Veterinary Microbiology 191 (2016) 60–64

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Original article

Multilocus sequence typing of *Mycoplasma mycoides* subsp. *capri* to assess its genetic variability in a contagious agalactia endemic area

Juan Tatay-Dualde, Miranda Prats-van der Ham, Christian de la Fe*, Ángel Gómez-Martín, Ana Paterna, Juan Carlos Corrales, Antonio Contreras, Antonio Sánchez

Ruminant Health Research Group, Department of Animal Health, Faculty of Veterinary Sciences, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, Campus de Espinardo s/n., 30100 Murcia, Spain

ARTICLE INFO

Article history:
Received 7 March 2016
Received in revised form 4 May 2016
Accepted 6 June 2016

Keywords:
Mycoplasma mycoides subsp. *capri*
Multilocus sequence typing
Epidemiology
Contagious agalactia
Surveillance
Goat

ABSTRACT

Mycoplasma mycoides subsp. *capri* (Mmc) is one of the main causative agents of caprine contagious agalactia. Besides, the absence of accurate control methods eases its dispersion between different herds within endemic areas of this disease. In this context, there is a need to implement molecular typing schemes which offer valuable information useful to establish control measures and enables the surveillance of this pathogen. The aim of this study was to assess the genetic variability of different strains of Mmc from a contagious agalactia endemic area through multilocus sequence typing (MLST).

For this purpose, five house-keeping genes (*fusA, glpQ, gyrB, lepA, rpoB*) from 39 field isolates were analysed. These isolates were obtained from different geographic areas of Spain, between the years 2004 and 2015. The results obtained in this study suggest that the selected MLST scheme could be a useful technique to monitor the genetic variability of Mmc in endemic areas. Despite the significant differences found between the assessed field isolates, they could be classified according to their geographical origin. Moreover, it was also possible to detect genetic differences between Mmc strains coming from the same herd at the same sampling time, which may need to be taken into consideration when designing or arranging prophylactic strategies.

1. Introduction

Contagious agalactia (CA) is an infectious disease affecting small dairy ruminants and which may be caused by four different mycoplasma species, including *M. mycoides* subsp. *capri* (Mmc). This pathogen is the main causative agent of caprine CA in some regions, as it has been reported in France (Chazel et al., 2010) and in the Canary Islands (Spain) (Assuncao et al., 2004). Mmc belongs to the *M. mycoides* cluster, a group of five closely related mycoplasmas that are pathogenic to ruminants. Apart from Mmc, this group also comprises *M. mycoides* subsp. *mycoides* "Small Colony", *M. capricolum* subsp. *capricolum*, *M. putrefaciens* and *M. leachii* (Manso-Silvan et al., 2007). Nevertheless, the absence of accurate control methods of this disease and the possible dispersion between different herds due to asymptomatic carriers of the agent (Amores et al., 2011), highlight the need to implement molecular typing schemes to offer robust and reproducible epidemiological information, providing valuable data that may be used to establish control measures and also allowing to trace back the source of infection after an outbreak of CA caused by Mmc. In this sense, Mmc isolates have been previously characterized by using several molecular typing methods such as pulsed-field gel electrophoresis (PFGE) (Corona et al., 2013), suggesting a low phenotypic stability.

From all the molecular typing tools available nowadays, multilocus sequence typing (MLST) is the most suitable technique to genotype bacterial isolates, as it allows an unambiguous and highly reproducible discrimination between different strains (Maiden, 2006). In this sense, an MLST scheme to distinguish the different mycoplasma species within this cluster has been reported (Manso-Silvan et al., 2007). This scheme is based on the genomic sequences of five housekeeping genes (*fusA, glpQ, gyrB, lepA* and *rpoB*), which have proved their ability to assign phylogenetic positions to all the members of the *M. mycoides* cluster.

Previous works have demonstrated the utility of MLST techniques to analyse evolutionary relationships among different mycoplasma species, including *M. bovis* (Rosales et al., 2015), *M. arginini* (Olaogun et al., 2015) or other CA-causing agents such as

* Corresponding author.
E-mail address: cdelafe@um.es (C. de la Fe).

http://dx.doi.org/10.1016/j.vetmic.2016.06.002
0378-1135/

Table 1
Description of the 39 field isolates of Mmc assessed in the present study.

Strain	Source	Geographical origin	Year
LC68	Bulk tank milk	Andalusia, Málaga[3]	2012
LC71	Bulk tank milk	Andalusia, Málaga[3]	2012
LC72	Auricular swab	Andalusia, Granada	2012
LC76	Bulk tank milk	Andalusia, Málaga	2013
LC79	Bulk tank milk	Andalusia, Málaga[4]	2014
LC80	Bulk tank milk	Andalusia, Málaga	2014
LC81	Bulk tank milk	Andalusia, Málaga	2014
LC82	Bulk tank milk	Andalusia, Málaga	2014
LC83	Bulk tank milk	Andalusia, Málaga[5]	2014
LC88	Auricular swab	Andalusia, Málaga	2015
LC96	Bulk tank milk	Andalusia, Málaga[4]	2014
LC97	Bulk tank milk	Andalusia, Málaga[4]	2015
LC98	Bulk tank milk	Andalusia, Málaga[5]	2015
LC100	Bulk tank milk	Andalusia, Málaga	2015
LC101	Mastitic milk	Andalusia, Málaga[5]	2015
LC102	Bulk tank milk	Andalusia, Málaga[3]	2013
LC104	Bulk tank milk	Andalusia, Málaga[3]	2013
LC50	Auricular swab	Canary Islands, Gran Canaria	2009
LC62	Mastitic milk	Canary Islands, Lanzarote	2004
LC84	Mastitic milk	Canary Islands, Lanzarote	2014
LC89	Auricular swab	Canary Islands, Lanzarote[6]	2015
LC90	Auricular swab	Canary Islands, Lanzarote[6]	2015
LC91	Auricular swab	Canary Islands, Tenerife	2015
LC92	Auricular swab	Canary Islands, Tenerife	2015
LC93	Auricular swab	Canary Islands, La Palma	2015
LC94	Auricular swab	Canary Islands, Gran Canaria	2015
LC95	Bulk tank milk	Canary Islands, Lanzarote	2015
LC45	Semen	Catalonia	2009
LC105	Auricular swab	Murcia[7]	2011
LC106	Auricular swab	Murcia[7]	2011
LC107	Auricular swab	Murcia[7]	2011
LC1	Auricular swab	Murcia[1]	2007
LC5	Auricular swab	Murcia[1]	2007
LC7	Auricular swab	Murcia[1]	2007
LC33	Bulk tank milk	Murcia	2008
LC53	Auricular swab	Murcia	2011
LC63	Mastitic milk	Murcia[2]	2011
LC73	Artritis	Murcia[2]	2012
LC77	Mastitic milk	Murcia[2]	2012

Strains with the same number were isolated from the same herds.

M. agalactiae (Manso-Silvan et al., 2012; McAuliffe et al., 2011). Those studies were able to relate the genetic variability of different field isolates with their geographical origin, as they came from separate areas (McAuliffe et al., 2011; Rosales et al., 2015), and also to detect genetic variations while studying different strains within the same herd (Olaogun et al., 2015). Furthermore, other authors have performed MLST schemes to assess the genetic variability of *M. agalactiae* (Ariza-Miguel et al., 2013) and *M. bovis* (Becker et al., 2015; Lysnyansky et al., 2016) within endemic areas, highlighting the importance of this technique not only to detect differences between strains from faraway territories, but also to monitor the evolution of these pathogens in closer areas.

Hence, considering the usefulness of genetic typing techniques to study the epidemiology of CA in endemic areas, in addition to the lack of MLST studies of Mmc, the aim of this work was to evaluate the genetic variability of different field isolates coming from different regions of Spain, where this disease is considered endemic.

2. Material and methods

2.1. Mmc strains

In the present study, 40 strains of Mmc were analysed: 39 were field isolates obtained from 25 different herds between 2004 and 2015, together with the reference strain PG3 (NCTC, 10137), whose sequences were retrieved from GenBank (Manso-Silvan et al., 2007). Some of the studied field strains came from the same farms, as it is shown in Table 1. All these field strains were previously identified by PCR (Woubit et al., 2007) and by assessing their *fusA* sequence (Manso-Silvan et al., 2007).

2.2. Multilocus sequence typing

Genomic DNA of each Mmc isolate was obtained applying a classical phenol-chloroform extraction method on 25 ml volume of mycoplasmal culture.

The MLST study was based on the scheme previously described for the M. mycoides cluster (Manso-Silvan et al., 2007). Therefore, the genomic sequences of five housekeeping genes (fusA, glpQ, gyrB, lepA and rpoB) of each strain were analysed. PCRs were performed with Phusion High-Fidelity PCR Kit (Thermo Scientific, USA) in a final volume of 25 μl containing 0.5 μM of each primer, 0.1 mM dNTPs, 1X Phusion Buffer and 0.02 units of Phusion DNA Polymerase, and applying the following incubation conditions: one cycle of 95 °C for 30 s; 35 cycles of 95 °C for 10 s, variable annealing temperatures (Table 2) for 1 min and 72 °C for 1 min, and a final incubation at 72 °C for 10 min. The reactions were performed in an i-Cycler (Biorad, USA) thermal cycler. Subsequently, PCR products were subjected to electrophoresis in 1% agarose gels containing 0.005% of RedSafe (iNtRON Biotechnology, Korea) DNA staining, and visualized under UV light. Afterwards, they were purified using QIAquick PCR Purification Kits (Qiagen, The Netherlands) and sequenced, comparing both forward and reverse directions with the same primers as used for the PCR. The sizes of MLST loci were the same as analysed by Manso-Silvan et al. (2007). The partial nucleotide sequences assessed in this study for each of the five protein-coding target genes, together with their GenBank entrance number, are detailed in the Supplementary Table S1 (see Supplementary material Table S1 in the online version at DOI: 10.1016/j.vetmic.2016.06.002). As for the reference strain of Mmc PG3, these sequences were retrieved from GenBank (Manso-Silvan et al., 2007). All the 40 strains sequences were aligned and trimmed to the same size, providing gene fragments ranging from 628 to 880 bp, and the number of single nucleotide polymorphisms (SNP) observed within each individual locus, as well as in the sequence resulting from concatenation of the five loci, are shown in Table 2. Additionally, the discriminatory power of each MLST locus was calculated using Simpson's index of diversity (Hunter and Gaston, 1988) by VNTR DIversity and Confidence Extractor

Table 2
Multilocus sequence typing (MLST) loci, PCR details and variability of each locus.

Loci	Annealing T (°C)	Amplicon (bp)	Sequence analysed (bp)	Variable sites	Informative sites	Simpson's index
fusA	56.5	781	677	15	15	0.819
glpQ	57	695	628	37	34	0.945
gyrB	65.5	635	593	41	39	0.943
lepA	58	1097	785	26	26	0.930
rpoB	63	824	785	26	26	0.942
Concatenated set	–	–	3563	159	152	0.953