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Molecular typing of *Mycoplasma agalactiae*: Tracing European-wide genetic diversity and an endemic clonal population

Laurent-Xavier Nouvel[a,b], Marc Serge Marenda[a,b,1], Michelle Diane Glew[a,b,c,2], Eveline Sagné[a,b], Philippe Giammarinaro[d,b,a], Florence Tardy[e], François Poumarat[e], Renate Rosengarten[c], Christine Citti[b,a,c,*]

[a] *Université de Toulouse, INP-ENVT, UMR 1225, F-31076 Toulouse, France*
[b] *INRA, UMR 1225, F-31076 Toulouse Cedex 3, France*
[c] *Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine, A-1210 Vienna, Austria*
[d] *GDS 64, Chambre d'agriculture, F-64078 Pau, France*
[e] *Anses, Lyon Laboratory, UMR Mycoplasmoses of Ruminants, F-69364 Lyon, France*

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ABSTRACT

Mycoplasma agalactiae causes chronic infections in small ruminants and remains endemic in many regions of the world, despite intensive and costly eradication programs. In this study, the innate genomic plasticity of *M. agalactiae* was exploited to design and assess a combination of molecular epidemiological tools to trace the pathogen in different geographic locations and to understand its emergence or re-emergence after eradication campaigns. For this purpose, two collections of *M. agalactiae* isolates, representing European outbreaks or localized endemic disease in a single region of France, were subjected to RFLP (Restriction Fragment Length Polymorphism) analyses using two sets of DNA probes (distributed across the genome and specific for the *vpma* gene locus), and a previously described VNTR (Variable Number Tandem Repeats) analysis. A combination of four genome-specific DNA probes and two VNTRs gave the highest discriminative power. Molecular typing revealed that, while isolates from diverse geographical origins fell into clearly different groups, the endemic disease repeatedly observed in the Western Pyrenees region over the past 30 years has been caused by a unique subtype of *M. agalactiae*. This indicates that the re-emergence of the pathogen after seemingly successful eradication programs is not due to the importation of exotic strains, but to the persistence of local reservoirs of infection.

1. Introduction

Bacteria of the genus *Mycoplasma* belong to the Class *Mollicutes* and include a large number of species, several of which are important pathogens of man and a wide range of animals. In sheep and goats, *Mycoplasma* infections have important socio-economic consequences in regions of the world where small ruminants are an important resource for the population [1]. In Europe, the most common and most serious mycoplasmosis of small ruminants is the Contagious Agalactia (CA) syndrome, which is characterized by mastitis, arthritis and keratoconjunctivitis and is listed by the OIE (World Organisation for Animal Health). Four mycoplasma species are responsible for CA, *Mycoplasma mycoides* subspecies *capri*, *M. capricolum* ss. *capricolum*, *M. putrefaciens* and *M. agalactiae* [2], with the latter considered the causative agent *sensu stricto*.

* Corresponding author. Tel.: +33 0 5 61 19 38 56; fax: +33 0 5 61 19 32 73.
E-mail address: c.citti@envt.fr (C. Citti).
[1] Current address: School of Veterinary Science, 250 Princes Highway, Werribee, Victoria 3030, Australia.
[2] Current address: Bio21 Institute, Level 3 North, Rm 375, Melbourne Dental School, 30 Flemington Rd, Parkville 3010, Australia.

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Some geographic regions are free of *M. agalactiae* infection, while in others it occurs sporadically or is endemic [3]. In France, CA due to *M. agalactiae* has been eradicated from the department of Savoy since 2002 by strict sanitary measures, while it still persists in the Western Pyrenees (department of Pyrenees-Atlantic). In this area, sanitary measures implemented in the 1990s, including systematic laboratory detection (serology, bulk milk cultures), isolation or slaughtering of positive flocks and contact tracing, were successful in reducing the number of new infected flocks from 58 in 1992 to none between 2003 and 2005. However, since 2006, despite continued sanitary measures, the incidence of the disease has dramatically increased, with 28 new infected flocks in 2007 and 95 in 2008 [4]. Whether such infections are due to the introduction of a new strain or to the re-emergence of the endemic strain that had previously been contained is a major issue in improving CA control.

Assessing the nature and extent of genetic diversity between different isolates of *M. agalactiae* is a prerequisite when choosing appropriate molecular epidemiology tools that can be further used to trace the history and the route of dissemination of a pathogen, as well as the efficiency of sanitary measures. Diversity was first assessed with a set of fifty-nine DNA probes (AGA probes) specific for the PG2 type strain, which were produced by Suppression Subtractive Hybridization between *M. agalactiae* and *M. bovis*, two closely related mycoplasmas [5]. Data collected in this study by Southern blot analyses of field isolates (included in the collection of 101 isolates studied here) showed that approximately 70% of the panel of isolates were very homogenous and resembled the PG2 type strain, while the remaining members showed a high level of diversity. The recent sequencing of two *M. agalactiae* genomes, representative of the genetic spectrum of the species [6,7], allowed the development of more conventional typing methods such as variable number of tandem repeats analysis (VNTR) and multi-locus sequence typing (MLST) [8,9]. Variable tandem repeats are similar to eukaryotic microsatellites and have been detected in many bacterial species, including *Bacillus anthracis* [10], *Escherichia coli* O157:H7 [11], *Staphylococcus aureus* [12] and *Yersinia pestis* [13]. The power of discrimination of VNTR analysis has been shown to be high and it has provided information on the evolutionary diversity of a number of bacteria [14]. For animal mycoplasmas, VNTR typing has been applied to *M. mycoides* ss. *mycoides* SC [15], and more recently to *M. agalactiae* [8]. Four VNTRs were identified using a panel of 88 European isolates and analysis of these was shown to have a higher power of discrimination than analysis of pulse field gel electrophoresis (PFGE) patterns or random amplified polymorphic DNA patterns (RAPD) [8]. VNTR typing discriminated the 88 isolates into 9 profiles, with a majority of strains (64%) falling into one group. Recently, a MLST scheme was developed for *M. agalactiae* that yielded a Simpson index of diversity almost identical to that of VNTR typing. House-keeping genes targeted by MLST render the method most appropriate for assessing evolutionary relationships of *M. agalactiae* strains, while VNTR analysis might be more reflective of local adaptations [9].

VNTR and Restriction Fragment Length Polymorphism (RFLP) approaches investigate genome wide variation, but the presence of particular loci with a high variability has been demonstrated in some mycoplasma species [16,17] and could offer one additional level for discrimination, if used appropriately. In *M. agalactiae*, one locus is subject to a number of distinct variations. It encodes a family of lipoproteins abundantly expressed on the cell surface, the Vpmas, that share a conserved signal sequence and repeated amino acid pattern [18,19]. The type strain PG2 contains six, clustered *vpma* genes [6,18], but a given cell expresses only one Vpma, which expression can be switched ON/OFF with a high frequency within the propagating population [18,19]. This hypervariability of expression leads to the potential expression of different Vpmas between different clones derived from the same parent cell and is driven by a cut-and-paste mechanism involving a site-specific recombinase (Xer1) to place alternate silent structural *vpma* genes behind a functional promoter [18,20]. While the *vpma* locus is very dynamic within clonal populations, the number and nature of the *vpma* genes that are clustered on the chromosome is stable in daughter cells [6,18]. Preliminary data obtained using a probe conserved between all PG2 *vpma* genes with a combination of DNA probes specific for each *vpma* gene [18,19] suggested that the size of the *vpma* gene repertoire can be estimated by Southern blot analyses and that it varies between strains [21].

The current study was undertaken to evaluate, improve and compare the potential of molecular epidemiological tools currently available for *M. agalactiae*. These tools were developed using collections of isolates obtained from various geographical locations, with the dates of isolation not being a criteria. We compared the genetic diversity of isolates from a range of European countries (except one isolate from Africa) with those obtained from in a geographically restricted endemic area (the French Western Pyrenees). The later collection was composed of isolates collected over 30 years from sheep in well defined area where outbreaks of CA have been successfully controlled until the disease unexpectedly re-emerged in 2006, causing severe economic losses. The suitability of VNTR and of the AGA and *vpma* probes as molecular epidemiological tools was assessed.

2. Materials and methods

2.1. Bacterial isolates, culture conditions and DNA isolation

The genome of the *M. agalactiae* type strain PG2 clone 55.5 used in this study was sequenced previously [6,18]. All isolates studied and their characteristics (year, host and country of isolation) are shown in Table 1 and in supplemental Table S1. These isolates are part of a collection maintained by the Anses laboratory of Lyon and were gathered mainly through the Vigimyc network [4]. Mycoplasmas were propagated in Aluotto [22] or SP4 [23] media and genomic DNA was extracted as previously described [24,25].