1 A dominant lineage of *Mycoplasma bovis* is associated with an increased number of severe
2 mastitis cases in cattle

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4 Sibylle Bürki^a, Joachim Spargser^b, Michèle Bodmer^c, Paola Pilo^a

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6 ^aInstitute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Laenggassstrasse
7 122, 3012, Bern, Switzerland, ^bInstitute of Microbiology, Department of Pathobiology,
8 University of Veterinary Medicine, Veterinaerplatz 1, 1210, Vienna, Austria, ^cClinic for
9 Ruminants, Vetsuisse Faculty, University of Bern, Bremgartenstrasse 109a, 3012, Bern,
10 Switzerland.

11
12 Corresponding author:

13 Paola Pilo: Tel, +41 31 631 2369; Fax, +41 31 631 2634; Email,
14 paola.pilo@vetsuisse.unibe.ch

Abstract

Mycoplasma bovis is the most frequent etiologic agent of bovine mycoplasmosis. It causes various diseases in bovines and considerable economic loss due to the lack of effective treatment or preventive measures such as vaccination. In contrast to the US, where *M. bovis*-mastitis has been reported for a long time, *M. bovis* infections in Switzerland and Austria were predominantly associated with pneumonia and subclinical mastitis. However, since 2007 the situation has changed with the emergence of severe *M. bovis*-associated mastitis cases in both countries. In order to evaluate the molecular epidemiology of the bacteria isolated from these infections, recent and old Swiss, along with recent Austrian *M. bovis* isolates were analyzed by a typing method displaying intermediate resolution of evolutionary relationships among isolates called Multi-Locus Sequence Typing (MLST). The analysis of Swiss and Austrian *M. bovis* isolates revealed two major lineages. Isolates collected since 2007 in both countries cluster in the lineage I including ST5, ST33, ST34, 36, and ST38-40 (clonal complex 1), while all Swiss isolates recovered before 2007 cluster in the lineage II comprising ST17 and ST35 (clonal complex 5). Further investigations are necessary to understand if lineage I has a higher predilection or virulence toward mammary gland cells than the old lineage or if other factors are involved in the increased number of severe mastitis cases.

Keywords: *Mycoplasma bovis*, MLST, mastitis, bovine

Mycoplasma (M.) bovis is one of the major causative agents of bovine mycoplasmosis. This disease manifests itself in various forms such as mastitis, pneumonia and arthritis (Bürki et al., 2015a). It is emerging in several countries where it results in enormous economic loss to both the beef and dairy industries (Nicholas, 2011). In Switzerland, *M. bovis* used to be predominantly associated with pneumonia and subclinical mastitis (Burnens et al., 1999). In the mid-2000s, an increase in the reported cases of severe mastitis due to *M. bovis* was detected (Aebi et al., 2012; Aebi et al., 2015). Interestingly, the same trend was observed in Austria, Northern Italy and Israel (Lysnyansky et al., 2016; Radaelli et al., 2011; Spargser et al., 2013). Following these observations, clinical and bacteriological studies were performed (Aebi et al., 2012; Aebi et al., 2015). Swiss isolates were typed by Insertion Sequence (IS) elements profiling and it was found that single isolates were present within herds and could affect several organs. Moreover, clinical mastitis was the most important manifestation induced by *M. bovis*. Interestingly, different isolates were present among herds (Aebi et al., 2012; Aebi et al., 2015) suggesting infection of animals when entering barns or the further colonization by a predominant strain within farms. However, the investigation of phylogenetic diversity existing among isolates and distinct populations is necessary to unveil the history and ecology of bacterial species (Kenefic et al., 2009; Petersen and Molins, 2010; Pilo and Frey, 2011). In recent years, several multilocus sequence typing (MLST) schemes were developed for *M. bovis* (Becker et al., 2015; Manso-Silván et al., 2012; Register et al., 2015; Rosales et al., 2015). In this study, we analyzed a large collection of *M. bovis* isolates by MLST in order to address the epidemiology of *M. bovis* infections and the emergence of severe mastitis associated with this bacterium (Register et al., 2015).

Seventy-six isolates of *M. bovis* from Austria, Germany, Switzerland and the UK along with the type strain PG45^T isolated in the US (n=77), were included in this study (Supplementary Table I). For clarity purposes, isolates collected before 2007 were specified as old, while

isolates collected after 2007 were defined as new or recent. Because of the recent increased severity of disease observed in the mammary gland of cattle, the majority of isolates were collected from severe mastitis cases occurring since 2007. Mycoplasmas were grown and the lysates were prepared as previously described (Bürki et al., 2015b; Freundt, 1983; Rossetti et al., 2010). The MLST scheme developed by Register and colleagues was employed since it is linked to a public database (Register et al., 2015). Sequence types (ST) were assigned to each isolate using the *M. bovis* MLST database (www.pubmlst.org/mbovis/). New ST profiles were submitted to the curator of the database for review and allocation of a new ST. Concatenated nucleotide sequences were further investigated in order to reconstruct a phylogenetic tree using maximum-likelihood, including 1000 bootstrap replications and the Kimura 2-parameter substitution model supplied in the MEGA 6.06 software package. In the resulting phylogenetic tree, the isolates investigated in this study clustered in 2 lineages, separating new (lineage I) from old isolates (lineage II) (Figure 1). While new Austrian, German and Swiss isolates belonged to ST5, ST33, ST34 and ST36-40, old Swiss isolates grouped into ST17 and ST35. Five new alleles (four new *gyrB*- and one new *tkt* alleles) and eight new STs (ST33-40) were recorded.

Furthermore, a categorical minimum spanning tree analysis using BioNumerics (Version 7.5, Applied Maths) was performed, including the 76 isolates presented in this study and 94 isolates present in the *M. bovis* MLST database (accessed in May 2016) plus the retrieved MLST data from the genome of strain NM2012 available in the GenBank database (accession number NZ_CP011348). Minimum spanning tree analysis revealed the presence of 6 clonal complexes (CC1-CC6) based on the maximum distance between nodes in the same partition of 2 loci (Figure 2). Our 76 newly characterized isolates were grouped into CC1, CC4 and CC5 with 62 of them belonging to CC1 (Figure 3, panels A, B) which were solely collected after 2007 (Figure 3, panel B) in Switzerland, Germany, or Austria (Figure 3, panel A). Forty

out of 62 isolates clustering in CC1 were recovered from milk or the mammary gland, 21 from the lung or the nasal cavity and only one isolate was recovered from synovial fluid. CC4 comprised only one newly analyzed strain isolated in the UK before 2007 (Figure 3, panel A, B) whereas CC5 included 14 isolates characterized in this study all of which were defined as old isolates (Figure 3, panel B). Interestingly, isolates belonging to CC5 (n=14) were collected in the US (PG45^T), Switzerland and Germany (Figure 3, panel A) of which 9 were isolated from milk and 5 from lung samples.

Results obtained in this study indicate that a shift in circulating *M. bovis* isolates occurred in 2007 at the eastern border of Western Europe and is associated with the emergence of severe clinical mastitis cases (Figure 1). In Israel, the pattern appears to be similar, with only rare isolations of *M. bovis* from milk in the past and a shift in 2008 when an increase of *M. bovis*-associated clinical mastitis cases was recorded (Lysnyansky et al., 2016). Interestingly, the emergence of a new single subtype circling in the cattle population was detected in France. However the situation in France seems to be different since the emerging ST (ST18 according to the Register MLST scheme) was only associated with pneumonia cases, whereas no increasing numbers of severe mastitis cases have been recorded (Khalil et al., 2015). These results highlight the importance of further examining the association of specific phylogenetic clusters with the emergence of new clinical manifestations. However, experimental data are necessary to confirm this hypothesis and to understand the molecular mechanisms leading to the recent changes observed in the development of disease due to *M. bovis*.

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Figure legends

Figure 1: Phylogenetic reconstruction of concatenated MLST sequence data of the 76 isolates investigated in this study using maximum-likelihood and Kimura 2-parameter model in the MEGA software package (version 6.06). Bootstrap confidence values (1000 replications) are given at the respective nodes. The scale bar indicates the length representing 0.002 nucleotide substitutions per site. Individual sequence types are shown as dots in distinct colors.

Figure 2: Categorical minimum spanning tree using allelic profiles of MLST data including 76 isolates of this study and 94 bovine isolates available in the *M. bovis* MLST database (www.pubmlst.org/mbovis/) plus the retrieved MLST data from the genome of strain NM2012 available in the GenBank database (accession number: NZ_CP011348). Circles represent isolates and their respective ST. Their size is proportional to the number of isolates per ST. Colored sectors around circles define clonal complexes created based on the maximum distance between nodes in the same partition of 2 loci (CC1-CC6).

Figure 3: Categorical minimum spanning tree using allelic profiles of MLST data of the 76 isolates of this study including the type strain PG45^T. Circles represent isolates and their ST. Their size is proportional to the number of isolates per ST. Colored sectors around circles define clonal complexes based on the maximum distance between nodes in the same partition of 2 loci, and their color represents the color from the majority of their isolates. All 77 isolates are colored according to their country of isolation (A), or whether they were collected before or after the year 2007 (B).

Figure

Figure 1

Figure 2

Figure 3

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B