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Comparison of Culture and PCR for Detection of Field Isolates of Bovine Milk Mollicutes

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Abstract

Mycoplasma mastitis raises significant concerns in the dairy industry worldwide. The study objective was to develop an accurate and rapid screening method for identification of field isolates of *Mycoplasma* and *Acholeplasma* species and investigate relative merits of conventional microbial culture versus PCR-based method for detecting *Mycoplasma* and *Acholeplasma* species in bovine milk. A total 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. DNA extracted directly from milk and used for amplification through specifically designed universal mollicutes PCR-based method. Of them, 269 (73%) tested positive. Sequencing results of 30 positive samples targeting the 16S rRNA gene, showed five different mollicutes species involved, including *Acholeplasma laidlawii, Acholeplasma axanthum, Mycoplasma arginini, Mycoplasma bovirhinis, 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. The 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. It is recommended surveying mollicutes using the universal PCR used in this study. The PCR system used in this study showed significant rapidity and sensitivity compared to the conventional bacteriological culture method for screening *Mycoplasma* and *Acholeplasma* species in dairy herds.

Keywords: *Mycoplasma, Mastitis, Dairy cattle, Acholeplasma*

Sığır Sütü Mollekütlerinin Saha İzolatlarının Tespiti Için Kültür ve PCR Yöntemlerinin Karşılaştırması

Öz

Mycoplasma mastitisleri dünya çapında süt endüstrisinde önemli sorunlara yol açmaktadır. Çalışmanın amacı, *Mycoplasma* ve *Acholeplasma* türlerinin saha izolatlarının tanımlanması için doğru ve hızlı bir tarama yöntemi geliştirmek ve inek sütünde *Mycoplasma* ve *Acholeplasma* türlerinin saptanması için PCR tabanlı yönteme karşı geleneksel mikrobiyal kültürün göreceli değerlerini araştırmaktı. Güney Avustralya'daki tek bir süt çiftliğinden toplanan toplam 368 bireysel süt örneğinden 192 (%52)'sinde geleneksel kültür bazlı yöntem ile mollikût varlığı pozitif bulundu. Doğrudan sütten ekstrakte edilen DNA, özel olarak tasarlanmış evrensel mollikût PCR bazlı yöntemle amplifikasyon için kullanıldı. Bu örneklerden 269'u (%73) pozitif bulundu. 16S rRNA genini hedefleyen 30 pozitif örneğin sekanslama sonuçlarına göre, *Acholeplasma laidlawii, Acholeplasma axanthum, Mycoplasma arginini, Mycoplasma bovirhinis, Mycoplasma bovis* olmak üzere beş farklı mollikût türü belirlendi. Bu sonuçlara göre, tüm numuneler üzerinde türe özgü PCR gerçekleştirilmiştir. Türe özgü PCR kullanılarak gerçekleştirilen DNA amplifikasyonları 256 (%70) örnekte mollikût pozitif sonuç verdi. Geliştirilen evrensel PCR, türe özgü PCR ile en iyi uyumu gösterdi (Cohen's Kappa = 0.747±0.031). Yukarıda belirtilen mollikütlerin iki veya daha fazlasının birlikte enfeksiyonu yüksek prevalans gösterdi. Bu çalışmada mollikütlerin araştırılmasında evrensel PCR kullanımı önerilmektedir. Çalışmada kullanılan PCR sistemi, süt sığırcılığında *Mycoplasma* ve *Acholeplasma* türlerinin taranmasında geleneksel bakteriyolojik kültür yöntemine kıyasla önemli bir hız ve hassasiyet göstermiştir.

Anahtar sözcükler: *Mycoplasma, Mastitis, Süt sığırı, Acholeplasma*

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INTRODUCTION

Mycoplasmas and *Acholeplasma* are the smallest bacteria, belonging to the mollicutes class, andsome of these mollicutes can cause many infections in cattle. Diseases caused by *Mycoplasma* species occur worldwide causing serious problems for the dairy and beef feedlot industries and impose significant economic impact [1]. Specific concerns regarding *Mycoplasma* arise from difficulty of detection, a wide range of transmission methods, long persistence in affected herds, poor response to antimicrobials and the tendency to cause mixed infections [2,3]. *Mycoplasma* species leads to clinical, subclinical or chronic mastitis in cattle [4]. Among 200 of *Mycoplasma* species discovered, several species have been identified to be responsible for mastitis in cattle, or isolated occasionally from milk including *Mycoplasma. arginine, M. bovirhinis, M. bovigenitalium, M. bovis*, *M. californium, M. canadense, M. dispar, Mycoplasma* species bovine group 7 and F-38 [5]. *M. bovis* is the most common pathogen causing mastitis [6]. Some studies claim that *Acholeplasma* species, another genus of the mollicutes class, considered as non-pathogenic saprophyte and milk contaminant [7,8]. However, other studies have shown that involving of *A. laidlawii* in mastitis cannot be excluded [9-11]. Additionally, *Acholeplasma axanthum* has been isolated from bovine milk harvested from cattle suffering from mastitis [4]. Identification of milk *Mycoplasma* is often achieved using conventional bacteriological culture method or through serological determination methods. However, both detection methods have the significant limitation of a prolonged sampling to results timeframe [12,13]. *Mycoplasma* species can cause bovine mastitis cases either individually or as co-*Mycoplasma* infection [14]. Currently, most molecular studies focus on a single *Mycoplasma* species invader (usually *M. bovis)* and disregard potential co-infection. Few recent studies have included multiple *Mycoplasma* and *Acholeplasma* species in milk using multiplex PCR [15,16]. However, the universal PCR detailed in this work expands to few more common milk mollicutes including species not been reported previously, like. *A. axanthum* and *M. bovirhinis*. Previous studies have reported co-infections of limited variety. Current knowledge does not inform the reader of the potential combinations and their effect on milk composition and yield.

Detection of *Mycoplasma* species using 16S rRNA as a molecular marker has been previously evaluated [17-19]. The usefulness of 16S rRNA gene was demonstrated in detecting slow-growing bacteria [20]. However, most previous studies targeted species-specific oligonucleotides and disregarded co-infection of *Mycoplasma* and *Acholeplasma* species. The clinical importance of co-infection with *Mycoplasma* has been reported [9,21]. Given that mollicutes have a small genome and low G-C content [22], a sensitive, accurate and broad-species detection is required. Implementation of a rapid, reliable and affordable screening method can be used in eradication strategies of *Mycoplasma* mastitis at quarter, cattle, herd and national level.

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

MATERIAL and METHODS

Samples Collection

Milk samples were collected aseptically in sterile 50 mL tubes at individual cow level from a single commercial dairy farm near Mount Gambier/South Australia. Cows had high somatic cell counts and the farm had experienced repeated failure of mastitis treatment at the time of sampling. A total of 368 milk samples were collected from each functional quarter. Milk samples were kept on ice and sent immediately to the laboratory at the School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia. Milk samples were subjected to conventional *Mycoplasma* culture, and remaining sample contents were frozen at -20°C and retained for molecular analysis.

Mollicutes Culture

Milk samples were subjected first to bacterial pre-enrichment process following the procedure described previously [23,24]. Detection of mollicutes colonies were performed using a stereomicroscope (Olympus SZ30, Vic, Australia) at 10x magnification. Positive culture samples were counted when there is growth on the mollicutes agar plate of at least one *Mycoplasma*-like colony [25]. The process of axenization of mollicutes was carried out by selecting 3-5 colonies from each plate then subcultured into the enriched *Mycoplasma* broth (Oxoid, Australia) and inoculated under the same conditions. When there was change of colour of phenol red indicator in *Mycoplasma* broth to yellow, the subculture onto a fresh broth and agar was carried out.

DNA Extraction

The DNA extraction was carried out directly, either from frozen milk or enriched samples and all tests were repeated on both type of samples. After thawing milk samples at ambient temperature, 2 mL of each milk sample was centrifuged at 8.000 g for 20 min to remove supernatant fat and excess liquid. The enriched samples in broth were used directly for DNA extraction. DNA was extracted using QIAmp DNA extraction kit (Qiagen, Germany) following the manufacturer's instructions. Genomic DNA concentration measurement was carried out using Nanodrop l000c (Thermofisher Scientific Inc., Waltham, MA, USA).

PCR Probes and Protocol

In our study, five different primers pairs were used for five separated PCR reactions. The universal primers, Mol-F: GGCGAAYGGGTGAGTAACAC and Mol-R: CATHG YCTTGGTRRGCYNTTA were designed targeting 16S rRNA gene at genus-level and generate amplicon (180 bp). Multiple sequence alignment of 16S rRNA gene was conducted on number of cattle-associated *Mycoplasma* and *Acholeplasma* using Clustal Omega software [26]. A block containing highly variable region, flanking by two conserve regions in upstream and downstream of the sequences, was selected. Based on general criteria for primer designing, forward and reverse primers were selected from conserved region of multiple blocks. The accuracy of the different primer sets for different blocks was checked by ATCC strains, PCR and sequencing. *Acholeplasma laidlawii* (Sabin) Edward and Freundt (ATCC® 23206-MINI-PACK™) and *Mycoplasma bovis* (ATCC® 25025™) were used as positive controls. *M. bovis*-specific 16S rRNA primers (442 bp), composed of PpSM5-1: 5′-CCAGCTCACCCTTATACATGAGCGC-3′ and PpSM5-2: 5′-TGACTCACCAATTAGACCGACTATTTCACC-3′ were used for *M. bovis* detection [12]; while the other three primers for *A. laidlawii*, *M. arginini* and *M. bovirhinis* were previously published elsewhere and cited in our previous work [9]. PCR reactions were carried out in 25 µL containing 0.25 µL Taq DNA polymerase (Bioline, UK), 5 µL of 5x reaction buffer (Bioline, UK), 1 µL (0.5 µM) of each forward and reverse primers (AGRF, Australia), 1 µL (approximately 20 ng) of template, and 16.75 µL of DEPC-treated water. The negative control was prepared from the same reagents of Master Mix (Bioline, UK), except DNA template, and the volume was compensated with DEPC water [27]. DNA was amplified for 35 PCR cycles conditions using T100™ Thermal Cycler (Biorad thermocycler, Australia), and consisted of pre-heating activation for 5 min at 95ºC, denaturation at 95ºC for 30 sec, annealing at 60ºC for the universal primer, *M. bovis* and A. *laidlawii*; 55ºC for *M. arginini* and 64ºC for *M. bovirhinis,* and primer extension at 72ºC for 45 sec. The final extension step was performed at 72ºC for 10 min. The PCR products were analysed by 1.5% agarose gel electrophoresis and visualised by staining with Gel Red (Biotium, US. Selected species for this study were nominated based on the 16S rRNA sequencing of the universal PCR. The same PCR methods have been done on all isolated mycoplasmas to identify the isolate in sequencing of the PCR products. All tests were carried out in duplicate. Six samples for each positive 16S rRNA PCR detected species were submitted to the Australian Genome Research Facility Ltd (AGRF, Adelaide, South Australia) for Sanger sequencing according to the method described previously [19].

Statistical Analysis

Positive results of conventional bacteriological culture method, universal PCR and species-specific PCR were reported as number and percentage. Cohen's Kappa coefficient test was used to identify the agreement between the abovementioned detection methods using (R version 3.1.1, R Development Core Team, New Zealand).

RESULTS

Of 368 milk samples collected at individual cow level from a single dairy farm in South Australia, the universal PCR used in this study showed higher prevalence of mollicutes in milk (73%) as compared to the conventional culture method (52%) *(Table 1)*. Samples were considered as positive for culture growth when at least a single colony of mollicutes was identified. PCR results were confirmed using species-specific primers (according to 16S rRNA sequencing results) for *A. laidlawii, M. arginini* and *M. bovirhinis*. Using species-specific primers, co-infection with two or more of the aforementioned mollicutes was detected in 165 (45%); *A. laidlawii* was the highest individual species detected followed by *M. bovis* and *M. bovirhinis*, *M. arginini* while *A. axanthum* had the lowest prevalence *(Fig. 1)*. In addition, 34% of samples were negative for culture and positive for either or both PCR methods (universal and species-specific). However, approximately 7% of positive samples were identified by culture but not by PCRs, 36 samples tested positive using the universal PCR, but negative using species-specific primers for *A. laidlawii*, *M. bovis*, *M. bovirhinis* and *M. arginini (Fig. 2)*. These were confirmed as *A. axanthum* via 16S rRNA sequencing. Cohen's Kappa coefficients showed good agreement between the universal PCR and species-specific PCRs and fair agreement between culture and both PCR tests *(Table 2)*.

DISCUSSION

The objective of this study was to develop an accurate, rapid, and reliable method for milk screening of *Mycoplasma* and *Acholeplasma* species and investigate the relative merits of microbiological and molecular detection of mollicutes in bovine milk.

Table 1. *Count of samples positive for detected mollicutes (Mycoplasma + Acholeplasma) species using conventional bacteriological culture method, universal PCR and species-specific PCR*

Test	Positive	Negative	Percentage
Conventional bacteriological culture method	192	176	52%
Universal PCR	269	99	73%
Species-specific PCR	256	112	70%

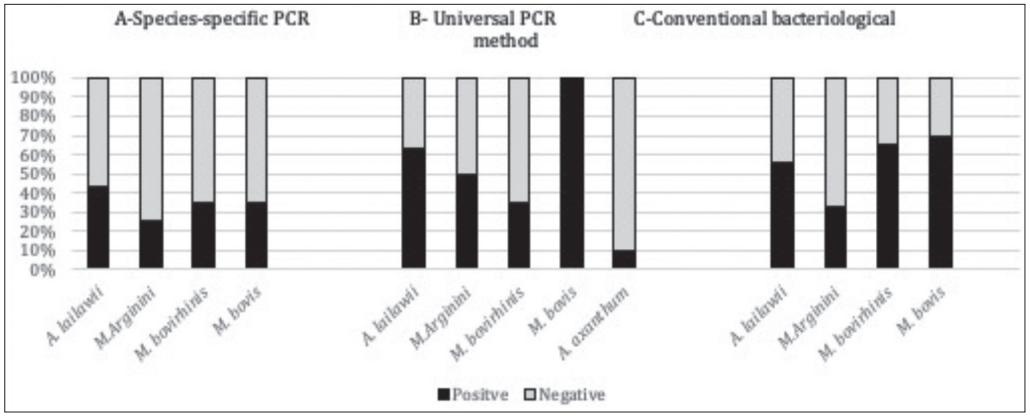

Fig 1. Percentage of positive/ negative samples detected by species-specific PCR, universal PCR and culture for each species from bovine milk samples

Table 2. *Concordance between three detecting tests for identification of Mycoplasma and Acholeplasma species from milk samples of cattle*

Tests	Concordant	Discordant	Concordant (%)	Cohen's Kappa (95% CE)	Concordance
Conventional bacteriological method versus Universal PCR	225	143	61%	0.298±0.049	Fair
Conventional bacteriological method versus Species-specific PCR	212	156	58%	0.213±0.048	Fair
Universal PCR versus Species-specific PCR	313	55	85%	0.747±0.031	Good

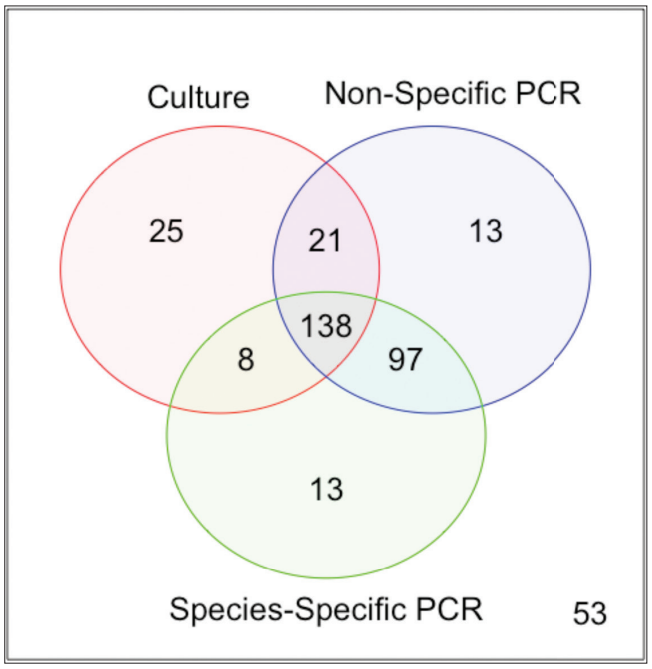

Fig 2. Venn diagram of the positive and negative samples of the three detection methods from 368 bovine milk samples from a single farm in South Australia

The studied farm had a history of treatment failure of mastitis with high somatic cell counts (~300.000 cells/ mL at bulk tank level). *Mycoplasma* mastitis has a wide range of transmission methods through milking machines and other fomites [28,29]. Another important reason for the relatively high prevalence of mastitis causing *Mycoplasma* is due to intermittent shedding of the infection from the chronic mastitic cattle [30]. It is understood that *Mycoplasma* species have the ability to form multiple micro-abscesses within the infected mammary gland leading to chronic mastitis [31]. Results of the current study can be the cornerstone for raising awareness of the consequence of mollicutes-induced mastitis for the dairy industry. The association of these mollicutes and mastitis in addition to their pathogenic significance have previously studied [9]. The study concluded that the co-infection with *Mycoplasma* and *Acholeplasma* species has similar effects on milk composition to other major mastitis pathogens [9]. Therefore, the developed universal PCR in this study is useful for milk mollicutes screening. Further research in affected herds is required to establish the current prevalence of *Mycoplasma* mastitis in Australian dairy herds. Our study found that sensitivity of mollicutes detection using the novel universal 16S rRNA amplification was significantly higher than detection using the culture-based method. Naturally, 16S rRNA demonstrates high copy numbers and low sequence diversity which can enhance sensitivity of PCR based tests [32,33]. Results of our study show that one third of samples returned negative *Mycoplasma* results for culture and positive for both PCR methods *(Fig. 1)*. This difference can be explained by the fastidious nature of *Mycoplasma* species, as failure to culture may occur due to lack of a cell wall [21], or due to involvement of multiple *Mycoplasma* species in a single case of mastitis that may have affected the growth requirements of each individual *Mycoplasma* colony. However, approximately 7% of positive samples were identified by culture, but not by PCR methods. This may be attributed to failure of DNA amplification due to existing inhibitors in milk samples or

due to failure of the developed universal 16S rRNA PCR to detect some of the species.

Although culture-based methods is still considered as a gold standard in the detection of *Mycoplasma* infection [34], the specificity of this test particularly for various genera and species of mollicutes is challenging. Morphologically, detected colonies, which grew on the specific *Mycoplasma* 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 have also been observed previously [7]. Hence, these authors developed biochemical and molecular differentiation techniques [7]. In this study, we have confirmed the different species using PCR/sequencing tests but not using the biochemical properties.

In conclusion, the newly developed universal PCR of 16S rRNA by this study showed significant sensitivity to detect various *Mycoplasma* and *Acholeplasma* at genus-level in milk. Direct extraction of DNA from milk for detection of *Mycoplasma* 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* species for farmers and laboratory staff.

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