoplasma
1707 mastitis in 3 dairy cattle herds]. *Arch Exp Veterinarmed,* 33**,** 685-97.
1708 PILLAI, S., KUNZE, E., SORDILLO, L. & JAYARAO, B. 2001. Application of differential
1709 inflammatory cell count as a tool to monitor udder health. *Journal of Dairy Science,* 84**,**
1710 1413-1420.
1711 PINNOW, C. C., BUTLER, J. A., SACHSE, K., HOTZEL, H., TIMMS, L. L. &
1712 ROSENBUSCH, R. F. 2001. Detection of Mycoplasma bovis in preservative-treated
1713 field milk samples. *J Dairy Sci,* 84**,** 1640-5.

1714 POTHMANN, H., SPERGSER, J., ELMER, J., PRUNNER, I., IWERSEN, M., KLEIN-
1715 JOBSTL, D. & DRILLICH, M. 2015. Severe Mycoplasma bovis outbreak in an
1716 Austrian dairy herd. *J Vet Diagn Invest*.
1717 PUNYAPORNWITHAYA, V., FOX, L. K., HANCOCK, D. D., GAY, J. M. & ALLDREDGE,
1718 J. R. 2010. Association between an outbreak strain causing mycoplasma bovis mastitis
1719 and its asymptomatic carriage in the herd: a case study from Idaho, USA. *Prev Vet Med,*
1720 93**,** 66-70.
1721 PYÖRÄLÄ, S. 2003. Indicators of inflammation in the diagnosis of mastitis. *Veterinary*
1722 *research,* 34**,** 565-578.
1723 RACCACH, M., ROTTEM, S. & RAZIN, S. 1975. Survival of frozen mycoplasmas. *Applied*
1724 *microbiology,* 30**,** 167-171.
1725 RADAELLI, E., CASTIGLIONI, V., LOSA, M., BENEDETTI, V., PICCININI, R.,
1726 NICHOLAS, R. A., SCANZIANI, E. & LUINI, M. 2011. Outbreak of bovine clinical
1727 mastitis caused by Mycoplasma bovis in a North Italian herd. *Res Vet Sci,* 91**,** 251-3.
1728 RAZIN, S. & HAYFLICK, L. 2010. Highlights of mycoplasma research--an historical
1729 perspective. *Biologicals,* 38**,** 183-90.
1730 RAZIN, S. & ROTTEM, S. 1978. Cholesterol in membranes: studies with mycoplasmas.
1731 *Trends in Biochemical Sciences,* 3**,** 51-55.
1732 RAZIN, S. 2012. *Methods in Mycoplasmology V1: Mycoplasma Characterization*, Elsevier.
1733 REBELO, A. R., PARKER, L. & CAI, H. Y. 2011. Use of high-resolution melting curve
1734 analysis to identify Mycoplasma species commonly isolated from ruminant, avian, and
1735 canine samples. *J Vet Diagn Invest,* 23**,** 932-6.
1736 REED, G. H., KENT, J. O. & WITTWER, C. T. 2007. High-resolution DNA melting analysis
1737 for simple and efficient molecular diagnostics. *Pharmacogenomics,* 8**,** 597.
1738 REITER, B. & BRAMLEY, A. 1975. Defence mechanisms of the udder and their relevance to
1739 mastitis control. *Doc Int Dairy Fed*.
1740 REN, X., FU, Y., XU, C., FENG, Z., LI, M., ZHANG, L., ZHANG, J. & LIAO, M. 2017. High
1741 resolution melting (HRM) analysis as a new tool for rapid identification of Salmonella
1742 enterica serovar Gallinarum biovars Pullorum and Gallinarum. *Poultry science,* 96**,**
1743 1088-1093.
1744 ROGER, V., FONTY, G., ANDRE, C. & GOUET, P. 1992. Effects of glycerol on the growth,
1745 adhesion, and cellulolytic activity of rumen cellulolytic bacteria and anaerobic fungi.
1746 *Current microbiology,* 25**,** 197-201.
1747 ROTTEM, S. 2003. Interaction of mycoplasmas with host cells. *Physiological reviews,* 83**,** 417-
1748 432.
1749 ROY, J. P., FRANCOZ, D. & LABRECQUE, O. 2008. Mastitis in a 7-week old calf caused by
1750 Mycoplasma bovigenitalium. Vet J, 176, 403-4.
1751 SAAD, N. & HAMEED, K. 2012. Detection of Mycoplasma species in raw milk of lactating
1752 animals in Assiut and Qena city of Egypt. *Veterinary World,* 5.
1753 SACHSE, K. & FREY, J. 2003. *PCR detection of microbial pathogens*, Springer Science &
1754 Business Media.
1755 SACHSE, K., PFUTZNER, H., HOTZEL, H., DEMUTH, B., HELLER, M. & BERTHOLD,
1756 E. 1993. Comparison of various diagnostic methods for the detection of Mycoplasma
1757 bovis. *Rev Sci Tech,* 12**,** 571-80.
1758 SACKS, D., LEDWABA, J., MORRIS, L. & HUNT, G. M. 2017. Rapid Detection of Common
1759 HIV-1 Drug Resistance Mutations by Use of High-Resolution Melting Analysis and
1760 Unlabeled Probes. *Journal of clinical microbiology,* 55**,** 122-133.
1761 SAHAR, E., METWALLY, A., AL-SAUD, N. & IBRAHIM, M. Molecular typing of different
1762 isolates of Mycoplasma bovis. Proceedings of the 6th Scientific Conference of Animal

1763 Wealth Research in the Middle East and North Africa, Hurghada, Egypt, 27-30
1764 September 2013, 2013. Massive Conferences and Trade Fairs, 277-290.
1765 SCHNEE, C., SCHULSSE, S., HOTZEL, H., AYLING, R. D., NICHOLAS, R. A.,
1766 SCHUBERT, E., HELLER, M., EHRICHT, R. & SACHSE, K. 2012. A novel rapid
1767 DNA microarray assay enables identification of 37 Mycoplasma species and highlights
1768 multiple Mycoplasma infections. *PLoS One,* 7**,** e33237.
1769 SCHUKKEN, Y., SMIT, J., GROMMERS, F., VANDEGEER, D. & BRAND, A. 1989. Effect
1770 of freezing on bacteriologic culturing of mastitis milk samples. *Journal of Dairy*
1771 *Science,* 72**,** 1900-1906.
1772 SCHULTZ, L. 1977. Somatic cells in milk-physiological aspects and relationship to amount
1773 and composition of milk. *Journal of Food Protection®,* 40**,** 125-131.
1774 SCHWARZ, D., DIESTERBECK, U., KÖNIG, S., BRÜGEMANN, K., SCHLEZ, K.,
1775 ZSCHÖCK, M., WOLTER, W. & CZERNY, C.-P. 2011. Flow cytometric differential
1776 cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and
1777 subclinically infected bovine mammary glands. *Journal of dairy science,* 94**,** 5033-
1778 5044.
1779 SEFFNER, W., PFUTZNER, H. & WEHNERT, C. 1983. [Mycoplasma mastitis of cattle. 11.
1780 Histological udder findings after experimental infection with Mycoplasma
1781 bovigenitalium and Acholeplasma laidlawii]. *Arch Exp Veterinarmed,* 37**,** 375-82.
1782 SHAHRIAR, F. M., CLARK, E. G., JANZEN, E., WEST, K. & WOBESER, G. 2002.
1783 Coinfection with bovine viral diarrhea virus and Mycoplasma bovis in feedlot cattle
1784 with chronic pneumonia. *Can Vet J,* 43**,** 863-8.
1785 SHUSTER, D., HARMON, R., JACKSON, J. & HEMKEN, R. 1991. Suppression of milk
1786 production during endotoxin-induced mastitis. *Journal of dairy science,* 74**,** 3763-3774.
1787 SIEVERS, F. & HIGGINS, D. G. 2014. Clustal omega. Current protocols in bioinformatics,
1788 48, 3.13. 1-3.13. 16.
1789 SILLIS, M. 1994. Mycoplasma arginini--a new human zoonosis? *Clin Infect Dis,* 18**,** 488.
1790 SILLO, P., PINTER, D., OSTORHAZI, E., MAZAN, M., WIKONKAL, N., PONYAI, K.,
1791 VOLOKHOV, D. V., CHIZHIKOV, V. E., SZATHMARY, S., STIPKOVITS, L. &
1792 KARPATI, S. 2012. Eosinophilic Fasciitis associated with Mycoplasma arginini
1793 infection. *J Clin Microbiol,* 50**,** 1113-7.
1794 SINGH, A., GUPTA, P. & BANGA, H. 1990. Pathogenicity of Acholeplasma laidlawii for the
1795 goat udder. *Australian veterinary journal,* 67**,** 155-156.
1796 SNEATH, P. H. & SOKAL, R. R. 1973. *Numerical taxonomy. The principles and practice of*
1797 *numerical classification*.
1798 SONG, Q., WANG, L., FANG, R., KHAN, M. K., ZHOU, Y. & ZHAO, J. 2013. Detection of
1799 Mycoplasma wenyonii in cattle and transmission vectors by the loop-mediated
1800 isothermal amplification (LAMP) assay. *Trop Anim Health Prod,* 45**,** 247-50.
1801 SONG, Z., LI, Y., LIU, Y., XIN, J., ZOU, X. & SUN, W. 2012. alpha-Enolase, an adhesion-
1802 related factor of Mycoplasma bovis. *PLoS One,* 7**,** e38836.
1803 SOSA, C., TIRANTE, L., CHAVES, J., POL, M., AMBROGI, A., GIRAUDO, J. A. &
1804 TAMIOZZO, P. 2018. [Identification of species of Mycoplasma and Ureaplasma
1805 diversum from Argentinian dairy herds]. *Rev Argent Microbiol,* 50**,** 31-35.
1806 SRINIVASAN, R., KARAOZ, U., VOLEGOVA, M., MACKICHAN, J., KATO-MAEDA, M.,
1807 MILLER, S., NADARAJAN, R., BRODIE, E. L. & LYNCH, S. V. 2015. Use of 16S
1808 rRNA gene for identification of a broad range of clinically relevant bacterial pathogens.
1809 *PloS one,* 10**,** e0117617.
1810 STELWAGEN, K. & SINGH, K. 2014. The role of tight junctions in mammary gland function.
1811 Journal of mammary gland biology and neoplasia, 19, 131-138.

1812 STEIM, J. M., TOURTELLOTTE, M. E., REINERT, J. C., MCELHANEY, R. N. & RADER,
1813 R. L. 1969. Calorimetric evidence for the liquid-crystalline state of lipids in a
1814 biomembrane. *Proceedings of the National Academy of Sciences,* 63**,** 104-109.
1815 STIPKOVITS, L., RADY, M. & GLAVITS, R. 1993. Mycoplasmal arthritis and meningitis in
1816 calves. *Acta Veterinaria Hungarica,* 41**,** 73-88.
1817 STIPKOVITS, L., ROMVARY, J., NAGY, Z., BODON, L. & VARGA, L. 1974. Studies on
1818 the pathogenicity of Acholeplasma axanthum in swine. *Epidemiology & Infection,* 72**,**
1819 289-296.
1820 STIPKOVITS, L., SOMOGYI, M., ASVANYI, B., TOTH, A. & SZATHMARY, S. 2013.
1821 Short communication: role of Mycoplasma arginini in mastitis caused by Streptococcus
1822 dysgalactiae. *J Dairy Sci,* 96**,** 1661-7.
1823 SULEMAN, M., CYPRIAN, F. S., JIMBO, S., MAINA, T., PRYSLIAK, T., WINDEYER, C.
1824 & PEREZ-CASAL, J. 2018a. Mycoplasma bovis-Induced Inhibition of Bovine
1825 Peripheral Blood Mononuclear Cell Proliferation Is Ameliorated after Blocking the
1826 Immune-Inhibitory Programmed Death 1 Receptor. *Infection and Immunity,* 86**,**
1827 e00921-17.
1828 SULEMAN, M., CYPRIAN, F. S., JIMBO, S., MAINA, T., PRYSLIAK, T., WINDEYER, C.
1829 & PEREZ-CASAL, J. 2018b. Mycoplasma bovis induced inhibition of bovine PBMC
1830 proliferation is ameliorated after blocking the immune-inhibitory programmed death-1
1831 (PD-1) receptor. *Infection and immunity***,** IAI. 00921-17.
1832 SULYOK, K. M., BEKŐ, K., KREIZINGER, Z., WEHMANN, E., JERZSELE, Á., RÓNAI,
1833 Z., TURCSÁNYI, I., MAKRAI, L., SZEREDI, L. & JÁNOSI, S. 2018. Development
1834 of molecular methods for the rapid detection of antibiotic susceptibility of Mycoplasma
1835 bovis. *Veterinary microbiology,* 213**,** 47-57.
1836 SULYOK, K. M., KREIZINGER, Z., FEKETE, L., HRIVNAK, V., MAGYAR, T., JANOSI,
1837 S., SCHWEITZER, N., TURCSANYI, I., MAKRAI, L., ERDELYI, K. &
1838 GYURANECZ, M. 2014. Antibiotic susceptibility profiles of Mycoplasma bovis strains
1839 isolated from cattle in Hungary, Central Europe. *BMC Vet Res,* 10**,** 256.
1840 SULYOK, K. M., KREIZINGER, Z., WEHMANN, E., LYSNYANSKY, I., BANYAI, K.,
1841 MARTON, S., JERZSELE, A., RONAI, Z., TURCSANYI, I., MAKRAI, L., JANOSI,
1842 S., NAGY, S. A. & GYURANECZ, M. 2017. Mutations Associated with Decreased
1843 Susceptibility to Seven Antimicrobial Families in Field and Laboratory-Derived
1844 Mycoplasma bovis Strains. *Antimicrob Agents Chemother,* 61.
1845 SUN, P., LUO, H., ZHANG, X., XU, J., GUO, Y. & HE, S. 2018. Whole-Genome Sequence
1846 of Mycoplasma bovis Strain Ningxia-1. *Genome Announc,* 6.
1847 SUN, Z., FU, P., WEI, K., ZHANG, H., ZHANG, Y., XU, J., JIANG, F., LIU, X., XU, W. &
1848 WU, W. 2014. Identification of novel immunogenic proteins from Mycoplasma bovis
1849 and establishment of an indirect ELISA based on recombinant E1 beta subunit of the
1850 pyruvate dehydrogenase complex. *PLoS One,* 9**,** e88328.
1851 SZACAWA, E., NIEMCZUK, K., DUDEK, K., BEDNAREK, D., ROSALES, R. & AYLING,
1852 R. 2015. Mycoplasma bovis infections and co-infections with other Mycoplasma spp.
1853 with different clinical manifestations in affected cattle herds in eastern region of Poland.
1854 *Bulletin of the Veterinary Institute in Pulawy,* 59**,** 331-338.
1855 TAMIOZZO, P. J., ESTANGUET, A. A., MAITO, J., TIRANTE, L., POL, M. & GIRAUDO,
1856 J. A. 2014. Detection of Mycoplasma canadense and Mycoplasma californicum in dairy
1857 cattle from Argentina. *Rev Argent Microbiol,* 46**,** 119-21.
1858 TAMURA, K., NEI, M. & KUMAR, S. 2004. Prospects for inferring very large phylogenies
1859 by using the neighbor-joining method. *Proceedings of the National Academy of*
1860 *Sciences of the United States of America,* 101**,** 11030-11035.

1861 THOMAS, A., BALL, H., DIZIER, I., TROLIN, A., BELL, C., MAINIL, J. & LINDEN, A.
1862 2002. Isolation of mycoplasma species from the lower respiratory tract of healthy cattle
1863 and cattle with respiratory disease in Belgium. *Vet Rec,* 151**,** 472-6.
1864 THOMPSON, C. C., VIEIRA, N. M., VICENTE, A. C. P. & THOMPSON, F. L. 2011.
1865 Towards a genome based taxonomy of Mycoplasmas. *Infection, Genetics and*
1866 *Evolution,* 11**,** 1798-1804.
1867 THURMOND, M. C., TYLER, J. W., LUIZ, D. M., HOLMBERG, C. A. & PICANSO, J. P.
1868 1989. The effect of pre-enrichment on recovery of Streptococcus agalactiae,
1869 Staphylococcus aureus and mycoplasma from bovine milk. Epidemiol Infect, 103, 465-
1870 74.
1871 TIMONEN, A. A., KATHOLM, J., PETERSEN, A., MÕTUS, K. & KALMUS, P. 2017.
1872 Within-herd prevalence of intramammary infection caused by Mycoplasma bovis and
1873 associations between cow udder health, milk yield, and composition. *Journal of Dairy*
1874 *Science*.
1875 TODARO, M., PULEIO, R., SABELLI, C., SCATASSA, M., CONSOLE, A. & LORIA, G.
1876 2015. Determination of milk production losses in Valle del Belice sheep following
1877 experimental infection of Mycoplasma agalactiae. *Small Ruminant Research,* 123**,** 167-
1878 172.
1879 TONG, S. Y. & GIFFARD, P. M. 2012. Microbiological applications of high-resolution
1880 melting analysis. *Journal of clinical microbiology,* 50**,** 3418-3421.
1881 TULLY, J. G. & RAZIN, S. 1970. Acholeplasma axanthum, sp. n.: a new sterol-nonrequiring
1882 member of the Mycoplasmatales. *Journal of bacteriology,* 103**,** 751-754.
1883 UHAA, I. J., RIEMANN, H. P., THURMOND, M. C. & FRANTI, C. E. 1990. The use of the
1884 enzyme-linked immunosorbent assay (ELISA) in serological diagnosis of Mycoplasma
1885 bovis in dairy cattle. *Vet Res Commun,* 14**,** 279-85.
1886 VAN KUPPEVELD, F. J., JOHANSSON, K. E., GALAMA, J. M., KISSING, J., BOLSKE,
1887 G., HJELM, E., VAN DER LOGT, J. T. & MELCHERS, W. J. 1994a. 16S rRNA based
1888 polymerase chain reaction compared with culture and serological methods for diagnosis
1889 of Mycoplasma pneumoniae infection. *Eur J Clin Microbiol Infect Dis,* 13**,** 401-5.
1890 VAN KUPPEVELD, F. J., JOHANSSON, K. E., GALAMA, J. M., KISSING, J., BOLSKE,
1891 G., VAN DER LOGT, J. T. & MELCHERS, W. J. 1994b. Detection of mycoplasma
1892 contamination in cell cultures by a mycoplasma group-specific PCR. *Appl Environ*
1893 *Microbiol,* 60**,** 149-52.
1894 VILLANUEVA, M., TYLER, J. & THURMOND, M. 1991. Recovery of Streptococcus
1895 agalactiae and Staphylococcus aureus from fresh and frozen bovine milk. *Journal of the*
1896 *American Veterinary Medical Association,* 198**,** 1398-1400.
1897 WATERS, A. & MCCUTHAN, T. 1990. Ribosomal RNA: nature's own polymerase-amplified
1898 target for diagnosis. *Parasitology Today,* 6**,** 56-59.
1899 WATTS, J. L. 1988. Etiological agents of bovine mastitis. *Veterinary microbiology,* 16**,** 41-66.
1900 WAWEGAMA, N. K. & BROWNING, G. F. 2017. Improvements in diagnosis of disease
1901 caused by Mycoplasma bovis in cattle. *Animal Production Science,* 57**,** 1482-1487.
1902 WAWEGAMA, N. K., BROWNING, G. F., KANCI, A., MARENDA, M. S. & MARKHAM,
1903 P. F. 2014. Development of a recombinant protein-based enzyme-linked
1904 immunosorbent assay for diagnosis of Mycoplasma bovis infection in cattle. *Clin*
1905 *Vaccine Immunol,* 21**,** 196-202.
1906 WAWEGAMA, N. K., MARKHAM, P. F., KANCI, A., SCHIBROWSKI, M., OSWIN, S.,
1907 BARNES, T. S., FIRESTONE, S. M., MAHONY, T. J. & BROWNING, G. F. 2016.
1908 Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for
1909 detection of Mycoplasma bovis infection in feedlot cattle. *Journal of clinical*
1910 *microbiology,* 54**,** 1269-1275.

1911 WILSON, D. J., GONZALEZ, R. N. & DAS, H. H. 1997. Bovine mastitis pathogens in New
1912 York and Pennsylvania: prevalence and effects on somatic cell count and milk
1913 production. *Journal of Dairy Science,* 80**,** 2592-2598.
1914 WINDSOR, H. M., WINDSOR, G. D. & NOORDERGRAAF, J. 2010. The growth and long
1915 term survival of Acholeplasma laidlawii in media products used in biopharmaceutical
1916 manufacturing. *Biologicals,* 38**,** 204-210.
1917 WITTWER, C. T., REED, G. H., GUNDRY, C. N., VANDERSTEEN, J. G. & PRYOR, R. J.
1918 2003. High-resolution genotyping by amplicon melting analysis using LCGreen.
1919 *Clinical chemistry,* 49**,** 853-860.

1920 YECHOURON, A., LEFEBVRE, J., ROBSON, H. G., ROSE, D. L. & TULLY, J. G. 1992.
1921 Fatal septicemia due to Mycoplasma arginini: a new human zoonosis. *Clin Infect Dis,*
1922 15**,** 434-8.

1923 YONG, T. B., HASHIM, R., NOOR, A. M., HAMZAH, S. H. & AHMAD, N. 2015.
1924 Identification of Brucella spp. isolated from human brucellosis in Malaysia using high-
1925 resolution melt (HRM) analysis. *Diagnostic microbiology and infectious disease,* 81**,**
1926 227-233.

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1932 **Appendix 1**

1933 Raw data of mollicutes culture, conventional PCR, qPCR, and ELISA; conventional mastitis

1934 bacterial culture and effects on milk composition for each sample

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cow_id	Mollicutes	PCR-16S rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
13640	0	0	0	0	0	0			32	83	2	CNS	nega	43.7	3.94	3.07	3.06	64	3365	134	102	236	102
15053	0	0	0	0	0	0			33	83	2	CNS	nega	37.7	4.08	3.24	2.76	14	1772	72	57	129	101
11574	1	1	0	0	0	0	0	25.2			2	ND	ques	28.2	3.58	3.01	1.86	39	8919	270	265	535	116
10833	0	0	0	0	0	0	1	470.5	34.1	83	2	ND	nega	37	2.92	2.97	2.18	114	9175	270	272	542	104
14222	1	1	1	1	0	0	0	81.9			2	CNS	mixd	35.2	4.35	3.32	2.7	1234	1654	72	55	127	98
14695	1	1	0	0	0	0	1	143.7	30	80.4	2	ND	ques	36.6	3.28	3.2	2.37	36	1867	61	60	121	96
12626	0	1	0	1	0	0	1	367.809	20.08		2	CNS	mbov	28.9	2.87	3.29	1.78	1011	12943	369	408	777	88
15818	0	0	0	0	0	0	0	43.145			2	CNS	nega	31.5	3.71	3.17	2.17	21	7594	279	251	530	118
14703	0	0	0	0	0	0	1	159.7			2	ND	nega	65	2.45	2.83	3.43	11	2860	70	81	151	112
14411	1	1	0	1	0	0	1	811.249	25.18		2	CNS	mbov	26.6	4.44	3.83	2.2	60	12773	501	423	924	0
13691	1	1	0	1	1	0	1	244	28.1		2	ND	mixd	13.4	3.66	3.21	0.92	726	3310	87	106	193	47
11692	0	1	0	0	0	0			27.29		2	CNS	ques	40.3	2.58	2.95	2.23	21	2176	56	64	120	94
15387	1	1	1	1	1	0	1	282.6	29	80.4	2	ND	mixd										
15444	1	1	1	0	1	0	0	14.9	24.11		2	ND	mixd	51.1	3.25	2.88	3.13	26	2811	91	81	172	110
14437	1	1	0	0	0	0	0	6.6			2	ND	ques	45.8	2.66	2.73	2.47	18	1878	50	51	101	0
16262	0	0	0	0	0	0	1	184.7			2	ND	nega	24.3	3.95	2.8	1.64	52	1365	59	44	103	88
15341	1	1	1	0	1	0	1	239.3	24.86		2	ND	mixd	34.9	3.3	2.92	2.17	108	1501	49	44	93	95

cow_id	Mollicutes	PCR_16S_rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
16196	1	0	0	0	0	0	0	0			2	CNS	nega										
15499	0	0	0	0	0	0					2	ND	nega	18.5	3.41	3.24	1.23	210	1055	36	34	70	83
16429	0	1	1	0	0	0					2	ND	acho										
15731	1	1	0	1	0	0	0	0			2	ND	mbov	27.1	3.14	3.21	1.72	23	7337	228	245	473	107
12823	1	1	0	0	0	0	0	0			2	ND	ques	51.4	2.08	2.68	2.45	7	2416	50	65	115	97
10856	1	0	0	0	0	0					2	ND	nega	40.6	2	3.2	2.11	49	12207	378	378	756	117
13167	1	1	0	0	0	0			29.78		2	ND	ques	45.1	3.88	3.06	3.13	556	2255	88	69	157	102
12638	0	0	0	0	0	0					2	ND	nega	38.4	2.53	3.18	2.19	45	8480	226	275	501	97
12901	1	1	0	1	0	0	0	112.5	23.27		2	CNS	mbov										
19494	0	0	0	0	0	0			27.7		2	ND	nega	22.9	2.71	3.1	1.33	1582	1168	32	36	68	82
15435	1	1	0	1	0	0					2	CNS	mbov	49.8	4.06	3.23	3.63	28	2341	95	76	171	116
13108	1	1	0	1	0	0					2	CNS	mbov	36.1	2.55	2.52	1.83	952	1841	47	46	93	87
	0	0	0	0	0	0			28.64		2	ND	nega										
12707	0	0	0	0	0	0					2	ND	nega	48.6	2.14	2.94	2.47	35	1798	38	53	91	97
15423	0	0	0	0	0	0	1	222.1			2	ND	nega	47.2	3.16	3.05	2.93	12	1841	58	56	114	107
13708	0	0	0	0	0	0					2	CNS	nega										
15637	0	1	0	0	0	0	1	161.8			2	CNS	test	56.2	2.24	3.1	3	36	2360	53	73	126	113
13406	0	1	0	1	0	0					2	ND	mbov	19.2	3.75	3.13	1.32	176	14727	482	425	907	103
14342	1	1	0	0	0	0	1	272.016	32	81	2	ND	ques	27.1	3.06	3.58	1.8	59	16795	486	561	1047	120
12949	1	1	0	1	0	0			29	83-78	2	CNS	mbov										
17436	1	0	0	0	1	0	1	384.9	27.08	77.4	2	ND	mbrh	34.7	5.07	3.11	2.84	52	1527	77	48	125	97

cow_id	Mollicutes	PCR_16S rRNA	A. laidlawii	M. bovis	M. bovirhinis	M. arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
10222	1	1	1	1	0	0	0	0	29	83	2	ND	mixd										
14610	1	1	0	0	1	0	0	85.9			2	CNS	mbrh	48.1	2.87	2.91	2.78	27	1828	52	53	105	101
16208	1	1	0	0	0	0	1	460.9	33	83	2	ND	ques	14.6	3.7	3.08	0.99	339	511	19	16	35	81
14211	1	0	0	0	0	0			37	83.4	2	ND	nega	14.8	2.43	3.85	0.93	1179	7013	164	237	401	70
14326	1	0	0	0	0	0			34	83	2	ND	nega	41.7	2.09	2.95	2.1	13	1585	33	47	80	93
11577	1	1	1	0	0	0	1	225.1	17.11		2	ND	acho	40.7	3.51	2.85	2.59	54	10034	330	294	624	113
15668	1	1	1	0	1	0			24.41		2	ND	mixd	33.6	3.51	3.1	2.22	136	1770	64	57	121	90
15587	0	0	0	0	0	0	0	18.9			2	ND	nega	41.4	2.15	2.8	2.05	484	2111	45	59	104	98
15627	1	1	0	0	0	0	1	207.6	30	83	2	ND	ques	48.1	2.37	2.89	2.53	20	2597	62	75	137	105
12667	0	1	1	0	0	0	0	0	31	83	2	ND	acho	29.4	3.3	3.23	1.92	80	9899	240	286	526	89
15203	1	1	0	0	1	0			27.6		2	ND	mbrh	45.6	4.5	3.14	3.48	671	2143	96	67	163	110
13962	1	1	0	0	0	0			26.36		2	CNS	ques	52.8	4.07	3.31	3.9	127	1901	77	63	140	112
14906	1	1	0	1	1	0	1	370.195			2	ND	mixd	37	3.3	3.16	2.39	21	1628	54	51	105	97
12155	1	1	1	1	0	1	1	440.5			2	ND	mixd	33.8	3.17	2.81	2.02	108	1589	50	45	95	89
11133	1	1	0	1	0	0			21.12		2	ND	mbov										
13169	1	1	0	1	0	0	1	350.822	28.05		2	ND	mbov	40.3	4.96	2.73	3.1	24	1572	78	43	121	98
11256	0	1	1	1	0	0	0	83.73	25.93		2	ND	mixd										
13561	1	1	0	1	0	0			27.1		2	CNS	mbov	35.4	3.73	3.19	2.45	91	1239	46	40	86	94
16155	1	1	0	0	0	0	0	48.6			2	ND	ques	7.6	2.76	3.42	0.47	4242	3607	122	129	251	62
14293	1	0	0	0	0	0	0	90.6			2	ND	nega	48.4	2.81	2.91	2.77	379	2130	60	62	122	100

cow_id	Mollicutes	PCR_16S_rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
15536	1	0	0	0	1	0			27.46		2	ND	mbrh	16.4	5.12	3.66	1.44	253	9164	359	295	654	90
15392	1	1	0	0	1	1	1	175	26.39		2	ND	mixd	51.1	2.19	2.97	2.64	15	2708	59	81	140	106
14592	0	1	0	0	0	0	0	0	27.26		2	ND	ques	15.3	5.16	4.05	1.41	91	10823	426	366	792	85
15647	1	1	1	1	1	0	0	40.549			2	ND	mixd	28.5	3.93	3.02	1.98	16	7172	266	234	500	110
13502	1	0	0	0	1	0	0	93.1	27.14		2	ND	mbrh	50.5	2.63	2.71	2.7	181	2424	64	66	130	100
11960	0	1	0	1	0	0	1	272.6	20.77		2	CNS	mbov	26.4	3.26	3.3	1.73	3710	1267	41	42	83	86
12464	1	1	1	0	0	0	1	389.99	25.72		2	ND	acho	22.7	5.02	4.01	2.05	112	10258	459	367	826	92
14794	1	0	0	0	0	0			30	84.3 - 77.7	2	CNS	nega	45.4	3.06	3.06	2.78	4321	1907	58	58	116	102
14006	0	1	0	1	1	1					2	ND	mixd	19.2	3.28	3.28	1.26	202	7638	195	236	431	84
12478	1	1	1	1	1	1	1	457.4	15.86	83.1	2	ND	mixd	16.2	3.27	2.65	0.96	32	664	22	18	40	76
12227	1	1	0	0	0	1	0	69.8			2	CNS	marg	56.9	2.55	2.92	3.11	258	10705	252	290	542	112
12360	0	0	0	0	0	0	1	759.5	32	83	2	CNS	nega	19.7	3.91	3.25	1.41	977	11939	340	345	685	99
12977	1	1	0	1	0	0	1	932.1	13.82		2	ND	mbov	49.5	2.91	3.21	3.03	56	1881	55	60	115	104
11325	0	0	0	0	0	0	0	0			2	ND	nega										
16558	1	0	0	0	0	0	0	85.59			2	ND	nega	12.5	3.84	2.88	0.84	17	650	25	19	44	80
11679	0	1	0	1	0	0	0	0	23.67		2	ND	mbov										
11158	0	1	0	0	0	0			30	83.1	2	ND	ques	51.5	2.95	2.91	3.02	228	1597	47	47	94	101

cow_id	Mollicutes	PCR_16S_rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
12539	1	1	0	1	0	0	1	1397.9	19.38		2	ND	mbov	39.3	4.3	2.67	2.74	380	1493	64	40	104	96
12114	1	1	1	0	1	1	0	0	17.69		9	ND	mixd	33.3	3.12	3.09	2.07	68	21064	601	602	1203	123
12334	0	1	1	1	0	1			12.42		9	ND	mixd	15.3	3.99	3.79	1.19	118	13139	554	433	987	105
16491	0	1	0	1	1	0	0	72.09			9	ND	mixd	23	3.43	3.7	1.64	18	1340	50	51	101	92
16331	1	0	0	0	0	0	0	0.3	fail		9	ND	nega	18.7	3.16	3.05	1.16	434	5355	174	160	334	71
16469	1	1	0	0	0	0			fail		9	ND	ques	26	2.46	3.73	1.61	803	6694	186	227	413	98
16337	0	0	0	0	0	0	1	197.9	29.5	78	9	CNS	nega	16.6	4.88	3.8	1.44	79	5283	232	178	410	86
14802	1	1	0	0	0	0					9	ND	ques	13.2	3.64	3.64	0.96	299	4908	156	176	332	61
19567	1	1	1	0	0	1	0	2	21.3		9	ND	mixd	28.5	3.4	3.33	1.92	531	8091	347	252	599	94
12492	0	1	1	0	0	0	0	5.2	38	78.2	9	ND	acho	30.5	3.84	3.64	2.28	241	8724	336	273	609	94
14711	0	0	0	0	0	1			12.42		9	CNS	marg	11.3	3.45	3.54	0.79	198	16913	606	544	1150	123
16415	0	0	0	0	0	0			29.47		9	ND	nega	24.4	2.66	3.28	1.45	30	5947	176	188	364	88
16479	0	1	1	1	0	0	1	135.8	25.05		9	ND	mixd	22.8	3.99	3.33	1.67	74	5791	213	193	406	86
12848	0	1	1	0	0	1	0	0	23.72		9	ND	mixd	15.9	6.48	4.03	1.67	322	15931	663	517	1180	111
13821	1	1	1	0	1	0			21.69		9	ND	mixd	9.6	3.13	3.65	0.65	318	13328	378	427	805	96
13094	0	0	0	0	0	0					9	ND	nega	9.3	3.55	3.66	0.67	381	20776	709	689	1398	130
16503	1	1	1	1	0	0	0	0	9.94		9	ND	mixd	21.4	2.85	3.64	1.39	84	6780	184	211	395	90
13838	1	1	0	1	0	0	0	0	16.02		9	ND	mbov	5.5	0	0	0	0	13484	365	373	738	83
12141	1	0	0	0	0	0	1	459.9	34		9	ND	nega	20.3	3.3	3.55	1.39	79	16293	416	527	943	101
12534	1	1	1	0	0	0	0	50.9			9	ND	acho	10.7	3.08	3.36	0.69	282	16719	481	493	974	105

cow_id	Mollicutes	PCR_16S_rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
11587	0	0	0	0	0	0					9	Klebsiella	nega	17.8	2.98	3.48	1.15	1983	12376	402	409	811	90
12690	0	0	0	0	0	1	0	6.1	26.5		9	Enterococcus	marg	9.1	1.54	3.3	0.44	744	11036	313	349	662	78
11197	0	1	1	0	0	0	0	5.7			9	ND	acho	18	4.44	3.5	1.43	1456	13810	435	419	854	98
13624	0	1	1	1	0	0	0	15.6			9	S.aureus	mixd	7.1	3.66	3.66	0.52	198	10758	361	384	745	86
16497	0	1	1	1	0	0	1	336.6	22	78.1	9	CNS	mixd	23.9	3.43	3.39	1.63	135	6380	262	212	474	100
14859	0	1	0	0	0	0					9	ND	ques	37.4	2.73	2.97	2.13	102	9848	344	288	632	101
13182	1	1	1	1	0	0					9	Strept	mixd	8	4.63	4	0.69	347	11372	453	403	856	104
17435	1	1	0	1	0	0	0	0	17.56		9	ND	mbov	17.5	4	3.66	1.34	47	11674	453	384	837	98
16445	1	1	1	1	0	0	0	0	26.2		9	ND	mixd	23.5	4.21	3.62	1.84	165	7285	268	237	505	106
16209	0	1	0	0	0	0			40		9	ND	ques	29.6	4.12	3.11	2.14	521	7371	294	244	538	111
16472	1	0	0	0	0	0					9	ND	nega	3	0	0	0	0	3971	139	126	265	78
16345	1	1	0	0	0	0			fail		9	ND	ques	23.2	3.15	3.32	1.5	305	5558	197	174	371	84
16225	1	1	0	0	1	1	0	26.7			9	ND	mixd	28	4.11	3.75	2.2	564	6223	272	222	494	108
16216	0	0	0	0	0	0					9	ND	test	22.3	3.05	3.5	1.46	47	5927	160	192	352	77
16168	1	0	0	0	0	0	0	0			9	ND	nega	36	2.86	3.5	2.29	61	7350	270	239	509	109
16302	0	0	0	0	0	0			25.34		9	ND	nega	29.4	3.13	3.4	1.92	126	7374	234	234	468	104
11109	1	1	1	0	1	1	0	8.1	19.29		9	ND	mixd	20.7	3.53	3.53	1.46	175	6958	283	226	509	79
15104	1	1	0	1	0	0	0	43.2	25.1		9	ND	mbov	23.2	3.75	3.79	1.75	67	13916	476	479	955	116
16230	0	1	1	0	1	0					9	ND	mixd	6.6	3.03	3.48	0.43	1209	6118	202	198	400	80
11577	1	1	0	0	0	0					9	ND	ques	55.4	2.15	2.92	2.81	154	1496	32	44	76	100

cow_id	Mollicutes	PCR_16S rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
12590	0	0	0	0	0	0	1	177.5			9	ND	nega	39.6	2.4	3.18	2.21	679	1228	29	39	68	92
14510	1	1	1	0	0	0	1	361.4	10.93		9	ND	acho	6.2	4.19	3.39	0.47	175	6059	270	182	452	64
16305	1	1	1	0	0	0	0	0	28.72		9	ND	acho	19.6	3.93	3.27	1.41	244	4409	158	134	292	74
16227	1	1	1	1	1	1					9	ND	mixd	24.6	3.37	3.5	1.69	290	6082	201	181	382	88
13379	1	1	1	0	0	0			21.35		9	ND	acho	43.7	2.22	3.09	2.32	123	1879	42	58	100	94
	0	1	0	0	0	0					9	ND	ques										
14357	0	1	1	0	1	0					9	ND	mixd	39	2	3.51	2.15	380	11841	343	367	710	116
13341	1	1	1	1	1	0	1	186.1	29.5		9	Enterococcus	mixd										
13325	1	1	1	0	0	1	0	27.6			9	Strept	mixd	49.7	1.99	2.96	2.46	1158	11284	276	317	593	105
12535	1	1	1	0	0	0	0	17.7	25.7		9	CNS	acho										
13928	0	1	1	1	0	1			26.49		9	ND	mixd	28.7	3.21	3.07	1.8	166	7022	252	212	464	0
	1	1	0	0	0	0	1	267.7			9	ND	ques										
13126	1	1	1	1	0	0			32.67		9	ND	mixd	29.4	2.69	3.16	1.72	1285	16814	549	501	1050	106
11222	0	1	0	1	1	1	1	267.65	23.83		9	CNS	mixd	34.6	3.03	3.01	2.09	1676	7885	312	248	560	95
11404	1	1	1	0	0	0	0	0	27.97		9	ND	acho	54.9	2.6	2.9	3.02	334	9748	303	279	582	112
15587	0	0	0	0	0	0			25.3		9	ND	nega	34.4	2.06	3.02	1.75	49	9718	209	293	502	97
14322	0	1	0	1	0	0	0	0			9	Strept	mbov	39	2.77	3.28	2.36	76	10684	341	341	682	108
14302	0	1	1	1	0	0	0	74.7			9	Strept	mixd	21.4	4.02	3.41	1.59	174	12802	494	433	927	104

cow_id	Mollicutes	PCR_16S_rRNA	A._laidlawii	M._bovis	M._bovirhinis	M._arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
12414	0	1	1	0	1	0	0	0	21.25		9	ND	mixd	38.5	3.14	3.27	2.47	1015	10939	376	330	706	109
13152	1	1	1	0	0	1			31.36		9	ND	mixd	34.6	4.13	3.5	2.64	1695	9775	370	304	674	104
	1	1	0	1	0	1	1	167.4	34.52		9	ND	mixd										
19191	0	1	0	1	0	0	1	154.4	29	83	9	ND	mbov	24.6	3.25	3.13	1.57	174	15757	472	469	941	98
11746	1	1	0	1	1	0	1	1198.1			9	S.aureus	mixd	14.1	2.77	3.19	0.84	240	22494	545	677	1222	104
12400	1	1	1	0	1	0	0	0			9	ND	mixd	43.3	2.66	3.14	2.51	839	9034	292	285	577	102
11669	0	1	0	0	1	1			16.91		9	S.aureus	mixd	19.8	2.53	3.64	1.22	3065	7213	208	237	445	78
13655	1	1	0	1	0	0	0	0			9	Strept	mbov	21.2	5.24	3.49	1.85	92	212	11	7	18	84
14655	1	1	0	1	0	0	0	0	31.3		9	ND	mbov	33	3.85	3.48	2.42	214	8455	361	293	654	110
12773	1	1	1	0	1	1	0	67			9	CNS	mixd										
15011	0	1	1	0	0	0	0	0	29.37		9	ND	acho	21.9	3.38	3.61	1.53	177	13430	377	439	816	104
13312	0	1	0	1	0	0	0	0			9	ND	mbov	31.9	1.94	3.29	1.67	118	9493	223	291	514	90
13353	1	1	1	1	0	1	1	11.2			9	ND	mixd	34.4	3.43	3.17	2.27	59	16193	492	499	991	103
15140	0	1	1	0	0	0	0	0			9	ND	acho	19.1	2.09	3.4	1.05	26	5370	130	188	318	58
12879	0	1	0	0	0	0					9	ND	ques	37.6	2.58	3.14	2.15	85	11846	291	326	617	102
12381	0	1	0	0	0	0					9	E.coli	test	40.6	3.67	3.35	2.85	25	8002	373	254	627	104
16215	1	0	0	0	0	0	0	0	28..32		9	ND	nega	25.5	3.45	3.25	1.71	41	6364	240	204	444	93
13478	1	1	1	0	0	0	0	121.9	26.74	78.3 - 82.6	9	ND	acho	38.5	3.84	3.04	2.65	360	10537	423	297	720	109

cow_id	Mollicutes	PCR_16S_rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
13870	0	1	1	0	1	0			27.92		9	ND	mixd	46.9	2.92	2.86	2.71	73	9268	356	284	640	108
14076	0	1	1	0	0	0	0	0	26.54		9	ND	acho	44.4	2.27	3	2.34	80	11602	367	362	729	113
13662	0	1	1	0	0	0	0	0			9	Strept	acho	48.1	3.35	3.64	3.36	77	8616	310	291	601	112
13171	1	1	0	1	1	0	0	74.4	25.41		9	ND	mixd	28.5	4.53	3.51	2.29	2412	16770	647	523	1170	100
13108	0	0	0	0	1	0	0	39.9	28.6		9	ND	mbrh	21.4	2.8	3.04	1.25	2282	7233	190	201	391	66
14111	0	1	1	1	0	0	0	0	20.1		9	ND	mixd	15	3.6	3.53	1.07	232	15771	521	527	1048	123
11398	0	1	0	1	0	1	1	364	24.73		9	ND	mixd	23.9	4.14	3.22	1.76	1185	18007	684	525	1209	121
14489	1	0	0	0	0	0	0	0			9	ND	test	33.3	3.21	3.33	2.18	37	15303	539	469	1008	115
14288	1	1	1	1	1	1	0	87.7			9	ND	mixd	30.8	3.57	3.41	2.15	299	16894	523	546	1069	117
11798	1	1	0	1	0	1	0	30.2			9	ND	mixd	54.2	2.82	3.32	3.33	89	10259	322	326	648	118
14405	0	1	1	0	1	0	0	27.4	30.63		9	ND	mixd	25.3	3.24	3.72	1.76	129	14622	532	495	1027	0
12364	1	1	1	1	1	1	0	0			9	ND	mixd										
14675	1	1	1	0	0	0	1	245.6	fail		9	S.aureus	acho	43.7	2.2	3.48	2.48	44	12072	243	376	619	114
14484	0	1	1	0	1	1	0	0			9	ND	mixd	30.8	2.79	3.54	1.95	95	16708	464	519	983	115
13394	0	1	1	1	0	1	0	0	26.57		9	ND	mixd	34.6	2.63	3.27	2.04	64	10452	338	306	644	100
11808	0	0	0	0	1	0	1	220.9	29.1	79	9	E.coli	mbrh	33.7	3.06	3.35	2.16	611	7580	262	270	532	98
	0	1	0	1	0	0					9	ND	mbov										
15483	0	1	0	0	0	0					9	ND	ques	34.9	1.98	3.12	1.78	56	9820	241	303	544	108
10044	1	1	1	0	1	0	0	0			9	ND	mixd										
10502	1	1	1	0	1	0	0	0			9	ND	mixd	29.4	2.21	3.03	1.54	91	18003	445	506	951	102
12717	1	1	0	1	0	1	0	0	19.46		9	ND	mixd	36.9	2.3	2.98	1.95	629	18940	494	548	1042	106
13109	1	1	1	0	1	0					9	ND	mixd	30.5	3.67	3.34	2.14	340	19369	568	562	1130	113

cow_id	Mollicutes	PCR_16S rRNA	A._laidlawii	M._bovis	M._bovirhinis	M._arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
12818	0	1	0	0	1	1	0	0			9	Enterococcus	mixd	40.3	2.13	3.28	2.18	35	11693	308	360	668	107
	0	0	0	0	0	0	0	25.2			9	CNS	nega										
13125	0	1	0	0	0	0	0	0			9	ND	ques	39.9	2.58	3.63	2.48	258	12385	423	411	834	122
11617	0	1	1	0	1	0			34.7		9	ND	mixd	36.5	1.95	3.23	1.89	271	10051	267	306	573	101
13746	1	1	1	1	0	1	0	0			9	ND	mixd	47.9	2.32	2.94	2.52	65	11210	291	330	621	108
14283	1	1	1	0	0	0	0	0	26.59		9	ND	acho	36.5	2.9	3.45	2.32	28	9688	322	309	631	103
10292	1	1	0	1	1	1	0	30.7			9	ND	mixd	49.2	2.3	3.27	2.74	44	9279	286	296	582	114
15302	1	1	0	1	0	1	0	0			9	ND	mixd	34.6	2.83	3.47	2.18	36	7805	304	260	564	102
13652	1	1	1	1	0	1	0	0			9	ND	mixd	25.1	4.3	3.9	2.06	77	7638	321	261	582	94
12484	1	1	1	1	1	0			10.5		9	ND	mixd	23.7	4.05	3.54	1.8	705	19582	709	607	1316	131
10067	0	1	1	0	1	0	0	0			9	ND	mixd										
13235	1	1	1	1	1	1	1	234.6	28	70.6	9	E.coli	mixd	12.8	3.91	3.28	0.92	646	538	21	18	39	75
15413	0	0	0	0	0	0					9	ND	nega	52.2	1.88	3.22	2.66	1229	3550	67	114	181	104
15362	1	1	0	1	1	0	0	0	29.78		9	CNS	mixd										
12060	1	0	0	0	0	0	0	0	29.34		9	ND	nega	66.5	2.12	3.05	3.44	101	2727	58	83	141	109
18876	1	1	1	0	1	0	1	555.9	12.36		9	E.coli	mixd										
15962	1	1	1	1	1	0	0	11.7	27.91		9	Enterococcus	mixd	23	3.83	3.39	1.66	125	11518	494	395	889	121
14289	0	0	0	0	0	0	1	678.8			9	ND	nega	23	3.43	3.74	1.65	1339	8347	325	284	609	101
13959	0	1	0	0	1	1			25.5		9	ND	mixd	23.9	2.72	3.22	1.42	2562	717	20	23	43	82
15649	1	1	1	0	0	0	0	0	28.54		9	ND	acho	57.4	3.05	3.4	3.7	50	1722	53	59	112	116

cow_id	Mollicutes	PCR_16S_rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
19911	1	0	1	0	0	0	0	0.9	31.65		9	ND	acho	41.7	2.73	2.76	2.29	32	1209	33	33	66	92
14044	0	1	1	0	0	1	1	1006			9	ND	mixd	35.1	3.02	3.28	2.21	42	1018	31	33	64	91
13251	0	1	1	0	0	1					9	ND	mixd	52.4	3.57	3.19	3.54	218	1310	47	42	89	105
11828	0	0	0	0	1	0	0	0	28.01		9	E.coli	mbrh	49.9	3.11	3.25	3.17	1411	2395	74	78	152	104
10835	0	0	0	0	0	0					9	ND	nega	37	3.86	2.7	2.43	51	9763	297	275	572	97
12292	1	1	0	1	1	1	1	135.2	27.28		9	ND	mixd	50.8	1.79	3.01	2.44	135	2896	52	87	139	98
16465	0	0	0	0	0	0					9	ND	nega	13.9	4.39	3.45	1.09	155	4141	168	140	308	72
14726	0	1	0	1	0	0			18.22		9	ND	mbov										
14064	0	1	1	0	1	0			21.61		9	E.coli	mixd	47.4	2.11	2.74	2.3	15	1375	29	38	67	94
13243	1	0	0	0	0	0			33.15		9	ND	nega	43.7	2.93	3.02	2.6	620	1967	58	59	117	96
14114	1	0	0	0	0	0	0	0	29.34		9	ND	nega	69.7	2.53	2.77	3.69	12	2021	51	56	107	110
11480	0	0	0	0	0	0					9	ND	nega										
16823	0	0	0	0	0	0	0	0			9	ND	nega	34.2	3.48	3.04	2.23	10	1265	44	38	82	103
16379	1	1	0	0	0	0			34.23		9	ND	ques	16.2	4.14	3.21	1.19	25	4849	189	156	345	75
13958	0	0	0	0	0	0	0	0			9	ND	nega	44.2	2.04	3.12	2.28	50	11384	312	323	635	109
13185	0	0	0	0	0	0					9	ND	test	42.8	2.8	3.13	2.54	82	1541	43	48	91	94
15268	1	1	0	0	0	0					9	ND	ques	39	2.05	3.31	2.09	1065	10477	256	330	586	107
14108	0	0	0	0	0	0					9	ND	nega										
13831	0	1	1	0	1	1	1	1127.9	fail		9	ND	mixd	49.9	2.3	3.19	2.74	16	2395	55	76	131	100
15468	0	0	0	0	0	0			31.16		9	ND	nega	26.4	3.3	3.33	1.75	3588	7504	273	256	529	93
16593	0	0	0	0	0	0					9	ND	nega	21	3.48	3.29	1.42	138	1470	51	48	99	90
16342	1	1	0	0	1	0	1	833.3	9.39	78.3	9	ND	mbrh	37.8	3.7	3.04	2.55	437	907	34	28	62	101

cow_id	Mollicutes	PCR_16S rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
13640	1	1	1	0	0	0	0	0			9	ND	acho	33.3	2.64	3.51	2.05	116	10737	406	357	763	106
15468	1	0	0	0	0	0	0	0			9	ND	nega	26.4	3.3	3.33	1.75	3588	7504	273	256	529	93
12782	1	1	0	0							9	ND	test	36.2	2.35	3.23	2.02	62	10547	272	336	608	99
16840	0	0	0	0	0	0					9	ND	nega	32.8	3.17	3.26	2.11	9	2034	64	66	130	103
11498	0	1	0	1	1	0	0	25.6			9	ND	mixd	42.8	2.87	3.6	2.77	209	3735	174	138	312	106
12366	0	1	0	1	1	0	0	65.5	30.6		9	ND	mixd	46.9	2.96	3.09	2.84	839	1548	46	48	94	99
14859	0	0	0	0	0	0			37.88		9	ND	nega	37.4	2.73	2.97	2.13	102	9848	344	288	632	
16414	0	1	0	0	0	0					9	ND	test	35.1	3.53	2.76	2.21	76	5638	179	157	336	87
11410	0	1	1	0	1	0	1	175.7			9	ND	mixd	24.8	2.06	3.15	1.29	121	794	16	25	41	81
13698	0	0	0	0	0	0					9	ND	test	28.7	2.93	3.21	1.76	95	8788	280	251	531	90
12593	1	1	1	1	1	0	0	0.2	28.84		9	ND	mixd	45.3	2.12	3.02	2.33	435	2492	53	75	128	95
12300	0	0	0	0	1	1			30.48		9	ND	mixd	32.8	3.66	3.17	2.24	82	9984	374	287	661	101
15795	1	0	0	0	1	1	0	63.8	41		9	ND	mixd	33	2.82	2.94	1.9	52	2013	57	59	116	92
15860	0	0	0	0	0	0	0	0			9	ND	nega	44.7	2.26	2.95	2.33	65	2280	52	67	119	99
15037	1	1	1	0	0	0					9	ND	acho	50.4	1.63	3.08	2.37	243	13531	329	380	709	120
17010	1	0	0	0	1	0	0	0			9	ND	mbrh	17.5	3.03	3.43	1.13	152	630	19	22	41	85
14098	1	0	0	0	1	1			31.02		9	CNS	mixd	25.5	3.33	3.06	1.63	73	1403	47	43	90	84
13640	1	1	0	0	1	1	0	0			9	CNS	mixd	33.3	2.64	3.51	2.05	116	10737	406	357	763	106
15632	1	0	0	0	0	0	0	0			9	ND	nega	31.4	2.71	3.12	1.83	942	7811	209	247	456	92
14176	0	1	1	0	1	1	0	15.1			9	ND	mixd	54.9	2.77	3.26	3.31	127	1647	46	54	100	105
13303	0	1	1	1	0	0	0	36.7			9	E.coli	mixd										
13406	0	0	0	0	1	1			26.19		9	ND	mixd	38.7	2.25	2.89	1.99	101	1393	31	40	71	89
16685	0	0	0	0	1	0			29.02		9	ND	test	21	4.29	3.43	1.62	103	672	29	23	52	91
13391	1	1	0	1	1	0					9	CNS	mixd	19.4	1.8	3.25	0.98	226	15197	391	434	825	92

cow_id	Mollicutes	PCR_16S_rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
16069	0	0	0	0	0	0	1	308.9	26.24		9	ND	nega	43.3	2.98	3.16	2.66	22	1472	44	47	91	102
15855	1	1	0	0	1	0	0	0			9	ND	mbrh	27.6	3.59	3.26	1.89	562	11508	410	359	769	107
15027	0	0	0	0	0	0					9	CNS	nega	11.4	2.89	3.07	0.68	225	14028	442	427	869	104
14498	0	1	1	1	1	0	0	0			9	ND	mixd	21.9	3.01	3.38	1.4	58	16165	475	522	997	118
16464	0	1	1	0	1	1	0	0			9	CNS	mixd	18.5	4.38	3.41	1.44	1802	6436	253	206	459	94
14879	1	1	1	1	0	0	1	503.8	24.96		9	ND	mixd	26.2	3.89	3.51	1.94	85	13342	505	434	939	98
12978	0	0	0	0	1	1	0	0			9	ND	mixd	30.3	3.37	3.37	2.04	613	8510	283	284	567	93
13525	1	1	1	1	1	0	0	97.7			9	ND	mixd										
13962	1	1	1	0	1	1	0	56.5			9	Enterococcus	mixd	29.8	3.69	3.49	2.14	3585	9155	362	300	662	102
10883	1	1	1	1	1	0	0	0	29.2		9	ND	mixd	16.9	3.02	2.96	1.01	279	6251	170	185	355	64
11486	1	1	0	1	1	0	0	0			9	ND	mixd	24.4	3.85	3.4	1.77	630	13782	565	414	979	104
15857	1	1	0	0	0	0					9	CNS	test	30.8	3.51	3.28	2.09	122	12329	453	387	840	95
12119	1	1	0	1	1	1	0	94.2			9	ND	mixd	18.7	4.39	4.22	1.61	121	15530	559	580	1139	120
15046	0	1	0	1	1	1	0	12.1			9	ND	mixd	9.1	3.3	3.41	0.61	234	12032	451	420	871	100
13241	0	1	1	0	1	1	0	92.5	17.75		9	ND	mixd	19.8	3.79	3.54	1.45	144	12623	413	385	798	78
15862	1	1	1	1	1	1	0	0			9	ND	mixd	23	3.83	3.39	1.66	125	11518	494	395	889	121
13571	0	1	1	0	0	0			28.46		9	ND	acho	21.9	4.11	3.56	1.68	102	14717	511	478	989	107
14651	0	1	1	0	1	1	0	0			9	ND	mixd	28.9	2.42	3.18	1.62	67	8094	201	240	441	81
14239	0	1	1	1	1	0	0	18.4	21.7		9	ND	mixd	21	4.29	4.14	1.77	191	19433	757	678	1435	117
10694	0	1	1	0	1	1	0	12.7			9	ND	mixd	25.3	4.19	3.87	2.04	447	10665	394	370	764	96
16272	1	1	1	1	0	0	0	0			9	ND	mixd	31.7	2.21	3.44	1.79	1166	7620	235	222	457	103
14682	0	1	1	1	1	0	0	0	30.97		9	CNS	mixd	26.9	4.54	3.83	2.25	562	13807	608	508	1116	115
12926	0	1	1	0	1	1	0	0			9	ND	mixd	34.9	2.12	2.78	1.71	592	8711	236	238	474	85

cow_id	Mollicutes	PCR_16S_rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
10892	1	1	1	0	1	1	0	0	25.46		9	ND	mixd	29.4	2.59	3.16	1.69	136	13650	443	417	860	94
12539	0	1	0	1	1	1	1	137.6	fail		9	ND	mixd	26.2	2.94	3.36	1.65	355	8925	316	263	579	90
16494	0	0	0	0	0	0	0	0			9	CNS	nega	26.4	2.46	3.3	1.52	462	7268	208	242	450	99
12136	0	0	0	0	0	0					9	ND	nega	4.6	0	0	0	0	12979	447	447	894	108
15550	0	1	0	1	1	0	0	1.7	29.56		9	CNS	mixd	20.5	2.39	3.61	1.23	147	6594	171	237	408	73
11778	1	0	0	0	0	0	1	277.8	25.45		9	ND	test	18.1	3.87	3.48	1.33	244	13402	423	427	850	103
11229	1	1	1	1	1	0	1	840.3	25.02		9	ND	mixd	28.9	3.6	3.53	2.06	279	19250	675	616	1291	127
15370	0	1	1	0	1	0	0	0	fail		9	ND	mixd	15.7	3.12	3.38	1.02	448	16591	499	533	1032	107
14118	1	1	1	1	1	0	0	7.9	18.06		9	ND	mixd	11.4	2.98	3.6	0.75	194	8320	245	275	520	58
14442	1	1	1	0	0	0					9	ND	acho	36.9	2.98	3.33	2.33	25	8466	319	265	584	97
13563	0	1	1	0	1	1			34	82.8-78.4	9	ND	mixd	9.8	4.49	4.08	0.84	1094	10301	359	332	691	72
10616	0	1	1	1	1	0	0	104.8	28	78.2-81.5	9	S.aureus	mixd	18.2	4.07	3.96	1.46	466	29246	1051	924	1975	129
15061	1	1	1	1	1	0	1	140.1	25.06	82.8	9	ND	mixd	17.1	3.74	3.63	1.26	157	12869	424	426	850	95
14196	1	1	1	1	1	0	0	0			9	ND	mixd	20.7	4.4	3.91	1.72	79	13561	446	445	891	103
13582	1	1	0	0	1	0	0	72.09	34	78.4-82.5	9	ND	mbrh	12.3	3.74	3.33	0.87	156	11710	410	390	800	84
12116	1	0	0	0	0	0	0	121.9			9	ND	nega	23.9	3.43	3.43	1.64	42	18977	490	579	1069	123

cow_id	Mollicutes	PCR_16S_rRNA	A._laidlawii	M._bovis	M._bovirhinis	M._arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
15549	0	1	1	1	1	0	1	304.7	27.44		9	ND	mixd	31.9	2.26	3.7	1.9	39	10253	269	344	613	109
14885	1	1	0	1	0	0	0	0			9	ND	mbov	15	3	3.33	0.95	137	15493	439	489	928	117
15548	0	1	1	1	0	0			17.16		9	ND	mixd	31.7	3.19	3.41	2.09	56	7565	259	262	521	100
12546	1	1	1	1	1	0	1	135.2			9	S.aureus	mixd	25.5	2.78	3.45	1.59	133	17970	449	535	984	110
10665	0	0	0	0	1	0	0	0			9	ND	mbrh	41.5	2.72	3.33	2.51	58	10200	346	331	677	111
15012	1	1	1	0	0	0	0	0			9	ND	acho	19.8	3.59	3.48	1.4	76	14641	460	463	923	110
11393	1	1	0	1	1	0	0	20	fail		9	ND	mixd	29.6	3.28	3.07	1.88	1363	17705	486	482	968	96
16464	1	1	0	1	1	0	0	57.3	30.87		9	CNS	mixd	18.5	4.38	3.41	1.44	1802	6436	253	206	459	94
14413	1	1	0	1	0	0	0	0	17.66		9	CNS	mbov	5.2	3.08	3.08	0.32	188	3208	117	105	222	0
16194	0	1	1	0	0	1			25.58		9	ND	mixd	24.6	2.93	2.89	1.43	24	6294	219	182	401	83
12786	1	1	1	1	1	1	0	0	28.4		9	S.aureus	mixd	24.8	3.1	3.43	1.62	506	5415	179	190	369	77
14162	0	0	0	0	1	0	0	23.21	23.99		9	ND	mbrh	36.7	2.83	3.38	2.28	67	9660	286	296	582	98
12676	0	1	0	1	1	1			21.2	83	9	ND	mixd	16.6	3.43	3.55	1.16	123	18278	537	560	1097	121
11847	0	1	1	1	0	0	0	0	29.23	78.3 - 83.1	9	ND	mixd	15.7	2.68	3.25	0.93	161	17655	596	551	1147	119
13311	1	1	1	0	1	0			26.08	78.3	9	ND	mixd	14.4	3.75	3.54	1.05	1407	14050	440	443	883	95
13131	1	0	0	0	0	0	0	0			9	S.aureus	nega	27.6	3.44	3.33	1.87	169	15972	578	481	1059	115
16181	0	0	0	0	0	0			25.5		9	ND	nega	22.6	5.53	3.72	2.09	170	5587	246	189	435	92
15366	0	1	1	1	1	1	0	0	27.5	78-83	9	ND	mixd	25.5	2.78	3.37	1.57	89	7036	203	231	434	84
14816	1	1	0	0	1	0	0	0			9	ND	mbrh	25.1	2.95	3.51	1.62	67	14514	374	472	846	105

cow_id	Mollicutes	PCR_16S rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
14127	1	1	0	1	1	1	0	10.6	28.8	78.1	9	ND	mixd	42.6	2.3	3.15	2.32	199	13089	403	371	774	115
13790	0	0	0	0	0	0			fail		9	ND	nega	41.9	2.22	3.1	2.23	103	12598	334	372	706	110
15321	0	1	1	0	1	1	0	0	27.44	78.1	9	CNS	mixd	24.4	3.07	3.4	1.58	271	17095	630	536	1166	116
13663	0	0	0	0	1	1	0	6.6			9	ND	mixd	15.3	2.48	3.27	0.88	281	8439	311	263	574	81
14475	0	0	0	0	0	0	1	295.7			9	ND	nega	23.2	2.76	3.28	1.4	63	10323	267	290	557	90
12646	1	0	0	0	0	0			28.05		9	ND	nega	44.7	2.26	3.27	2.47	598	12550	404	381	785	117
15188	1	1	0	0	0	0					9	ND	test	21.4	2.2	3.27	1.17	739	12805	351	403	754	93
16309	0	0	0	0	0	0	0	0			9	ND	nega	6.8	4.26	3.24	0.51	85	2456	99	64	163	54
14185	0	1	1	0	0	0	0	0			9	ND	acho	47.4	2.09	2.85	2.34	668	8319	238	239	477	97
10913	1	1	0	0	0	0					9	ND	ques	33.7	3.18	3.2	2.15	190	20419	607	607	1214	128
13193	1	1	0	1	1	1	0	11.7			9	CNS	mixd	8.9	2.7	3.37	0.54	202	11235	301	349	650	78
16032	1	1	1	0	0	1			27.03		9	ND	mixd	16.2	4.01	3.58	1.23	66	8546	291	292	583	84
12293	1	1	1	1	1	0	0	14.7	26.6		9	ND	mixd	18	4.22	3.67	1.42	121	13347	410	399	809	92
15298	0	1	1	1	0	0	0	0	12.16		9	ND	mixd	22.8	3.03	3.16	1.41	182	8129	243	241	484	84
13387	0	1	1	0	0	1	0	5.9	29.26		9	ND	mixd	24.6	3.17	3.25	1.58	157	7693	180	231	411	74
13794	1	1	1	1	0	0	0	14.9			9	CNS	mixd	26.9	2.12	3.35	1.47	365	14904	318	448	766	93
12304	0	0	0	0	0	0					9	ND	nega	27.6	2.64	3.19	1.61	456	18922	554	519	1073	112
11134	1	0	0	0	0	0	1	522.6			9	ND	nega	29.2	4.73	3.97	2.54	76	19651	856	696	1552	119
13804	1	1	0	1	0	0	0	0			9	ND	mbov	21.8	4.77	4.08	1.93	1952	15539	725	535	1260	108
13737	0	1	1	0	0	0	0	0			9	CNS	acho	29.8	3.46	3.59	2.1	111	18589	614	616	1230	128
14473	0	1	1	0	0	0	0	0			9	ND	acho	39.9	3.58	3.43	2.8	216	15727	560	491	1051	113
13181	0	1	1	0	0	1	0	0	27.87		9	ND	mixd	42.4	2.88	3.14	2.55	1513	12751	398	388	786	120
12776	1	1	1	0	1	1	0	0			9	ND	mixd	40.1	2.42	2.97	2.16	104	7429	199	233	432	88
13683	1	1	1	1	0	1			33.25		9	ND	mixd	27.6	2.36	3.22	1.54	53	16512	503	521	1024	111
16092	0	1	1	0	0	1	0	0			9	CNS	mixd	21.1	4.36	3.93	1.75	449	2801	132	100	232	85

cow_id	Mollicutes	PCR_16S rRNA	A. laidlawii	M. bovis	M. bovirhinis	M. arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
14304	1	1	1	0	0	0			30.34		9	ND	acho	36.2	1.71	3.48	1.88	175	10328	227	342	569	97
10906	1	0	0	0	0	0	1	563.6			9	CNS	test	24.2	3.14	3.72	1.66	750	15067	545	503	1048	114
12172	1	1	1	0	0	0	0	0	31.56		9	ND	acho	29.4	2.52	3.27	1.7	51	6452	215	219	434	80
12098	0	1	1	1	0	0	0	0	32.72		9	CNS	mixd	32.8	3.05	3.41	2.12	145	15197	466	498	964	112
16301	1	1	1	1	0	1	0	0			9	CNS	mixd	27.3	4.95	3.88	2.41	904	6511	275	231	506	114
13024	0	1	1	0	0	0	0	6	28.6		9	ND	acho	26.9	4.68	3.64	2.24	262	9103	327	287	614	97
17013	1	1	1	1	0	1	1	220.8			9	ND	mixd	25.5	2.98	3.18	1.57	377	816	24	26	50	93
14099	0	1	1	0	1	0					9	ND	mixd	33.5	2.51	3.19	1.91	72	12077	335	357	692	107
13632	0	1	1	0	0	1	0	11.2			9	ND	mixd	27.6	3.33	3.55	1.9	70	16935	483	571	1054	116
14521	0	0	0	0	0	0					9	Enterococcus	nega	38.1	2.15	3.15	2.02	191	17420	566	509	1075	122
10653	1	1	1	0	0	0	1	399.3	28.2		9	ND	acho	19.4	3.51	3.51	1.36	178	17082	616	533	1149	94
15207	0	0	0	0	0	1	1	243.2	29	78	9	ND	marg	15	3.8	3.93	1.16	189	13316	444	436	880	100
14199	1	1	1	1	0	1	0	106.9			9	ND	mixd	45.6	2.02	3.09	2.33	42	12483	296	367	663	113
15115	0	1	1	0	1	1	0	0			9	ND	mixd	34.6	2.86	3.61	2.24	187	13955	437	527	964	120
14754	0	1	0	1	0	0	0	0	23.05		9	ND	mbov	26.9	3.87	3.42	1.96	30	5875	252	184	436	77
14012	0	1	1	1	0	1	0	0			9	ND	mixd	31.2	3.53	3.21	2.1	801	6940	241	226	467	84
12870	0	1	1	0	1	1	0	0			9	CNS	mixd	13	2.46	3.08	0.72	363	16185	554	460	1014	115
13661	0	1	0	1	1	1	0	2.3			9	ND	mixd	29.2	2.91	3.15	1.77	264	7955	277	241	518	94
13561	0	1	1	1	0	0	0	0			9	ND	mixd	33.7	4.01	3.38	2.49	56	7687	290	255	545	91
12903	0	0	0	0	0	0					9	ND	nega	33	2.24	3.09	1.76	745	8738	245	265	510	93

cow_id	Mollicutes	PCR_16S_rRNA	A._laidlawii	M._bovis	M._bovirhinis	M._arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
15186	0	1	1	0	1	0	0	0.8			9	ND	mixd	53.3	2.2	2.91	2.72	169	2025	44	59	103	101
14743	1	0	0	0	1	0	0	19.1	23.07		9	ND	mbrh	30.3	2.15	3.56	1.73	52	7847	362	265	627	104
14804	1	1	0	0	0	1	0	0	24.9		9	ND	marg	23.7	2.62	3.84	1.53	394	9674	289	330	619	100
13749	1	1	0	1	1	0	1	160.9	27.21		9	CNS	mixd	41.7	2.73	3.05	2.41	186	8122	230	237	467	86
12666	1	1	0	1	1	0	0	0	28.3		9	ND	mixd	26	2.77	3.08	1.52	290	15670	389	434	823	92
12865	1	1	1	0	0	1	0	0			9	ND	mixd	21.6	3.01	3.52	1.41	152	20428	538	596	1134	122
13965	0	1	1	1	0	1			29.72		9	ND	mixd	31.9	3.07	3.35	2.05	535	9073	297	287	584	94
14525	1	1	1	0	1	0	0	0	26.53		9	ND	mixd	31.4	2.29	3.18	1.72	50	9939	347	304	651	109
11933	0	1	1	0	0	0	0	51.9			9	ND	acho	25.3	3.68	3.68	1.86	116	15408	603	501	1104	108
13158	0	0	0	0	0	0			28.9		9	ND	nega	40.6	2.56	3.37	2.41	167	12755	420	402	822	121
13833	1	1	1	0	0	1	0	16.8	22.77		9	ND	mixd	25.5	2.24	3.18	1.38	3444	9427	265	267	532	90
10855	0	1	1	1	1	1	0	0	24.77		9	ND	mixd	39.6	3.94	3.38	2.9	53	9200	284	291	575	102
13819	0	1	0	1	1	1	0	0			9	ND	mixd	26	2.54	3.38	1.54	338	17139	480	522	1002	106
19576	1	1	1	1	1	1			32.49		9	ND	mixd	31.9	3.42	3.26	2.13	257	9630	399	300	699	105
14834	0	1	0	1	1	0	1	271.3			9	ND	mixd	28.3	3.67	3.53	2.04	163	12678	436	420	856	100
	0	1	1	1	0	1	0	0	24.8		9	ND	mixd										
13027	1	1	1	0	1	0	0	0	26.82	83	9	ND	mixd	37.6	3.11	3.22	2.38	60	10604	340	316	656	104
15998	0	1	0	0	0	1	0	0	37.83		9	ND	marg	30.3	3.8	3.43	2.19	93	11903	477	383	860	116
19459	1	1	1	1	0	0	1	621.4	23.55		9	ND	mixd	20.5	3.12	3.46	1.35	116	13775	445	426	871	102
14992	1	1	0	1	1	0	0	63.7	21.78		9	ND	mixd	23.7	3.08	3.08	1.46	191	13605	355	416	771	100
	0	1	1	0	0	0					9	ND	acho	23.7	3.08	3.08	1.46	191	13605	355	416	771	100
12085	0	1	1	0	0	0					9	ND	acho	56.5	3.59	3.33	3.91	123	15617	515	492	1007	99
14335	0	0	0	0	0	0					9	ND	nega	22.3	3.72	3.86	1.69	65	16885	541	591	1132	130
11838	1	1	1	0	1	0					9	CNS	mixd	31.9	3.48	3.2	2.13	135	9008	344	269	613	93

cow_id	Mollicutes	PCR_16S rRNA	A_laidlawii	M_bovis	M_bovirhinis	M_arginini	Elisa_results	ELISA_value	qPCR	melting temp	month_of_sap	Conventional mastitis	agto	Milk yield	Fat percentage	Protein %	Milk solid%	SCC	Milk yield total	Fat total	Protein total	Milk solid total	pi
11328	0	0	0	0	0	0					9	ND	nega	56.5	2.81	3.1	3.34	261	1356	38	42	80	107

Appendix 2

Multiple alignment sequences of field isolated mollicutes with other referenced mollicutes

created by Mega 7 software based on 16S rRNA gene sequencing

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                                     205        215        225        235        245        255        265
275        285        295        305        315        325        335        345        355        365
375        385        395
LC158831.2_Mycoplasma_alkalesc   GTAACACGTG CTTAATCTAC CCTTTAGATT CACTAGAGGA TGAGGGTGCG GAACATTAGT TAGTTGGTAG
GGTAATGGCC TACCAAGACT ATGATGTTTA GCCGGGTCGA GAGACTGAAC GGCCACATTG GGACTGAGAT ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATATT CCACAATGAG CGAAAGCTTG
U44764.1_Mycoplasma_alkalescen   GTAACACGTG CTTAATCTAC CCTTTAGATT CACTAGAGGA TGAGGGTGCG GAACATTAGT TAGTTGGTAG
GGTAATGGCC TACCAAGACT ATGATGTTTA GCCGGGTCGA GAGACTGAAC GGCCACATTG GGACTGAGAT ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATATT CCACAATGAG CGAAAGCTTG
KU870649.1_Acholeplasma_laidla   ---------- ---------- ---------- CGGTTTAAGA GGGGTCTGCG GCGCATTAGT TAGTTGGTGG
GGTAAAAGCC TACCAAGACG ATGATGCGTA GCCGGACTGA GAGGTCTACC GGCCACATTG GGACTGAGA- ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
LC201977.1_Acholeplasma_laidla   ---------- ---------- ---------- CGGTTTAAGA GGGGTCTGCG GCGCATTAGT TAGTTGGTGG
GGTAAAAGCC TACCAAGACG ATGATGCGTA GCCGGACTGA GAGGTCTACC GGCCACATTG GGACTGAGA- ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
NR_074448.2_Acholeplasma_laidl   ---------- ---------- ---------- CGGTTTAAGA GGGGTCTGCG GCGCATTAGT TAGTTGGTGG
GGTAAAAGCC TACCAAGACG ATGATGCGTA GCCGGACTGA GAGGTCTACC GGCCACATTG GGACTGAGA- ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
JN935887.1_Acholeplasma_laidla   ---------- ---------- ---------- CGGTTTAAGA GGGGTCTGCG GCGCATTAGT TAGTTGGTGG
GGTAAAAGCC TACCAAGACG ATGATGCGTA GCCGGACTGA GAGGTCTACC GGCCACATTG GGACTGAGA- ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
MH259813.1                       ---------- ---------- ---------- CGGTTTAAGA GGGGTCTGCG GCGCATTAGT TAGTTGGTGG
GGTAAAAGCC TACCAAGACG ATGATGCGTA GCCGGACTGA GAGGTCTACC GGCCACATTG GGACTGAGA- ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
FJ226570.1_Acholeplasma_laidla   ---------- ---------- ---------- CGGTTTAAGA GGGGTCTGCG GCGCATTAGT TAGTTGGTGG
GGTAAGAGCC TACCAAGACG ATGATGCGTA GCCGGACTGA GAGGTCTACC GGCCACATTG GGACTGAGA- ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
FJ226559.1_Acholeplasma_laidla   ---------- ---------- ---------- CGGTTTAAGA GGGGTCTGCG GCGCATTAGT TAGTTGGTGG
GGTAAGAGCC TACCAAGACG ATGATGCGTA GCCGGACTGA GAGGTCTACC GGCCACATTG GGACTGAGA- ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
JN935888.1_Acholeplasma_laidla   ---------- ---------- ---------- CGGTTTAAGA GGGGTCTGCG GCGCATTAGT TAGTTGGTGG
GGTAAGAGCC TACCAAGACG ATGATGCGTA GCCGGACTGA GAGGTCTACC GGCCACATTG GGACTGAGA- ACGGCCCAAA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
FJ876270.1_Acholeplasma_axanth   ---------- ---------- ---------- TACTTTGGGA GGGACCTGCG TCGCATTAGC TAGTTGGTGA
GGTAGTAGCT CACCAAGGCG ATGATGCGTA GCCGGACTGA GAGGTTGAAC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
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NR_028829.1_Acholeplasma_axant   ---------- ---------- ---------- TACTTTGGGA GGGACCTGCG TCGCATTAGC TAGTTGGTGA
GGTAGTAGCT CACCAAGGCG ATGATGCGTA GCCGGACTGA GAGGTTGAAC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
LC158834.1_Mycoplasma_bovirhin   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_025986.1_Mycoplasma_bovirhi   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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HM751093.1_Mycoplasma_bovirhin   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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U04656.1_Mycoplasma_bovirhinis   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KP972459.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH259845.1                       ---------- ---------- ---------- ---------- ---------- ---------- ----------
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LC158831.2_Mycoplasma_alkalesc   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MG564233.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_025984.Mycoplasma_alkalesce   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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LC158832.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JQ903578.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MG564230.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_025182.1_Mycoplasma_dispar_   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KX462415.1_Mycoplasma_bovis_MY   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KX462395.1_Mycoplasma_bovis_PG   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KM576849.1_Mycoplasma_bovis_GD   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_044667.2_Mycoplasma_agalact   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JX193908.1_Mycoplasma_bovis_Sa   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MF101760.1_Mycoplasma_bovis_Eg   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JN624309.1_Mycoplasma_bovis_As   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH266037.1                       ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH259849.1                       ---------- ---------- ---------- TACTTTGGGA GGGACCTGCG TCGCATTAGC TAGTTGGTGA
GGTAGTAGCT CACCAAGGCG ATGATGCGTA GCCGGACTGA GAGGTTGAAC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
AGGGAATTTT CGGCAATGGG GGAAACCCTG
KX462374.1_Mycoplasma_bovis_MY   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JN644755.1_Mycoplasma_bovis_st   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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LT578453.1_Mycoplasma_bovis_is   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CP023663.1_Mycoplasma_bovis_Ni   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CP019639.1_Mycoplasma_bovis_st   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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                                      405        415        425        435        445        455        465
475        485        495        505        515        525        535        545        555        565
575        585        595
LC158831.2_Mycoplasma_alkalesc   ATGGAGCGAC ACAGCGTGCA CGATGAAGGT CTTCGGATTG TAAAGTGCTG TTATAGGGAA AGAAAACTTG
GTTGAGGAAA TGCTTCCAAG CTGACGGTAC CCTGTCAGAA AGCGATGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGTCGCAAG CGTTATCCGG
AATTATTGGG CGTAAAGCGT TTGTAGGCTG
U44764.1_Mycoplasma_alkalescen   ATGGAGCGAC ACAGCGTGCA CGATGAAGGT CTTCGGATTG TAAAGTGCTG TTATAGGGAA AGAAAACTTG
GTTGAGGAAA TGCTTCCAAG CTGACGGTAC CCTGTCAGAA AGCGATGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGTCGCAAG CGTTATCCGG
AATTATTGGG CGTAAAGCGT TTGTAGGCTG
KU870649.1_Acholeplasma_laidla   ACCGAGCAAC GCCGCGTGAA CGACGAAGTA CTTCGGTATG TAAAGTTCTT TTATATGGGA AGAA------
AAATTAAAAA T--------- -TGACGGTAC CATATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCGAG CGTTATCCGG
ATTTACTGGG CGTAAAGGGT GCGTAGGTGG
LC201977.1_Acholeplasma_laidla   ACCGAGCAAC GCCGCGTGAA CGACGAAGTA CTTCGGTATG TAAAGTTCTT TTATATGGGA AGAA------
AAATTAAAAA T--------- -TGACGGTAC CATATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCGAG CGTTATCCGG
ATTTACTGGG CGTAAAGGGT GCGTAGGTGG
NR_074448.2_Acholeplasma_laidl   ACCGAGCAAC GCCGCGTGAA CGACGAAGTA CTTCGGTATG TAAAGTTCTT TTATATGGGA AGAA------
AAATTAAAAA T--------- -TGACGGTAC CATATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCGAG CGTTATCCGG
ATTTACTGGG CGTAAAGGGT GCGTAGGTGG
JN935887.1_Acholeplasma_laidla   ACCGAGCAAC GCCGCGTGAA CGACGAAGTA CTTCGGTATG TAAAGTTCTT TTATATGGGA AGAA------
AAATTAAAAA T--------- -TGACGGTAC CATATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCGAG CGTTATCCGG
ATTTACTGGG CGTAAAGGGT GCGTAGGTGG
MH259813.1                       ACCGAGCAAC GCCGCGTGAA CGACGAAGTA CTTCGGTATG TAAAGTTCTT TTATATGGGA AGAA------
AAATTAAAAA T--------- -TGACGGTAC CATATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCGAG CGTTATCCGG
ATTTACTGGG CGTAAAGGGT GCGTAGGTGG
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FJ226570.1_Acholeplasma_laidla   ACCGAGCAAC GCCGCGTGAA CGACGAAGTA CTTCGGTATG TAAAGTTCTT TTATATGGGA AGAA------
AAATTAAAAA T--------- -TGACGGTAC CATATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCGAG CGTTATCCGG
ATTTACTGGG CGTAAAGGGT GCGTAGGTGG
FJ226559.1_Acholeplasma_laidla   ACCGAGCAAC GCCGCGTGAA CGACGAAGTA CTTCGGTATG TAAAGTTCTT TTATATGGGA AGAA------
AAATTAAAAA T--------- -TGACGGTAC CATATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCGAG CGTTATCCGG
ATTTACTGGG CGTAAAGGGT GCGTAGGTGG
JN935888.1_Acholeplasma_laidla   ACCGAGCAAC GCCGCGTGAA CGACGAAGTA CTTCGGTATG TAAAGTTCTT TTATATGGGA AGAA------
AAATTAAAAA T--------- -TGACGGTAC CATATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCGAG CGTTATCCGG
ATTTACTGGG CGTAAAGGGT GCGTAGGTGG
FJ876270.1_Acholeplasma_axanth   ACCGAGCAAC GCCGCGTGAA TGAAGAAGCA CTTAGGTGCG TAAAATTCTT TTATTAGGGA AGAATAGCTA
GTATAGGAAA TGATATTAGT GTGACGGTAC CTAATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCAAG CGTTATCCGG
AATTATTGGG CGTAAAGGGT GAGTAGGCGG
NR_028829.1_Acholeplasma_axant   ACCGAGCAAC GCCGCGTGAA TGAAGAAGCA CTTAGGTGCG TAAAATTCTT TTATTAGGGA AGAATAGCTA
GTATAGGAAA TGATATTAGT GTGACGGTAC CTAATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCAAG CGTTATCCGG
AATTATTGGG CGTAAAGGGT GAGTAGGCGG
LC158834.1_Mycoplasma_bovirhin   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_025986.1_Mycoplasma_bovirhi   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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HM751093.1_Mycoplasma_bovirhin   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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U04656.1_Mycoplasma_bovirhinis   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KP972459.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH259845.1                       ---------- ---------- ---------- ---------- ---------- ---------- ----------
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LC158831.2_Mycoplasma_alkalesc   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MG564233.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_025984.Mycoplasma_alkalesce   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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LC158832.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JQ903578.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MG564230.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_025182.1_Mycoplasma_dispar_   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KX462415.1_Mycoplasma_bovis_MY   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KX462395.1_Mycoplasma_bovis_PG   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KM576849.1_Mycoplasma_bovis_GD   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_044667.2_Mycoplasma_agalact   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JX193908.1_Mycoplasma_bovis_Sa   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MF101760.1_Mycoplasma_bovis_Eg   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JN624309.1_Mycoplasma_bovis_As   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH266037.1                         ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH259849.1                         ACCGAGCAAC GCCGCGTGAA TGAAGAAGCA CTTAGGTGCG TAAAATTCTT TTATTAGGGA AGAATAGCTA
GTATAGGAAA TGATATTAGT GTGACGGTAC CTAATGAATA AGCCCCGGCT AACTATGTGC CAGCAGCCGC GGTAATACAT AGGGGGCAAG CGTTATCCGG
AATTATTGGG CGTAAAGGGT GAGTAGGCGG
KX462374.1_Mycoplasma_bovis_MY     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JN644755.1_Mycoplasma_bovis_st     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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LT578453.1_Mycoplasma_bovis_is     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CP023663.1_Mycoplasma_bovis_Ni     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CP019639.1_Mycoplasma_bovis_st     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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                                       605        615        625        635        645        655        665
675        685        695        705        715        725        735        745        755        765
775        785        795
LC158831.2_Mycoplasma_alkalesc     TTTATTAAGT CTGGAGTTAA ATCCCAGGGC TCAACCCTGG TTCGCTTTGG ATACTGGTAA ACTAGAGTTG
GATAGAGGTA AGCGGAATTC CATGTGAAGC GGTGAAATGC GTAGATATAT GGAAGAACAC CAAA--GGCG AAGGCAGCTT ACTGGGTCTA TACTGACGCT
GAGGGACGAA AGCGTGGGGA GCAAACAGGA
U44764.1_Mycoplasma_alkalescen     TTTATTAAGT CTGGAGTTAA ATCCCAGGGC TCAACCCTGG TTCGCTTTGG ATACTGGTAA ACTAGAGTTG
GATAGAGGTA AGCGGAATTC CATGTGAAGC GGTGAAATGC GTAGATATAT GGAAGAACAC CAAA--GGCG AAGGCAGCTT ACTGGGTCTA TACTGACGCT
GAGGGACGAA AGCGTGGGGA GCAAACAGGA
KU870649.1_Acholeplasma_laidla     TTATAAAAGT TTGTGGTGTA AGTGCAGTGC TTAACGCTGT GAGGCTATGA AAACTATATA ACTAGAGTGA
GACAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAAGCGGCTT GCTGGGTCTA TACTGACACT
GATGCACGAA AGCGTGGGGA GCAAACAGGA
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LC201977.1_Acholeplasma_laidla   TTATAAAAGT TTGTGGTGTA AGTGCAGTGC TTAACGCTGT GAGGCTATGA AAACTATATA ACTAGAGTGA
GACAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACACT
GATGCACGAA AGCGTGGGGA GCAAACAGGA
NR_074448.2_Acholeplasma_laidl   TTATAAAAGT TTGTGGTGTA AGTGCAGTGC TTAACGCTGT GAGGCTATGA AAACTATATA ACTAGAGTGA
GACAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACACT
GATGCACGAA AGCGTGGGGA GCAAACAGGA
JN935887.1_Acholeplasma_laidla   TTATAAAAGT TTGTGGTGTA AGTGCAGTGC TTAACGCTGT GAGGCTATGA AAACTATATA ACTAGAGTGA
GACAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACACT
GATGCACGAA AGCGTGGGGA GCAAACAGGA
MH259813.1                       TTATAAAAGT TTGTGGTGTA AGTGCAGTGC TTAACGCTGT GAGGCTATGA AAACTATATA ACTAGAGTGA
GACAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACACT
GATGCACGAA AGCGTGGGGA GCAAACAGGA
FJ226570.1_Acholeplasma_laidla   TTATAAAAGT TTGTGGTGTA AGTGCAGTGC TTAACGCTGT GAGGCTATGA AAACTATATA ACTAGAGTGA
GACAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACACT
GATGCACGAA AGCGTGGGGA GCAAACAGGA
FJ226559.1_Acholeplasma_laidla   TTATAAAAGT TTGTGGTGTA AGTGCAGTGC TTAACGCTGT GAGGCTATGA AAACTATATA ACTAGAGTGA
GACAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACACT
GATGCACGAA AGCGTGGGGA GCAAACAGGA
JN935888.1_Acholeplasma_laidla   TTATAAAAGT TTGTGGTGTA AGTGCAGTGC TTAACGCTGT GAGGCTATGA AAACTATATA ACTAGAGTGA
GACAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACACT
GATGCACGAA AGCGTGGGGA GCAAACAGGA
FJ876270.1_Acholeplasma_axanth   CTACTTAAGT TTGAGGTATA AGCACAGTGC TTAACGCTGT GAGGCTTTGA AAACTGGGTA GCTAGAGTTA
GATAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACGCT
GAGGCACGAA AGCGTGGGGA GCAAACAGGA
NR_028829.1_Acholeplasma_axant   CTACTTAAGT TTGAGGTATA AGCACAGTGC TTAACGCTGT GAGGCTTTGA AAACTGGGTA GCTAGAGTTA
GATAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACGCT
GAGGCACGAA AGCGTGGGGA GCAAACAGGA
LC158834.1_Mycoplasma_bovirhin   ---------- ---------- ---------- ---------- ---------- ---------- ----------
----GAGGTT AGCGGAATTC CTAGTGAAGC GGTGAAATGC GTAGATATTA GGAAGAACAC CAATTTGGCG AAGGCAGCTA ACTGGGCACA TATTGACACT
GAGAGACGAA AGCGTGGGGA GCAAACAGGA
NR_025986.1_Mycoplasma_bovirhi   ---------- ---------- ---------- ---------- ---------- ---------- ----------
----GAGGTT AGCGGAATTC CTAGTGAAGC GGTGAAATGC GTAGATATTA GGAAGAACAC CAATTTGGCG AAGGCAGCTA ACTGGGCACA TATTGACACT
GAGAGACGAA AGCGTGGGGA GCAAACAGGA
HM751093.1_Mycoplasma_bovirhin   ---------- ---------- ---------- ---------- ---------- ---------- ----------
----GAGGTA AACGGAATTC CATGTGAAGC GGTGAAATGG GTAAATATAT GGAAGAACAC CAAA--TGGG AAGGCAGCTT ACTGGGTCTA TACTGACACT
GAGGGACGAA AGCGTGGGGA GCAAACAGGA
U04656.1_Mycoplasma_bovirhinis   ---------- ---------- ---------- ---------- ---------- ---------- ----------
----GAGGTT AGCGGAATTC CTAGTGAAGC GGTGAAATGC GTAGATATTA GGAAGAACAC CAATTTGGCG AAGGCAGCTA ACTGGGCACA TATTGACACT
GAGAGACGAA AGCGTGGGGA GCAAACAGGA
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KP972459.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
----GAGGTA AGCGGAATTC CATGTGAAGC GGTGAAATGC GTAGATATAT GGAAGAACAC CAAA--GGCG AAGGCAGCTT ACTGGGTCTA TACTGACGCT
GAGGGACGAA AGCGTGGGGA GCAAACAGGA
MH259845.1                       ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
LC158831.2_Mycoplasma_alkalesc   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
MG564233.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
NR_025984.Mycoplasma_alkalesce   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
LC158832.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
JQ903578.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
MG564230.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
NR_025182.1_Mycoplasma_dispar_   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
KX462415.1_Mycoplasma_bovis_MY   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
KX462395.1_Mycoplasma_bovis_PG   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
KM576849.1_Mycoplasma_bovis_GD   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- --------AT ATAAAAGGAA
NR_044667.2_Mycoplasma_agalact   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JX193908.1_Mycoplasma_bovis_Sa   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- --------AT ATAAAAGGAA
9029                             ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- --------AT ATAAAAGGAA
MF101760.1_Mycoplasma_bovis_Eg   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- --------AT ATAAAAGGAA
JN624309.1_Mycoplasma_bovis_As   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
MH266037.1                       ---------- ---------- ---------- ---------- ---------- ---------- ----------
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---------- ----TGGGGA GCAAACAGGA
MH259849.1                       CTACTTAAGT TTGAGGTATA AGCACAGTGC TTAACGCTGT GAGGCTTTGA AAACTGGGTA GCTAGAGTTA
GATAGAGGCA AGTGGAATTC CATGTGTAGC GGTAAAATGC GTAAATATAT GGAGGAACAC CAGT--GGCG AAGGCGGCTT GCTGGGTCTA TACTGACGCT
GAGGCACGAA AGCGTGGGGA GCAAACAGGA
KX462374.1_Mycoplasma_bovis_MY   ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- ---------- ---------- ---------- ---------- ----AGATTG GGATAGCGGA TGGAAACATC CGATAATACC GAATACTTAT
TATTTTTGCA TGAAAGTAAT ATAAAAGGAA
JN644755.1_Mycoplasma_bovis_st   ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- ---------- ---------- ---------- ---------- ----AGATTG GGATAGCGGA TGGAAACATC CGATAATACC GAATACTTAT
TATTTTTGCA TGAAAGTAAT ATAAAAGGAA
LT578453.1_Mycoplasma_bovis_is   ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- ---------- ---------- ---------- ---------- ----TCATCG CCTTGGTGGG CCGTTACCTC ACCAACTAGC TAATGTTGCG
CACTCCGATC TTTTAGCGAA GCAAACGCTT
CP023663.1_Mycoplasma_bovis_Ni   ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- ---------- ---------- ---------- ---------- ----TCATCG CCTTGGTGGG CCGTTACCTC ACCAACTAGC TAATGTTGCG
CACTCCGATC TTTTAGCGAA GCAAACGCTT
CP019639.1_Mycoplasma_bovis_st   ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- ---------- ---------- ---------- ---------- ----TCATCG CCTTGGTGGG CCGTTACCTC ACCAACTAGC TAATGTTGCG
CACTCCGATC TTTTAGCGAA GCAAACGCTT
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---------- ---------- ---------- ---------- ---------- ----TCATCG CCTTGGTGGA CCGTTACATC ACCAACTAGC TAATGTTGCG
CACTCCGATC TTTTAGTAAT ATAAAAGCTT

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                                 805         815         825         835         845         855         865
875          885          895          905          915          925          935          945          955          965
975          985          995
LC158831.2_Mycoplasma_alkalesc   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAATT C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGGAGAAC CTTACCCACT
U44764.1_Mycoplasma_alkalescen   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAATT C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGGAGAAC CTTACCCACT
KU870649.1_Acholeplasma_laidla   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGAAC TAAGT-GTTG GCCAAAAGGT C-AGTGCTGC
AGTTAACGCA TTAAGTTCTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CACGAAAAAC CTTACCAGGT
LC201977.1_Acholeplasma_laidla   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGAAC TAAGT-GTTG GCCAAAAGGT C-AGTGCTGC
AGTTAACGCA TTAAGTTCTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CACGAAAAAC CTTACCAGGT
NR_074448.2_Acholeplasma_laidl   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGAAC TAAGT-GTTG GCCAAAAGGT C-AGTGCTGC
AGTTAACGCA TTAAGTTCTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CACGAAAAAC CTTACCAGGT
JN935887.1_Acholeplasma_laidla   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGAAC TAAGT-GTTG GCCAAAAGGT C-AGTGCTGC
AGTTAACGCA TTAAGTTCTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CACGAAAAAC CTTACCAGGT
MH259813.1                       TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGAAC TAAGT-GTTG GCCAAAAGGT C-AGTGCTGC
AGTTAACGCA TTAAGTTCTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CACGAAAAAC CTTACCAGGT
FJ226570.1_Acholeplasma_laidla   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGAAC TAAGT-GTTG GCCAAAAGGT C-AGTGCTGC
AGTTAACGCA TTAAGTTCTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CACGAAAAAC CTTACCAGGT
FJ226559.1_Acholeplasma_laidla   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGAAC TAAGT-GTTG GCCATAAGGT C-AGTGCTGC
AGTTAACGCA TTAAGTTCTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CACGAAAAAC CTTACCAGGT
JN935888.1_Acholeplasma_laidla   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGAAC TAAGT-GTTG GCCATAAGGT C-AGTGCTGC
AGTTAACGCA TTAAGTTCTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CACGAAAAAC CTTACCAGGT
FJ876270.1_Acholeplasma_axanth   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGTAC TAAGT-GTCG G--AAGAATT C-GGTGCTGT
AGTTAACGCA ATAAGTACTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CGCGAAGAAC CTTACCAGGT
NR_028829.1_Acholeplasma_axant   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGTAC TAAGT-GTCG G--AAGAATT C-GGTGCTGT
AGTTAACGCA ATAAGTACTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CGCGAAGAAC CTTACCAGGT
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LC158834.1_Mycoplasma_bovirhin   TTAGATACCC TGGTAGTCCA -CGCTGTAAA CGATGATGAT TAGCT-GATA G---AGAGGT CTATCGGCGC
AGCTAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_025986.1_Mycoplasma_bovirhi   TTAGATACCC TGGTAGTCCA -CGCTGTAAA CGATGATGAT TAGCT-GATA G---AGAGGT CTATCGGCGC
AGCTAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
---------- ---------- ----------
HM751093.1_Mycoplasma_bovirhin   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTCTGGTG G---AGAGTT C-ACTGACGC
AGCTAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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U04656.1_Mycoplasma_bovirhinis   TTAGATACCC TGGTAGTCCA -CGCTGTAAA CGATGATGAT TAGCT-GATA G---AGAGGT CTATCGGCGC
AGCTAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KP972459.1_Mycoplasma_arginini   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAGTT C-ACTGACGC
AGCTAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH259845.1                       TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATATT TAGTG-GGTG G---CCAAT- C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTAAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAGGATA CGCGGAGAAC CTTACCCACT
LC158831.2_Mycoplasma_alkalesc   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAATT C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGGAGAAC CTTACCCACT
MG564233.1_Mycoplasma_arginini   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAGTT C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGGAGAAC CTTACCCACT
NR_025984.Mycoplasma_alkalesce   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAATT C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGGAGAAC CTTACCCACT
LC158832.1_Mycoplasma_arginini   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAGTT C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGGAGAAC CTTACCCACT
JQ903578.1_Mycoplasma_arginini   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAGTT C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGGAGAAC CTTACCCACT
MG564230.1_Mycoplasma_arginini   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTC-GGTG G---AGAGTT C-ACTGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGGAGAAC CTTACCCACT
NR_025182.1_Mycoplasma_dispar_   TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTT-GGTG GC--AAAAGT C-ACTAGCAC
AGCTAACGCG TTAAATGATC CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGAA CCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGTAGAAC CTTACCCACT
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KX462415.1_Mycoplasma_bovis_MY   TTAGATACCC TGGTAGTCCA -CGCCCTAAA CGATGATCAT TAGTT-GATG G---GGAACT C-ATCGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTACGTTCGC AAGAATAAAA CTTAAAGGAA TTGACGGGGA TCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGATGATA CGCGTAGAAC CTTACCCACT
KX462395.1_Mycoplasma_bovis_PG   TTAGATACCC TGGTAGTCCA -CGCCCTAAA CGATGATCAT TAGTT-GATG G---GGAACT C-ATCGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTACGTTCGC AAGAATAAAA CTTAAAGGAA TTGACGGGGA TCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGTAGAAC CTTACCCACT
KM576849.1_Mycoplasma_bovis_GD   GCGTTTGCTT CGCTAAAAGA TCGGAGTGCG CAACATTAGC TAGTT-GGTG AGGTAACGGC CCACCAAGGC
GATGA----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_044667.2_Mycoplasma_agalact   GCGYTY-CTT CGCTAGAAGA TCGGAGTGCG CAACATTAGC TAGTT-GGTG AGGTAACGGC CCACCAAGGC
GATGA----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JX193908.1_Mycoplasma_bovis_Sa   GCGTTTGCTT CGCTAAAAGA TCGGAGTGCG CAACATTAGC TAGTT-GGTG AGGTAACGGC CCACCAAGGC
GATGA----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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9029                             GCGTTTGCTT CGCTAAAAGA TCGGAGTGCG CAACATTAGC TAGTT-GGTG AGGTAACGGC CCACCAAGGC
GATGA----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MF101760.1_Mycoplasma_bovis_Eg   GCGTTTGCTT CGCTAAAAGA TCGGAGTGCG CAACATTAGC TAGTT-GGTG AGGTAACGGC CCACCAAGGC
GATGA----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JN624309.1_Mycoplasma_bovis_As   TTAGATACCC TGGTAGTCCA -CGCCCTAAA CGATGATCAT TAGTT-GATG G---GGAACT C-ATCGACGC
AGCTAACGCA TTAAATGATC CGCCTGAGTA GTACGTTCGC AAGAATAAAA CTTAAAGGAA TTGACGGGGA TCCGCACAAG CGGTGGAGCA TGTGGTTTAA
TTTGAAGATA CGCGTAGAAC CTTACCCACT
MH266037.1                       TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGATCAT TAGTCTGGTG G---AGAGTT C-ACTGGCGC
AGCTAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH259849.1                       TTAGATACCC TGGTAGTCCA -CGCCGTAAA CGATGAGTAC TAAGT-GTCG G--AAGAATT C-AGTGCTGT
AGTTAACGCA ATAAGTACTC CGCCTGAGTA GTACGTACGC AAGTATGAAA CTCAAAGGAA TTGACGGGAC CCCGCACAAG CGGTGGATCA TGTTGTTTAA
TTCGAAGATA CGCGAAGAAC CTTACCAGGT
KX462374.1_Mycoplasma_bovis_MY   GCGTTTGCTT CGCTAAAAGA TCGGAGTGCG CAACATTAGC TAGTT-GGTG AGGTAACGGC CCACCAAGGC
GA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JN644755.1_Mycoplasma_bovis_st   GCGTTTGCTT CGCTAAAAGA TCGGAGTGCG CAACATTAGC TAGTT-GGTG AGGTAACGGC CCACCAAGGC
GA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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LT578453.1_Mycoplasma_bovis_is   CCTTTTATAT TACT--TTCA --TGCAAAAA TAATAAGTAT TCGGT-ATTA TCGGATGTTT CCATCCGCTA
TCCCAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CP023663.1_Mycoplasma_bovis_Ni   CCTTTTATAT TACT--TTCA --TGCAAAAA TAATAAGTAT TCGGT-ATTA TCGGATGTTT CCATCCGCTA
TCCCAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CP019639.1_Mycoplasma_bovis_st   CCTTTTATAT TACT--TTCA --TGCAAAAA TAATAAGTAT TCGGT-ATTA TCGGATGTTT CCATCCGCTA
TCCCAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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40                               CCTTTTATAT TACT--TTCA --TGCAAAAA TAATAAGTAT TCGGT-ATTA TCGGATGTTT CCATCCGCCA
TCCCAA---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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....|....| ....|....| ....|....|
                                     1005       1015       1025       1035       1045       1055       1065
1075       1085       1095       1105       1115       1125       1135       1145       1155       1165
1175       1185       1195
LC158831.2_Mycoplasma_alkalesc   CTTGACATCC TTCGCAAAGG AGTGACAGAT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG AGATGTTTGG
TCAAGTCCTG CAACGAGCGC AACCCCTATC TTTAGTTACT AACGAGTCAT GTCGAGGACT CTAGAGATAC TGCCTGGGTA ACTGGGAGGA AGGTGGGGAT
GACGTCAAAT CATCATGCCT CTTACGAGTG
U44764.1_Mycoplasma_alkalescen   CTTGACATCC TTCGCAAAGG AGTGACAGAT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG AGATGTTTGG
TCAAGTCCTG CAACGAGCGC AACCCCTATC TTTAGTTACT AACGAGTCAT GTCGAGGACT CTAGAGATAC TGCCTGGGTA ACTGGGAGGA AGGTGGGGAT
GACGTCAAAT CATCATGCCT CTTACGAGTG
KU870649.1_Acholeplasma_laidla   CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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LC201977.1_Acholeplasma_laidla   CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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NR_074448.2_Acholeplasma_laidl   CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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JN935887.1_Acholeplasma_laidla   CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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MH259813.1                       CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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FJ226570.1_Acholeplasma_laidla   CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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FJ226559.1_Acholeplasma_laidla   CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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JN935888.1_Acholeplasma_laidla   CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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FJ876270.1_Acholeplasma_axanth   CTTGACATCC CCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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NR_028829.1_Acholeplasma_axant   CTTGACATCC CCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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LC158834.1_Mycoplasma_bovirhin   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_025986.1_Mycoplasma_bovirhi   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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HM751093.1_Mycoplasma_bovirhin   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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U04656.1_Mycoplasma_bovirhinis   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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KP972459.1_Mycoplasma_arginini   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH259845.1                       CT-GACATCC TC-------- ---------- ---------- ---------- ---------- ----------
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LC158831.2_Mycoplasma_alkalesc   CTTGACATCC TT-------- ---------- ---------- ---------- ---------- ----------
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MG564233.1_Mycoplasma_arginini   CTTGACATCC TT-------- ---------- ---------- ---------- ---------- ----------
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NR_025984.Mycoplasma_alkalesce   CTTGACATCC TT-------- ---------- ---------- ---------- ---------- ----------
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LC158832.1_Mycoplasma_arginini   CTTGACATCC TT-------- ---------- ---------- ---------- ---------- ----------
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JQ903578.1_Mycoplasma_arginini   CTTGACATCC TT-------- ---------- ---------- ---------- ---------- ----------
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MG564230.1_Mycoplasma_arginini   CTTGACATCC TT-------- ---------- ---------- ---------- ---------- ----------
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NR_025182.1_Mycoplasma_dispar_   CTTGACATCC TC-------- ---------- ---------- ---------- ---------- ----------
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KX462415.1_Mycoplasma_bovis_MY   CTTGACATCT TC-------- ---------- ---------- ---------- ---------- ----------
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KX462395.1_Mycoplasma_bovis_PG   CTTGACATCT TC-------- ---------- ---------- ---------- ---------- ----------
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KM576849.1_Mycoplasma_bovis_GD   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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NR_044667.2_Mycoplasma_agalact   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JX193908.1_Mycoplasma_bovis_Sa   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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9029                             ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MF101760.1_Mycoplasma_bovis_Eg   ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JN624309.1_Mycoplasma_bovis_As   CTTGACATCT TC-------- ---------- ---------- ---------- ---------- ----------
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MH266037.1                       ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MH259849.1                         CTTGACATAC TCTGCAAAG- ---------- ---------- ---------- ---------- ----------
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KX462374.1_Mycoplasma_bovis_MY     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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JN644755.1_Mycoplasma_bovis_st     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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LT578453.1_Mycoplasma_bovis_is     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CP023663.1_Mycoplasma_bovis_Ni     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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CP019639.1_Mycoplasma_bovis_st     ---------- ---------- ---------- ---------- ---------- ---------- ----------
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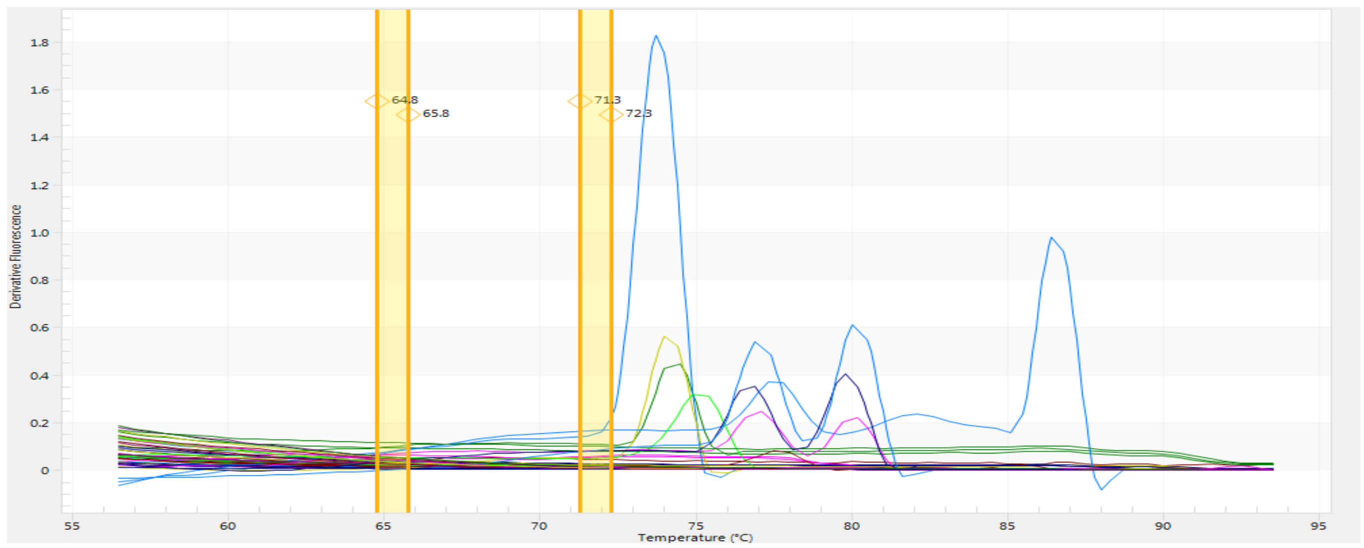
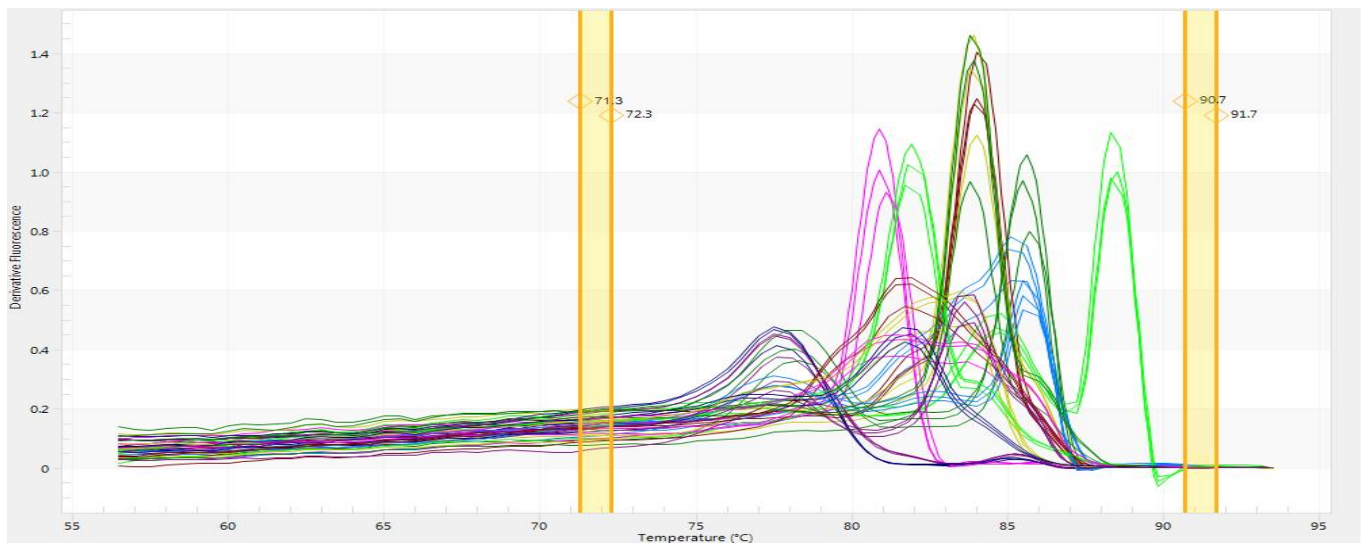
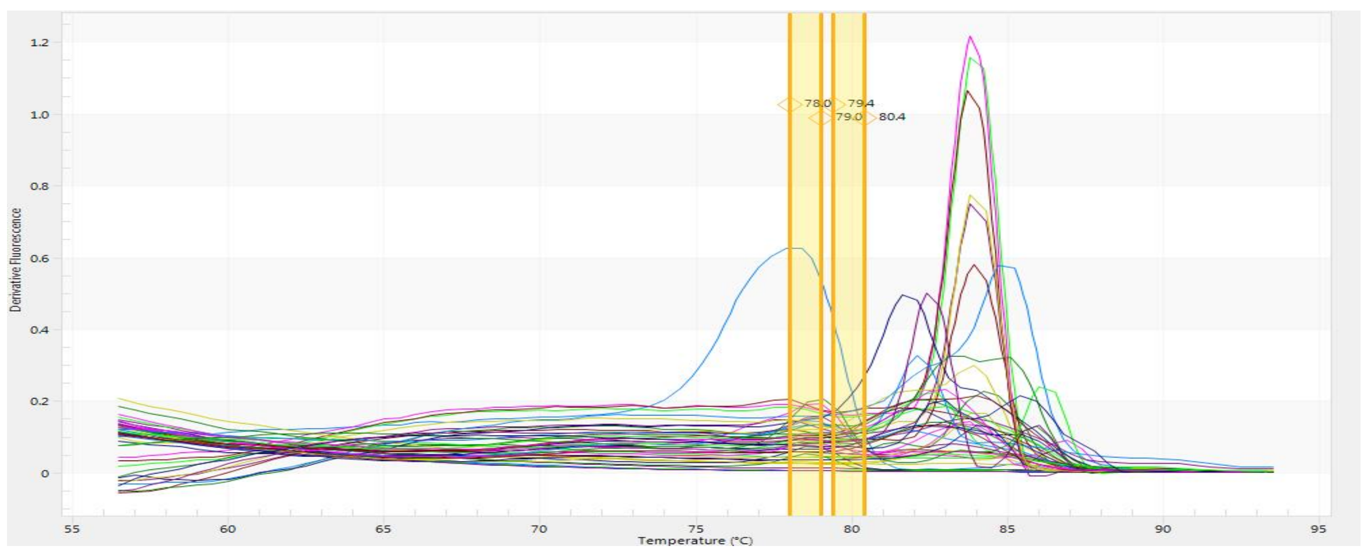
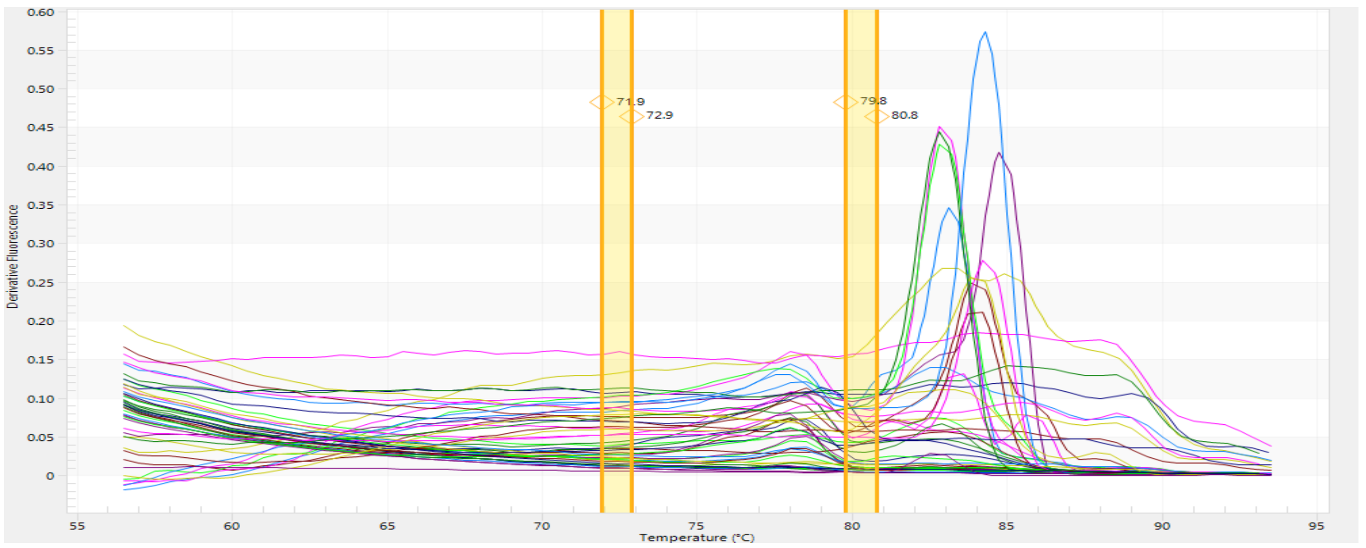
Appendix 3

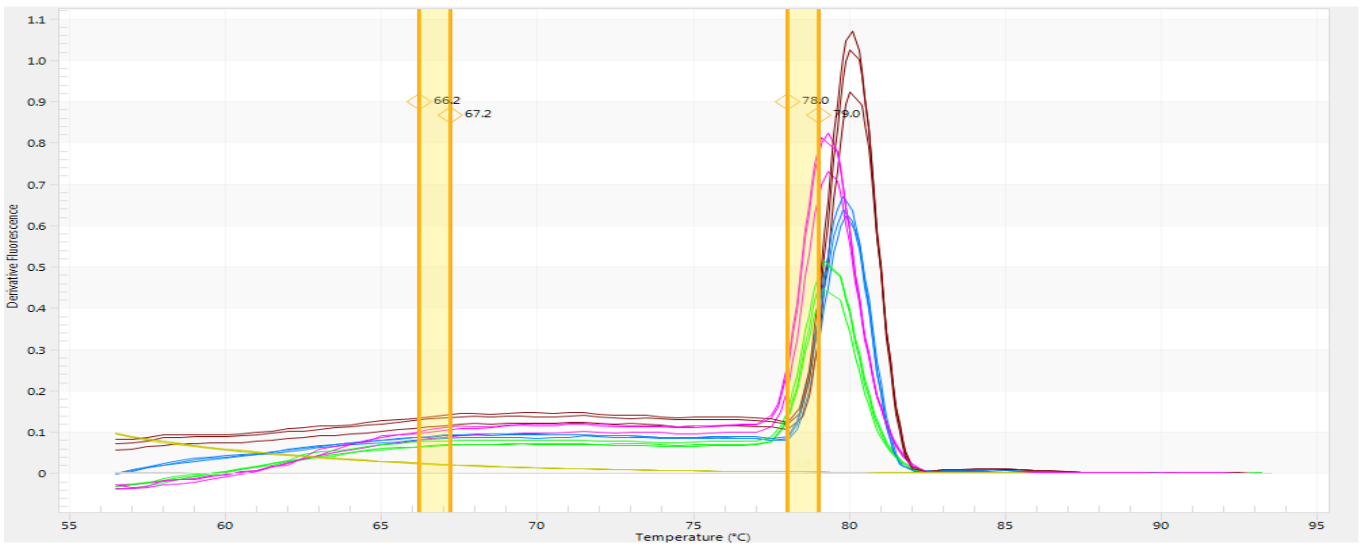
Selection of derivative melting curves related to different milk mollicutes generated by EcoStudy software (illumina).

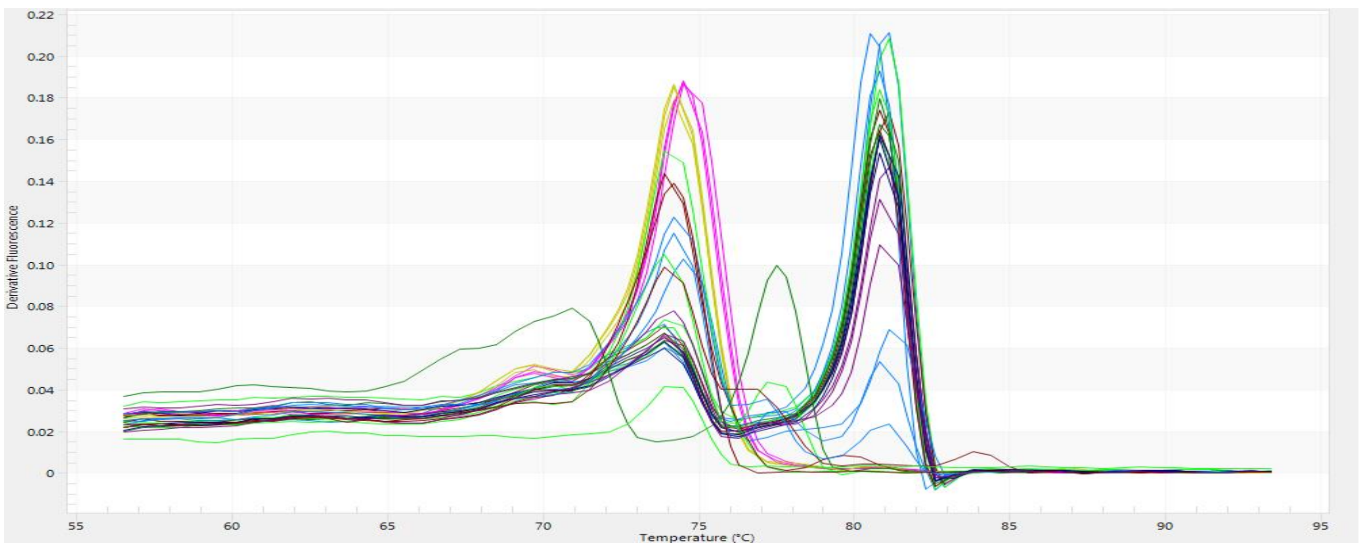

Derivative Fluorescence
Temperature (°C)
64.8
65.8
71.3
72.3


Derivative Fluorescence
Temperature (°C)
71.3
72.3
90.7
91.7


Derivative Fluorescence
Temperature (°C)
78.0
79.4
79.0
80.4


Derivative Fluorescence
Temperature (°C)
71.9
72.9
79.8
80.8


Derivative Fluorescence
Temperature (°C)
66.2
67.2
78.0
79.0


Derivative Fluorescence
Temperature (°C)

Appendix 4

Raw data of *M. bovis* survival experiment

Glycerol+DMSO @ 4°							FBS+DMSO @ 4º						Gelatin @ 4º						control					
Sample		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16
164		1	1	0	0	0		1	1	0	0	0		1	1	1	1	0		1	1	1	0	0
535		1	1	0	0	0		1	1	1	1	0		1	1	1	1	0		0	0	0	0	0
144		1	1	0	0	0		1	1	1	1	0		1	1	0	1	0		1	1	1	0	0
105		1	1	1	1	1		1	1	0	0	0		1	1	1	1	1		1	1	1	0	0
409		1	1	0	0	0		1	1	1	1	1		1	1	1	1	1		1	1	1	1	1
293		1	0	0	0	0		1	1	1	1	1		1	1	0	0	0		1	0	0	0	0
139		1	1	1	1	1		1	1	1	1	1		1	1	1	1	1		0	0	0	0	0
556		1	1	1	1	1		1	1	0	0	0		1	1	1	0	0		1	0	0	0	0
125		1	1	1	1	0		1	1	1	1	0		1	1	1	0	0		1	1	1	0	0
492		1	1	0	0	0		1	1	1	1	1		1	1	0	1	0		0	0	0	0	0
338		1	0	1	1	1		1	0	0	0	0		1	1	1	1	1		1	1	1	1	1
105		1	1	1	1	0		1	1	0	0	0		1	1	1	1	1		1	1	1	0	0
139		1	1	1	1	1		1	1	1	0	0		1	1	1	1	1		1	1	0	0	0
492		1	1	0	0	0		1	1	1	1	1		1	1	1	0	0		1	1	0	0	0
397		1	1	1	1	1		1	1	1	1	1		1	1	1	1	1		1	0	0	0	0
345		1	1	1	1	1		1	1	1	0	0		1	0	0	0	0		1	1	1	1	0
162		1	1	1	0	0		1	1	1	1	0		1	1	1	1	1		1	1	0	0	0
550		0	0	0	0	0		0	0	0	0	0		1	1	1	0	0		1	1	0	0	0
373		1	1	1	1	1		1	1	1	1	1		1	1	1	0	0		1	1	0	0	0
125		1	1	1	1	0		0	0	1	0	0		1	1	1	1	1		1	1	0	0	0
337		1	1	1	1	1		1	1	1	1	0		1	1	1	0	0		1	1	1	1	1
304		1	1	1	1	1		1	1	0	0	0		1	1	1	1	1		1	1	0	0	0
145		1	1	1	0	0		1	1	1	1	1		1	1	1	1	1		0	0	0	0	0
146		1	1	1	1	1		1	1	1	1	1		1	1	1	1	1		0	0	0	0	0
419		1	1	1	0	0		1	1	1	0	0		1	1	1	0	0		1	1	1	1	0
122		1	1	1	1	1		1	1	1	1	0		1	0	0	0	0		1	1	1	1	0
392		1	1	0	0	0		0	0	0	0	0		1	1	1	1	1		1	0	0	0	0
ATCC		1	1	1	1	0		1	1	1	1	0		1	1	1	1	1		1	1	1	1	1

		Glycerol+DMSO @ -20						FBS+DMSO @ -20						Gelatin @ -20						control @ -20					
Sample		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16	
164	m4	1	1	1	1	1		1	1	0	0	0		1	0	0	0	0		1	1	1	0	0	
535	a	1	1	1	0	0		1	0	0	0	0		1	0	0	0	0		0	0	0	0	0	
144	b	1	1	1	0	0		1	1	1	1	1		1	1	1	1	1		1	1	0	0	0	
105	n	1	1	1	1	1		1	0	0	0	0		1	0	0	0	0		0	0	0	0	0	
409	h	1	1	0	0	0		1	1	1	1	1		0	1	0	0	0		1	1	0	0	0	
293	b+c	1	1	1	1	1		1	0	0	0	0		1	0	0	0	0		1	1	1	1	1	
139	b+c	1	1	1	1	1		0	0	0	0	0		1	0	0	0	0		0	0	0	0	0	
556	b+h	1	1	1	1	1		1	1	0	0	0		1	1	1	1	1		1	1	1	0	0	
125	n	1	1	1	0	0		1	1	1	0	0		0	0	0	0	0		0	0	0	0	0	
492	n	1	1	1	0	0		1	1	1	1	1		0	0	0	0	0		1	1	1	1	0	
338	n	1	1	1	1	1		0	0	0	0	0		0	0	0	0	1		1	1	1	1	0	
105	n	1	1	1	0	0		1	1	0	0	0		1	0	0	0	0		1	1	1	1	1	
139	b+c	1	1	1	1	1		1	1	1	1	0		1	1	0	0	0		1	0	0	0	0	
492	n	1	1	0	0	0		1	1	1	1	0		1	0	0	0	0		1	1	1	1	1	
397	b+h	1	1	1	1	1		1	1	1	1	0		0	1	0	0	0		1	0	0	0	0	
345	n	1	1	1	1	0		1	1	1	0	0		1	1	1	1	0		0	0	0	0	0	
162	c	1	1	0	0	0		1	1	1	0	0		1	1	0	0	0		1	1	1	1	1	
550	m4	0	0	0	0	0		0	0	0	0	0		1	1	0	0	0		1	1	1	1	1	
373	b+h +a	1	1	1	1	1		1	1	1	1	1		0	0	0	0	0		1	1	0	0	0	
125	n	1	1	1	1	0		0	0	1	0	0		1	1	1	1	0		1	1	1	1	0	
337	b	1	1	1	1	1		1	1	0	0	0		0	0	0	0	0		1	1	0	0	0	
304	b+a	1	1	1	1	1		0	0	0	0	0		1	1	1	1	1		0	0	0	0	0	
145	n	1	1	1	0	0		1	1	1	1	1		1	0	0	0	0		1	1	1	0	0	
146	h+a	1	1	1	1	1		1	1	1	1	1		1	1	0	0	1		1	1	1	0	0	
419	b+h	1	1	0	0	0		1	1	1	1	0		0	0	0	0	0		1	1	1	1	0	
122	b+c	1	1	1	1	1		1	1	1	1	0		0	0	0	0	0		1	1	0	0	0	
392	C	1	1	1	1	0		0	0	0	0	0		1	1	1	0	0		0	0	0	0	0	

ATCC	b	1	1	1	1	1		1	1	1	1	0		1	1	1	1	1		1	1	1	1	1
Glycerol+DMSO @ -80								FBS+DMSO @ -80						Gelatin @ -80						control @ -80				
Sample		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16		W1	W2	W4	W8	W16
164	5	1	1	1	0	0		1	0	0	0	0		1	1	1	0	0		1	1	1	1	1
535	3	1	1	1	1	0		0	0	0	0	0		1	1	1	1	0		0	0	0	0	0
144	4	1	1	1	0	0		1	1	1	1	0		1	1	1	0	0		0	0	0	0	0
105	2	1	1	1	0	0		0	1	1	1	0		1	1	1	0	0		1	1	1	1	0
409	2	1	1	1	1	0		1	0	0	0	0		1	1	1	1	0		0	0	0	0	0
293	4	1	1	1	1	0		1	0	0	0	0		1	1	0	0	0		0	0	0	0	0
139	21	1	1	1	0	0		0	0	0	0	0		1	1	1	0	0		0	0	0	0	0
556	3	1	1	1	1	1		1	1	1	0	0		1	1	1	1	1		1	0	0	0	0
125	3	1	1	1	1	1		0	0	0	0	0		1	1	1	1	1		1	1	1	1	1
492	1	1	1	0	0	0		1	1	1	1	1		1	1	0	0	0		0	0	0	0	0
338	1	1	1	1	0	0		1	1	1	1	1		1	1	1	0	0		1	1	1	1	1
105	2	1	1	1	1	0		1	1	0	0	0		1	1	1	1	0		0	0	0	0	0
139	21	1	1	1	1	0		1	1	0	0	0		1	1	1	1	0		0	0	0	0	0
492	1	1	1	1	0	0		1	1	1	1	1		1	1	1	0	0		1	1	0	0	0
397	1	1	1	1	1	1		1	1	0	0	0		1	1	1	1	1		0	0	0	0	0
345	1	1	1	1	1	1		0	0	0	0	0		1	1	1	1	1		0	0	0	0	0
162	1	1	1	1	1	0		1	1	1	1	0		1	1	1	1	0		0	0	0	0	0
550	3	1	1	1	0	0		1	1	1	0	0		1	1	1	0	0		1	0	0	0	0
373	1	1	1	1	0	0		1	1	1	1	0		1	1	1	0	0		0	0	0	0	0
125	3	1	1	1	1	1		1	1	1	1	0		1	1	1	1	0		0	0	0	0	0
337	1	1	1	1	0	0		1	1	0	0	0		1	1	1	0	0		0	0	0	0	0
304	1	1	1	1	1	0		1	1	0	0	0		1	1	1	1	0		0	0	0	0	0
145	1	1	1	1	1	0		1	1	1	1	0		1	1	1	1	0		0	0	0	0	0
146	1	1	1	1	1	1		1	1	1	1	1		1	1	1	1	0		0	0	0	0	0
419	1	1	1	1	0	0		1	1	1	1	0		1	1	1	0	0		0	0	0	0	0

122	2	1	0	0	0	0		0	0	0	0	0		1	0	0	0	0		1	1	1	0	1
392	1	1	1	1	1	0		1	0	0	0	0		1	1	1	0	0		0	0	0	0	0
ATCC	3.3076	1	1	1	1	1		1	1	1	1	0		1	1	1	1	1		1	1	1	1	0