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

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

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

1 Introduction

Mastitis caused by Mycoplasma spp is among the emerging challenges facing the dairy industry worldwide, resulting in significant economic consequences. Similarly to the more common mastitis pathogens, Mycoplasma mastitis can affect milk quantity and quality [1-3] More than 200 spp. of mycoplasmas, acholeplasmas and other related mollicutes have been discovered [4]. Several of these mollicutes are of great concern in the dairy industry including M. bovis, M. bovigenetalium, M. alkalescens, M. californium, and M. canadense. Other isolates that are less

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frequently involved in bovine mastitis include: M. arginini, M bovirhinis, M. leachii, and M. dispar [5]. In addition, A. laidlawii has been reported to be less significant [4] or of equal significance to mastitis cases caused by mycoplasmas [1, 6]. Determining the species and strains involved should contribute to diagnostic and control strategies, in addition to providing a better understanding of the epidemiology of Mycoplasma mastitis in Australian dairy herds. The usefulness of 16S rRNA sequencing, due to the high copy numbers of sequenced data, has been proven and it is an efficacious, discriminatory, sensitive and accurate tool for studying the epidemiology of pathogens [7]. Copy numbers of some mollicutes have been estimated for the 16S rRNA gene to vary between 400 and 2000 [8].

Although the genotyping of Australian strains of M. bovis as the major pathogenic Mycoplasma in cattle has been reported recently [9], other mastitis causing mycoplasmas and non-pathogenic milk mollicutes are yet to be studied. Moreover, co-infection of mycoplasmas and Acholeplasma laidlawii has been recently reported to cause similar mastitis to other major mastitis pathogens [1]. This study aimed to determine the genotype distribution of Mycoplasma spp and other milk mollicutes isolated from two commercial dairy farms in South Australia, and compare their evolutionary relationship to isolates collected elsewhere.

2 Materials and Methods

Milk samples originated from two commercial dairy farms from the South East and Mid North regions of South Australia. A total of 368 milk samples were collected from Farm 1. Of them, one representative sample for each individual identified species was selected based on sequencing results [1]. The remaining 40 milk samples were collected from Farm 2. One sample of M. alkalescens isolated from Farm 2 was selected based on sequencing and 16S rRNA universal primer results. All samples were positive for Mycoplasma on conventional culture. Axenization of these bacteria was carried out. DNA was extracted directly from milk samples using DNA extraction kit (Qiagene, Germany). One set of universal primers included Mol-F: 5'-GGCGAAYGGGTGAGTAACAC-3' and reverse primer Mol-R: 5'-CATHGYCTTGGTRRGCYNTTA-3'. We have developed HRM analysis and qPCR assays to cover the variable parts of the 16S rRNA from mollicutes with the conserved primer binding sites. The PCR product is a 180 nucleotide and our submitted sequence for Acholeplasma has >700bp. We have amplified short part of the sequence to compare wider ranges of mollicutes; i.e., the primers and the sequences have been extracted from submitted sequences with different lengths but the assay has run on short 180bp sequence for test development and screening the archive. Real-time PCR-HRM analysis was performed to discriminate between some of these isolates using two pairs of genus-level universal primers targeting 16S rRNA [10]. Based on their HRM profile, one representative sample from each different profile was selected for 16S rRNA sequencing. Subsequently, DNA of the specific band (bp=180) was purified from gel using (Qiagene, Germany) for sanger sequencing. Sanger sequencing was performed at the Australian Genome Research Facility (AGRF, Adelaide, Australia). Assembling of sequences was performed using (ClustalX, 2.1) and compared with selected closest neighbors references identified by NCBI (www.ncbi.nlm.nih.gov). Evolutionary analyses at cluster level were conducted in MEGA7 (Molecular Evolutionary Genetics Analysis, version 7.0). The evolutionary history at the cluster level was inferred using the Neighbour-Joining method [11]. The optimal tree with the sum of branch length = 1.97698025 is shown. To build a reliable tree, huge length sequences were trimmed. The tree is drawn to scale, with branch lengths in the same

units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair. There were a total of 1738 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [12].

3 Results

In this study, six different field isolates of mycoplasmas and acholeplasmas have clustered in three distinct groups. The Acholeplasma group compromises two distinct clusters of field isolates of acholeplasmas. First, A. laidlawii (MH259813.1) showed 100% similarity to other bovine strains of this spp. isolated in The United States and South Korea (JN935887.1, NR074448.2 and LC201977.1), and were clearly distinct from other environmental A. laidlawii (FJ226559.1, J65556.1 and JN935888) presented in Figure 1. Second, A. axanthum (MH259849.1) showed 99% similarity to US (FJ876270.1) and Swedish (NR 028827.1) strains. Mycoplasma alkalescens (MH259845.1) was isolated from Farm 2, a 400 cow herd in the Mid North region of South Australia. The farm had a rising SCC over a period of four weeks with multiple antimicrobial treatment failures. Mycoplasma alkalescens clustered into the same clades of Swedish strain PG51 and other Japanese PG51 strains with reference number LC158831.1. The phylogenetic position of the M. bovirhinis strain in this study (MH266037.1) showed high similarity to an Egyptian strain: Fay. Bu1-10 (Fig 1). Two different strains of M. bovis has been recorded in this study, the first strain (40) clustered with M. bovis strains: 08M, Ningxia-1 and JF4278. The second field strain of M. bovis in this study (9029) was grouped with Egyptian and Hungarian strains.

4 Discussion

This study aimed to determine the genotypic distribution of some Mycoplasma and Acoleplasma spp isolated from two commercial dairy farms in South Australia based on 16S rRNA sequencing, and compare their evolutionary relationship to some similar isolates from elsewhere. The presence of A. laidlawii in milk samples from mastitis cows remains a controversial subject, however, with some authors considering it as pathogenic and others suggesting that it acts as an environmental milk saprophyte [1, 13-17] Acholepllasma axanthum was firstly reported in 1970 [18], and its pathogenicity in pneumonic swine has been studied [19]. A. axanthum has been isolated from mastitic milk [20, 21]. However, its role in mastitis is still unknown. The effects of some of the aforementioned mollicutes on milk quality and quantity have already been testified [1]. Co-infection with more than one species has severe consequences on milk compositions, similar to major conventional mastitis pathogens. Studying the correlation between genetic, observational and pathological effects of these genera/species may contribute in determination of their role in bovine mastitis.

Mycoplasma alkalescens (MH259845.1) was isolated from Farm 2, a 400 cow herd in the Mid North region of South Australia. The farm had a rising SCC over a period of four weeks with multiple antimicrobial treatment failures. Mycoplasma alkalescens clustered into the same clades of Swedish strain PG51 [27], and other Japanese PG51 strains with reference number LC158831.1 (unpublished).

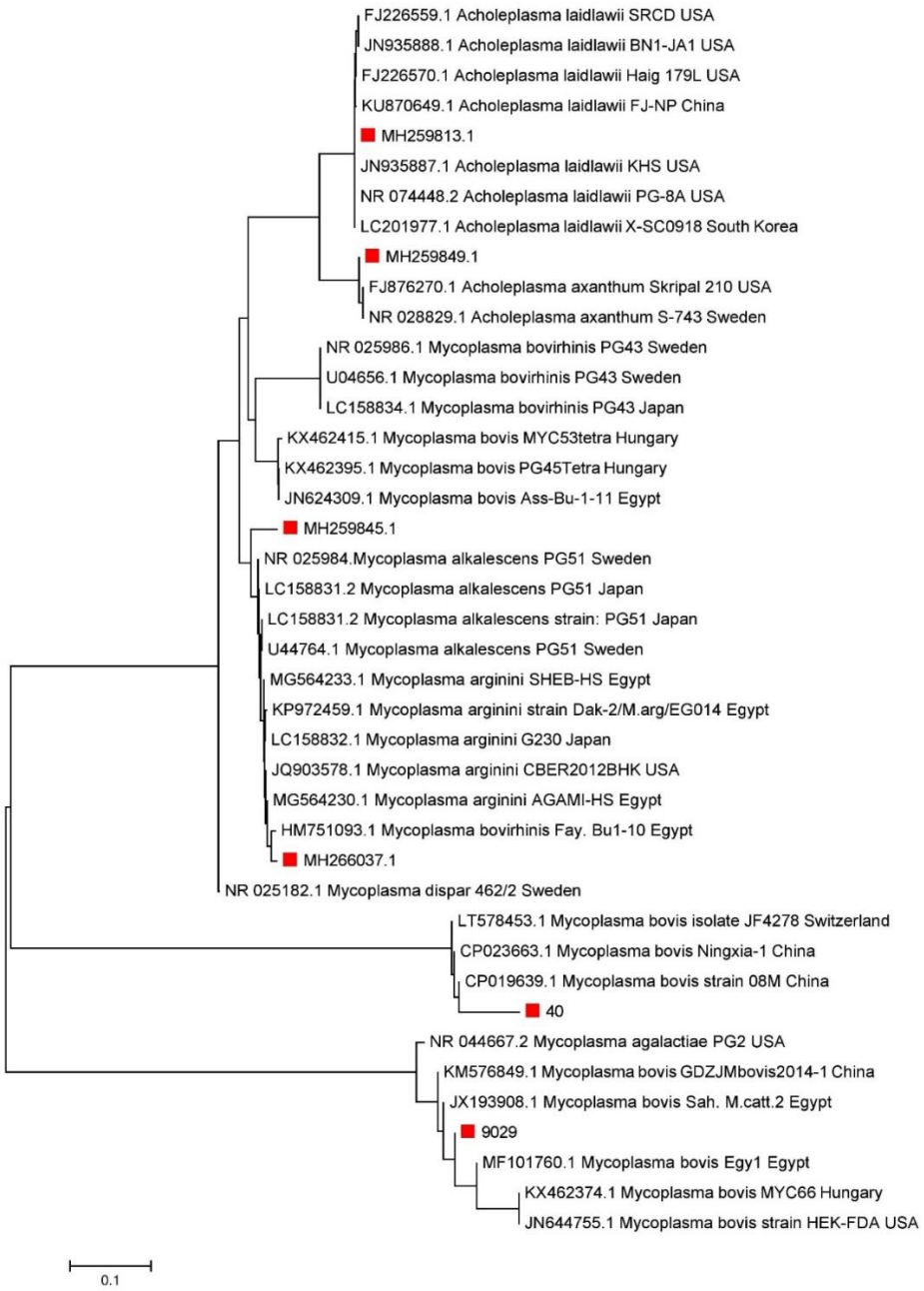

Fig. 1. Neighbor-joining consensus phylogenetic tree of six bovine milk mollicutes created by Mega 7 software based on 16S rRNA sequencing, and their relationship to referenced mollicutes.

Mycoplasma alkalescens was firstly identified in Australian cattle in 1963 [22], and has been isolated from the nasal cavity, joints affected by arthritis, mammary gland affected by mastitis, cows with dystocia and cows with endometritis [23-25]. This reflects the ability of this pathogen for colonization in a range of tissues. Further studies on the pathogenicity of these pathogens in mastitis cases are required. The phylogenetic position of the M. bovirhinis strain in this study (MH266037.1) showed high similarity to an Egyptian strain: Fay. Bu1-10 (Fig 1). Mycoplasma bovirhinis is well known to be isolated from the respiratory organs and causes secondary infections in cattle [26]. However, this pathogen has also been isolated from cows with mastitis [1, 27]. Mycoplasma bovirhinis strain PG51 has been reported previously as a secondary invader in mastitic cows in more than 70% of Australian dairy herds, which can be explained by the ascending transmission from the nasal secretions of calves during suckling [28]. Mycoplasma bovirhinis sequences in this study clustered with the M. arginini group which may clinically explain the usual co-invasion of these genetically related mycoplasmas in bovine diseases [1, 29].

Two different strains of M. bovis has been recorded in this study. The first strain (40) clustered with M. bovis strains: 08M, Ningxia-1 and JF4278. The first two of these strains have been isolated in China [30, 31], while the third strain was identified in Switzerland [3]. The second field strain of M. bovis in this study (9029) was grouped with Egyptian and Hungarian strains [32, 33]. The genetic relatedness between some of the strains isolated in this study and those previously isolated elsewhere indicate a correlation of the molecular epidemiologic distribution of mycoplasmas, and it is influenced by live animal movement interstate or foreign trade. Although the results of this study are not representative of all dairy herds in South Australia, the existing strains of pathogenic mycoplasmas may raise the awareness to include them in perspective epidemiological surveys and control strategies.

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