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Short Communication

Genetic diversity of *Mycoplasma arginini* isolates based on multilocus sequence typing

Olusola M. Olaogun, Anna Kanci, Stuart R. Barber, Kelly A. Tivendale, Philip F. Markham, Marc S. Marenda, Glenn F. Browning*

Asia-Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia

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ABSTRACT

The contribution of *Mycoplasma arginini* to mycoplasmosis in small ruminants remains unclear because it is recovered from both healthy and diseased animals. In order to gain a better understanding of any relationships between isolates from different sites and different geographical locations, we developed a method for genotyping *M. arginini* using multilocus sequence typing (MLST). A MLST scheme based on five housekeeping genes was used to characterize *M. arginini* isolates from flocks of sheep and goats. A high level of genetic variability was detected between strains and within herds.

1. Introduction

Members of the genus *Mycoplasma*, Class *Mollicutes*, are small self-replicating prokaryotes that lack a cell wall and are important bacterial pathogens of humans and animals, causing a number of debilitating and chronic illnesses (Citti and Blanchard, 2013). In small ruminants, mycoplasmosis is of considerable economic and clinical importance (Nicholas et al., 2008). The World Organisation for Animal Health (OIE) lists two mycoplasmal diseases of small ruminants, contagious caprine pleuropneumonia (CCPP), caused by *Mycoplasma capricolum* subsp. *capripneumoniae*, and contagious agalactia (CA), caused by four mycoplasma species (*M. agalactiae*, *M. mycoides* subspecies *capri*, *M. capricolum* subspecies *capricolum* and *M. putrefaciens*), as notifiable diseases because of their severe economic impact (Nicholas et al., 2008).

Mycoplasma arginini is commonly isolated from many animal species and humans, and is a frequent contaminant of tissue cultures (Chazel et al., 2010; Nicholas et al., 2008; Sillo et al., 2012). It has been isolated from both healthy and diseased sheep and goats alone, or in association with other pathogenic bacterial species, from a number of sites, including the respiratory tract, udder, joints, eyes, ears, blood and urogenital tract (Chazel et al., 2010; Stipkovits et al., 2013). No pathology has been attributed to infection with *M. arginini* in domestic animals and its clinical and economic significance remains uncertain. As a consequence it is often considered to be a commensal of warm-blooded animals (Nicholas et al., 2008). Although several reports have suggested that some strains may influence the severity of respiratory disease in small ruminants, experimental and epidemiological studies in small ruminants are yet to establish their pathogenicity (Goltz et al., 1986; Stipkovits et al., 2013; Weiser et al., 2012). Molecular epidemiological typing methods for *M. arginini* would facilitate strain differentiation and thus help in resolving some of the uncertainty surrounding the clinical significance of this mycoplasma species.

Several typing techniques have been developed to compare isolates of the same species and investigate the population genetics of bacterial species (Ranjbar et al., 2014). Of these tools, multilocus sequence typing (MSLT) is preferred for genotyping bacterial isolates as it allows unambiguous and highly reproducible strain discrimination, as well as the development of digital archives of strain genotypes, allowing both prospective and retrospective analyses (Maiden, 2006). In MLST, five to seven housekeeping genes are sequenced in each isolate. Each housekeeping gene is a locus and distinct sequences at each locus are arbitrarily assigned a unique allele number. A combination of allele numbers is designated a sequence type (ST). Sequence types (STs) that have similar alleles at a specified number of loci are assigned into lineages referred to as clonal complexes (CC) (Maiden, 2006). MLST has been applied to several mycoplasma species, and the schemes were able to successfully capture intermediate-level evolutionary relationships among the typed isolates (McAuliffe et al., 2011; Fischer et al., 2012; Manso-Silvan et al., 2012).

* Corresponding author. Fax: +61383447374.
E-mail address: glenfb@unimelb.edu.au (G.F. Browning).

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Recently the draft genome sequence of *M. arginini* strain 7264, an isolate from the lung of a calf with pneumonia in France, was published (Manso-Silvan et al., 2013). This allowed us to identify potential MLST loci and to design primers for their amplification. The study reported here was conducted with the aim of developing a MLST scheme to characterize *M. arginini* using a collection of field isolates from a variety of sites and disease conditions in sheep and goats in Victoria, Australia.

2. Materials and methods

2.1. Bacterial isolates, DNA extraction and species identification

A total of twenty *M. arginini* isolates recovered from milk, ear swabs, nasal swabs, bronchial lavages and lungs of sheep and goats from various regions in Victoria, Australia, during an epidemiological survey between 2011 and 2013 were used in this study (Table 1). The samples were collected from four small ruminant herds and a major small ruminant abattoir. Samples were transported on ice to the laboratory and inoculated on the day of collection into modified Hayflick's broth supplemented with thallium acetate (Kirchhoff and Rosengarten, 1984) and cultures were incubated for three to five days at 37 °C. When mycoplasma growth was suspected because of an increased turbidity, the cultures were inoculated onto solid modified Hayflick's medium and the plates were incubated at 37 °C until typical mycoplasma colonies appeared. One colony per isolate was then filter-cloned twice by passing broth through a 0.22 μm membrane filter and then immediately plating the filtrate on agar. A colony from this plate was suspended in broth and the procedure was repeated.

Mycoplasma genomic DNA was prepared using phenol–chloroform extraction from a 40 ml volume of broth culture (Sambrook et al., 1989). The DNA was suspended in 50 μl 2.5 M sodium acetate (pH 5.05), precipitated with ethanol at −20 °C and re-suspended in 200 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The concentration of the purified genomic DNA was determined using a NanoDrop spectrophotometer ND-1000 v3.7 (Thermo Fisher Scientific, USA). In order to confirm the species identity of the isolates, a PCR targeting the 16S rRNA of *Mycoplasma* species was performed on all samples using the primers GPO3 (5′-GGG AGC AAA CAG GAT TAG ATA CCC T-3′) and MGSO (5′-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3′) (van Kuppeveld et al., 1992). This was followed by a *M. arginini*-specific PCR using the primers MAGF (5′-GCA TGG AAT CGC ATG ATT CCT-3′) and GP4R (5′-GGT GTT CTT CCT TAT ATC TAC GC-3′) as described previously (Weiser et al., 2012). DNA sequencing of the 16S rRNA PCR product was performed on a selected set of isolates to confirm the specificity of the PCR assay for species identification.

2.2. Multilocus sequence typing

The MLST scheme was based on that developed for the *M. mycoides* cluster (Fischer et al., 2012), with some modifications introduced based on the partially assembled full genome sequence of *M. arginini* strain 7264 (Manso-Silvan et al., 2013), the only available *M. arginini* genome sequence at the time of the study. Primers sets were designed to amplify 500–700 nt internal fragments of the genes for adenylate kinase (*adk*), guanylate kinase (*gmk*), DNA gyrase subunit B (*gyrB*), DNA polymerase III (*polC*) and the RNA polymerase beta chain (*rpoB*) using Primer-BLAST (Ye et al., 2012) as detailed in Table 2.

PCRs were performed in a final volume of 50 μl containing 2 mM $MgCl_2$, 200 μM dNTPs (Promega, USA), 1.25 units of Taq DNA polymerase (Promega, USA), 2.5 pmol of each forward and reverse primer, approximately 50 ng of mycoplasma genomic DNA and 1 × GoTaq buffer as recommended by the manufacturer (Promega, USA). Amplifications were performed using the following incubation conditions: one cycle of 94 °C for 2 mins, 40 cycles of 94 °C for 45 s, 53 °C for 45 s and 72 °C for 1 min, then a final incubation at 72 °C for 7 mins. PCR products were analysed on a 1% agarose gel containing Sybr® Safe DNA gel stain (Life Technologies, USA) in 0.5 × TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0) using Hyperladder I (Bioline, Australia) as molecular weight markers. PCR products were purified using MoBio Ultraclean Gel Spin DNA purification kits (MoBio Laboratories Inc, USA) and purified DNA was quantified using a NanoDrop spectrophotometer ND-1000 v3.7. DNA sequencing reactions were performed using BigDye terminator technology at the Centre for Translational Pathology, University of Melbourne, Australia. All loci were sequenced in both forward and reverse directions with the same primers as were used for the PCRs (Table 2). Sequences are available in GenBank (Accession numbers KR708714–KR708818).

Table 1
M. arginini isolates used in this study.

Sample ID	Origin	Year of isolation	Sample type	Source	Clinical status
7264	France	2007	Lung	Calf	Pneumonia
93	Farm A	2011	Milk	Sheep	Subclinical mastitis
GE3	Abattoir	2012	Ear swab	Goat	Unknown
32RE	Abattoir	2011	Ear swab	Sheep	Unknown
GE10	Abattoir	2012	Ear swab	Goat	Unknown
3L	Abattoir	2011	Lung	Sheep	Pneumonia
33LE	Abattoir	2011	Ear swab	Sheep	Unknown
1011	Farm B	2012	Milk	Sheep	Subclinical mastitis
1066	Farm B	2012	Milk	Sheep	Subclinical mastitis
1033	Farm B	2012	Milk	Sheep	Subclinical mastitis
LS/L/3704	Farm C	2012	Bronchial lavage	Sheep	Pneumonia
LS/L/4533	Farm C	2012	Bronchial lavage	Sheep	Pneumonia
LS/L/4193	Farm C	2012	Bronchial lavage	Sheep	Pneumonia
MCL219	Farm D	2013	Milk	Sheep	Clinical mastitis
MCL220	Farm D	2013	Milk	Sheep	Clinical mastitis
MCL237	Farm D	2013	Milk	Sheep	Clinical mastitis
MCL222	Farm D	2013	Milk	Sheep	Clinical mastitis
MCL223	Farm D	2013	Milk	Sheep	Clinical mastitis
MCL239	Farm D	2013	Milk	Sheep	Clinical mastitis
M12693	Farm E	2012	Nasal swab	Sheep	Pneumonia
12900	Farm E	2012	Nasal swab	Sheep	Pneumonia