Manuscript Details

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Title	The moderate drift towards less tetracycline-susceptible isolates of contagious agalactia causative agents might result from different molecular mechanisms.
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Abstract

Contagious agalactia is a mycoplasmosis that affects small ruminants, is associated with loss of milk production and high morbidity rates, and is highly deleterious to dairy industries. The etiological agents are four mycoplasma (sub)species, of which the relative importance depends on the countries and the animal host. Tetracyclines are non-expensive, broad-spectrum antimicrobials and are often used to control mastitis in dairy herds. However, the in vitro efficiency of tetracyclines against each of the etiological agents of contagious agalactia has been poorly assessed. The aims of this study were i) to compare the tetracycline susceptibilities of various field isolates, belonging to different mycoplasma (sub)species and subtypes, collected over the years from different clinical contexts in France or Spain, and ii) to investigate the molecular mechanisms behind the decreased susceptibility of some isolates to tetracyclines. The Minimum Inhibitory Concentrations (MICs) of tetracyclines were determined in vitro on a set of 120 isolates. Statistical analyses were run to define the significance of any observed differences in MICs distribution. As mutations in the genes encoding the tetracycline targets (rrs loci) are most often associated with increased tetracycline MICs in animal mycoplasmas, these genes were sequenced. The loss of susceptibility to tetracyclines after year 2010 is not significant and recent MICs are higher in M. agalactiae, especially isolates from ovine mastitis cases, than in other etiological agents of contagious agalactia. The observed increases in MICs were not always associated with mutations in the rrs alleles which suggests the existence of other resistance mechanisms yet to be deciphered.

Keywords	antibioresistance; tetracycline; mycoplasmas; diversity; contagious agalactia
Manuscript category	Bacteria

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Submission Files Included in this PDF

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Supplementary table S1_vfOK_rev.xlsx [Table]

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Lyon, April 13rd, 2018

Dear Dr. Stefan Schwarz,

Please find enclosed a revised version of the manuscript entitled **"The moderate drift towards less tetracycline-susceptible isolates of contagious agalactia causative agents might result from different molecular mechanisms**" by Prats-van der Ham et al. and a detail point-by-point response to the reviewers. For your convenience, major changes in the manuscript have been highlighted in yellow in the revised version.

All issues raised by reviewer2 were carefully addressed and the manuscript has consequently improved. We would like to thank you for your time and efforts in handling our paper. We are grateful to the referees for their critical evaluation and for their helpful suggestions and corrections.

Sincerely yours,

On behalf of all co-authors,

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Answer to the reviewers' comments

-Reviewer 1

This paper describes the assessment and comparison of the tetracycline susceptibilities of different CA-causing mycoplasma species, collected up to 2010 (so called "old" isolates) and from 2011 onwards (current isolates) in France and Spain. In addition, molecular mechanisms responsible for the decreased susceptibilities to tetracyclines of some isolates were investigated. The subject is interesting as those mycoplasma species are very important pathogens of small ruminants causing to contagious agalactia, disease which has a substantial impact on dairy industries as well as on animal welfare. The study was well designed and performed. The data are clearly presented and interpreted. The discussion is helpful to understand the results and the significant of this study.

Answer: Thank you for this very positive review

-Reviewer 2

In this manuscript, the authors evaluated tetracycline resistance of 120 field isolates belonging to four Mycoplasma species collected in France and in Spain before 2010 and after 2011. Some resistant isolates with reduced susceptibility were characterized by amplification and sequencing of tetracycline target genes.

This study is of interest to assess tetracycline resistance in the Mycoplasma species responsible for contagious agalactia. However, the manuscript is too long, especially the discussion section, which should be significantly shortened to only discuss the important findings.

Major concern

- I don't agree with the author conclusion claiming that the loss of susceptibility to tetracycline over time remained limited. As tetracycline resistance was not significantly different between old and recent isolates, the conclusion should not be "our result revealed a limited loss of susceptibility to tetracyclines" (see line 412). The conclusion should be that there was no significant loss of susceptibility over time. This point should be corrected throughout the manuscript, especially in the title, abstract and conclusion.

Answer: we agree with this remark

Action taken: changed accordingly throughout the manuscript. However in the title, no reference is made to the time so we kept this notion of moderate drift to suggest that some isolates have slightly higher MICs than the wildtype.

- In this study, the field isolates were "chosen" or "selected" (see lines 193 and 195) because the authors aimed to balance isolates from both countries, collected before and after 2010, and isolates from symptomatic and asymptomatic infections. How do the authors make sure that the set of isolates was representative of the population? A systematic collection would be more appropriate to avoid selection bias.

Answer: we agree with the fact that a systematic collection is always better but it is not often feasible, especially when you include old samples collected years ago

Action taken: to clarify the meaning of "chosen" and "selected" we used the word "included" instead and we add a sentence to specify the absence of epidemiologically-related isolates.

Minor concerns:

- Line 94, line 97: italicize "tet"

Action taken: changed accordingly

- Lines 94-96: Is there a reference for this point?

Answer and action taken: As we are limited to approximately 35 references in the Instructions to authors, I choose to add a reference to a review by Waites et al 2014. Otherwise we should quote at least 3 references.

- Line 130: The #5632 strain of *M. agalactiae*: What does the # mean?

Answer and action taken: The # symbol was only meant here to distinguish the figure 5632 and the strain number 5632. Since it seems confusing to the reviewer, I removed it here and in other instance of the manuscript.

- Line 162: please specify the name of the DNA extraction kit.

Action taken: done

- Lines 266-268: Please rephrase this sentence for clarity.

Action taken: done. The sentence now reads "Mutations in the Tet-1 primary binding pocket of tetracycline were observed in only 7 isolates and only in helix 31(positions 965-967) as no mutation was detected in helix 34."

- Lines 275-276: What do the authors mean by "could be random"?

Answer: random is used opposite to "directed mutations".

Action taken: For clarity, it was changed to "neutral, weakly selected mutations"

- Lines 276-278: Please present these data in Table 2. It is important to the reader to easily see that these isolates harbor mutations outside the Tet-1 binding site. To my opinion, there are too many supplementary Tables. Notably, Table S4 (and S5, see below) should be included in Table 2 for clarity.

Action taken: Table2, S4 and S5 were merged accordingly. We feel it improves the readability of the results and thank the reviewer for this suggestion.

- Line 289: replace "no drastic increase" by " no significant difference"

Action taken: replace by "non-statistically significant difference"

- Line 298: Please specify these breakpoints here.

Action taken: done accordingly. We took this opportunity to update the CLSI reference

- Lines 329-332. There is confusion here between infected and symptomatic. All animals are infected but some are symptomatic and some are asymptomatic.

Action taken: corrected accordingly. The whole paragraph was rewritten for clarity.

- Lines 366-367: Please discuss the other mutations harbored by these strains.

Action taken: done, although briefly as we do not want to speculate further on the actual importance of these other mutation points

- Lines 373-376: This sentence is not clear.

Action taken: part of the paragraph was removed to shorten the discussion by removing this part that was poorly informative.

- Lines 377: MIC90 instead of IC90?

Action taken: this paragraph was removed from the discussion

- Table S5 should also be merged with Table 2. Table 2 could present a column for mutations in rrs1, a column for mutations in rrs2, a column for mutations outside the Tet-1binding side and a column for mutations in protein S10 along with the MIC values.

Action taken: Table2, S4 and S5 were merged accordingly.

- Lines 394-400: Where are the hotspots of mutation of the ribosomal protein S10 in other bacteria species?

Answer: There seems to be a great variability of mutated residues especially in Gram positive bacteria, with no clear pattern. However a recent publication associated reduced Tygecycline susceptibility to mutations in positions K57 and D60 in both Gram positive and negative bacteria (Beabout-K et al AAC 2015).

- Lines 401-404: The tet(M) gene is also associated with high level resistance to tetracycline in some mycoplasma species. Did the author look for the tet(M) gene in this field isolate collection?

Answer: no, we did not

- Table 2: Please add the PG2 reference strain in the Table for MIC comparison purpose. Isolates L16156 and L16160 also harbor mutations in other positions.

Action taken: the whole table was modified. The genotype and MICs of type strains are given in Tables 1 (MICs) or Table 2 and in the text (genotype or amino acid sequence).

The moderate drift towards less tetracycline-susceptible isolates of contagious agalactia causative agents might result from different molecular mechanisms.

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Keywords: antibioresistance; tetracycline; mycoplasmas; diversity; contagious agalactia

Abstract

Contagious agalactia is a mycoplasmosis that affects small ruminants, is associated with loss of milk production and high morbidity rates, and is highly deleterious to dairy industries. The etiological agents are four mycoplasma (sub)species, of which the relative importance depends on the countries and the animal host. Tetracyclines are non-expensive, broad-spectrum antimicrobials and are often used to control mastitis in dairy herds. However, the *in vitro* efficiency of tetracyclines against each of the etiological agents of contagious agalactia has been poorly assessed.

The aims of this study were i) to compare the tetracycline susceptibilities of various field isolates, belonging to different mycoplasma (sub)species and subtypes, collected over the years from different clinical contexts in France or Spain, and ii) to investigate the molecular mechanisms behind the decreased susceptibility of some isolates to tetracyclines.

The Minimum Inhibitory Concentrations (MICs) of tetracyclines were determined *in vitro* on a set of 120 isolates. Statistical analyses were run to define the significance of any observed differences in MICs distribution. As mutations in the genes encoding the tetracycline targets (*rrs* loci) are most often associated with increased tetracycline MICs in animal mycoplasmas, these genes were sequenced.

The loss of susceptibility to tetracyclines after year 2010 is not significant and recent MICs are higher in *M. agalactiae*, especially isolates from ovine mastitis cases, than in other etiological agents of contagious agalactia. The observed increases in MICs were not always associated with mutations in the *rrs* alleles which suggests the existence of other resistance mechanisms yet to be deciphered.

1. Introduction

Contagious agalactia (CA) is a syndrome affecting small ruminants and has a substantial economic impact on dairy industries due to its reduction/suppression of milk production and high morbidity rates. The three main clinical signs associated with CA are mastitis, arthritis and keratoconjunctivitis, but others like pneumonia or septicaemia have also been reported in young animals (Agnello et al., 2012; Corrales et al., 2007; Gomez-Martin et al., 2013). CA is caused by four *Mycoplasma* (sub)species: *M. agalactiae*, *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum* and *M. putrefaciens* (Corrales et al., 2007). In France, *M. agalactiae* is primarily isolated from

sheep herds reared in the Western Pyrenees, where it causes subclinical to acute mastitis, but is more rarely isolated from goats, and usually with no or few clinical signs (Poumarat et al., 2016). In contrast, *M. agalactiae* is the main mycoplasma species isolated from both Spanish ovine and caprine herds with CA (Ariza-Miguel et al., 2012), (De la Fe, personal communication). *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum* and *M. putrefaciens* are phylogenetically related, as they belong or are related to the *M. mycoides* cluster (Manso-Silvan et al., 2007). These mycoplasmas usually infect goats and are very seldom isolated from sheep. *M. mycoides* subsp. *capri* is the main species isolated from French herds with clinical CA (Chazel et al., 2010) and also the predominant species in some areas of Spain such as the Canary Islands (De la Fe et al., 2005). Although *M. capricolum* subsp. *capricolum* and *M. putrefaciens* are less frequently isolated, their presence in goat herds has been reported in severe CA outbreaks (De la Fe et al., 2007; Giadinis et al., 2008; Gil et al., 1999; Mercier et al., 2000).

Herds in endemic areas, such as Spain and France, tend to be chronically affected with CA-causing mycoplasmas and clinical outbreaks occur only sporadically. Due to the poor efficacy of currently available vaccines and their inability to prevent disease transmission or infection (Agnone et al., 2013), antibiotherapy is often used to control CA (Gomez-Martin et al., 2013). Tetracyclines are broad-spectrum, low cost antimicrobials and amongst the main ones used in veterinary medicine (De Briyne et al., 2014). Although three generations of tetracyclines now exist (Grossman, 2016), only first generation products such as oxytetracycline are specifically available for small ruminants in Spain and France (AEMPS, 2017; Summary of antimicrobials available in France for animals, accessible at http://www.ircp.anmv.anses.fr). The *in vitro* activity of oxytetracycline against CA-causing mycoplasmas has been demonstrated in several

76 studies (Antunes et al., 2007; Paterna et al., 2013; Paterna et al., 2016; Tatay-Dualde et
77 al., 2017).
78 Tetracyclines exert their bacteriostatic activity by binding preferentially to the 30S
79 ribosomal subunit and interacting with a highly conserved 16S rRNA target to prevent
80 binding of aminoacyl-tRNA to the ribosomal acceptor site and hence to stop bacterial
81 protein synthesis (Nguyen et al., 2014). Crystallographic studies have revealed a
82 primary tetracycline binding site (Tet-1), located in a pocket formed between helices
83 h31 (nucleotide positions 964-967, *E. coli* numbering) and h34 (positions 1052-1056
84 and 1196-1200). Five other minor binding sites in 16S rRNA have also been described
85 (Brodersen et al., 2000; Pioletti et al., 2001). In addition, the 30S ribosomal protein S10,
86 encoded by the *rpsJ* gene, forms a loop projecting towards the aminoacyl-tRNA-
87 binding site and may play a role in the interaction of tetracyclines and 16S rRNA
88 (Brodersen et al., 2000). Resistance to tetracyclines has been linked to several
89 mechanisms such as energy-dependent efflux, presence of ribosomal protection
90 proteins, mutations in the 16S rRNA encoding genes (the so-called *rrs* genes), and
91 enzymatic inactivation of the drug (Grossman, 2016; Nguyen et al., 2014).
92 Antimicrobial efflux in mycoplasmas has only been described *in vitro,* in association
93 with fluoroquinolone resistance (Antunes et al., 2015; Raherison et al., 2005).
94 Moreover, high level tetracycline resistance associated with the presence of the *tetM*
95 determinant, which encodes a protective protein conferring cross resistance to all
96 tetracyclines, has so far been reported only in *M. hominis* and *Ureaplasma* spp. (Waites
97 et al., 2014). No *tetM* determinants and/or derivatives have been found in other
98 mycoplasma species, such as *M. bovis* (Amram et al., 2015; Sulyok et al., 2017).
99 Target-based mutations in *rrs* genes conferring tetracycline resistance have been
100 reported in *in vitro* selected mutants of *M. hominis*, *M. pneumoniae* and *M. bovis*

(Degrange et al., 2008; Sulyok et al., 2017) and in field isolates of *M. bovis* (Amram et al., 2015; Khalil et al., 2017; Sulyok et al., 2017). However, the molecular mechanisms responsible for tetracycline resistance in CA-causing mycoplasmas have not yet been elucidated.

The aim of the present study was to assess and compare the tetracycline susceptibilities of different field isolates, belonging to different mycoplasma (sub)species, and obtained from subclinical or clinical infections in France and Spain. The susceptibility patterns were analysed in relation to the time of collection, sample origin (nature and place of collection), and the molecular subtypes of the isolates. In addition, the molecular mechanisms responsible for the decreased susceptibility to tetracyclines of some isolates were investigated.

2. Material and methods

2.1. Mycoplasma isolates and subtyping

A total of 120 field isolates of CA-causing mycoplasmas, namely 60 *M. agalactiae*, 30 *M. mycoides* subsp. *capri*, 18 *M. capricolum* subsp. *capricolum* and 12 *M. putrefaciens,* were analysed **(Supplementary Table S1)**. Isolates were defined as old when collected up to 2010 included (n = 64), or current, when they were isolated from 2011 included onwards (n = 56). They were retrieved from different regions of France (n = 59) or Spain (n = 61), and from different samples including mainly bulk tank milk (BTM, n = 50) and mastitic milk (n = 49) but also auricular swabs (n = 6), joints (n =6) and lungs (n = 6).

All isolates were identified by dot immunoblotting on a filtration membrane (Poumarat et al., 1991). In the case of ambiguous antigenic identification, complementary tests were conducted, including a species-specific PCR assay for *M. agalactiae* (Marenda et

126 al., 2005) or a *fusA* PCR followed by sequence analysis for species in the *M. mycoides*
127 cluster (Maigre et al., 2008).
128 In addition, the type strains of the four studied mycoplasma species, namely *M.*
129 *agalactiae* PG2^T (NCTC 10123); *M. mycoides* subsp. *capri* PG3^T (NCTC 10137); *M.*
130 *capricolum* subsp. *capricolum* California Kid^T (NCTC 10154); and *M. putrefaciens*
131 KS1^T (NCTC 10155) were used as controls. The 5632 strain of *M. agalactiae,* that
132 defines the other genetic extremity of the species, was also included (Nouvel et al.,
133 2010).
134 *M. agalactiae* isolates were subtyped using a previously described multilocus variable-
135 number tandem-repeat (VNTR) scheme which analyses VNTR17 and VNTR19, the
136 most discriminating loci (Nouvel et al., 2012). The sizes of the PCR products were
137 estimated using an automated capillary electrophoresis device from Qiagen (QIAxcel
138 System) and compared with those of previously described profiles (De la Fe et al.,
139 2012; Nouvel et al., 2012; Poumarat et al., 2016). PCR products corresponding to new
140 profiles were further sequenced using an external facility at Beckman Coulter Genomics
141 (Genewiz, United Kingdom).
142 Isolates belonging to the *M. mycoides* cluster were subtyped by analysing a 561bp-
143 partial sequence of their *fusA* gene, as previously described (Maigre et al., 2008; Manso-
144 Silvan et al., 2007).
145 *Tetracycline susceptibility testing*
146 The Minimum Inhibitory Concentrations (MIC) of oxytetracycline and doxycycline
147 (both purchased from Sigma-Aldrich, France) were determined using the agar dilution
148 method on modified PPLO agar, as previously described (Khalil et al., 2016; Poumarat
149 et al., 2016). Briefly, 1 µl of each isolate and the reference strains, diluted to 10^4 – 10^5
150 CFU/ml, were inoculated onto agar plates containing two-fold dilutions of each

151 antimicrobial, ranging from 0.0625 to 128 µg/ml. Plates were incubated at 37°C in 5%
152 CO_2 for 4 or 2 days for *M. agalactiae* and *M. mycoides* cluster isolates, respectively.
153 The MIC was defined as the minimum concentration of antimicrobial at which no
154 growth was observed. Analyses were repeated twice and, if the results were not
155 consistent, a third repetition was performed. In case of 3 assays, the final MIC value
156 was the mode of the 3 values **(Supplementary table S1)**.

157 *PCR amplification and sequence analysis of 16S rRNA tetracycline binding sites*

158 A selected subset of field isolates, showing high, intermediate or low MIC values, was
159 used to study the 16S rRNA encoding genes. This subset included 32 *M. agalactiae*
160 isolates and 24 isolates of different species belonging to the *M. mycoides* cluster
161 **(Supplementary table S1).** All these mycoplasma (sub)species have two *rrs* alleles,
162 hereafter designated *rrs1* and *rrs2*.

163 Genomic DNA was extracted from 2 ml of broth culture using the DNeasy
164 Blood&Tissue kit from Qiagen. Novel sets of primers were designed to separately
165 amplify the 2 *rrs* copies, based on long range PCRs using primers targeting flanking
166 genes, as previously proposed (Khalil et al., 2017), and the *rpsJ* gene of the four studied
167 mycoplasma species **(Supplementary table S2)**. The individual *rrs* copies were further
168 amplified by performing a nested PCR using the universal primers U1 and U8
169 (Johansson et al., 1998) on each long range PCR product diluted to 1/10. Final PCR
170 products were then sequenced using primers U7 and U3 (Johansson et al., 1998) to
171 cover all the previously described tetracycline binding sites (Brodersen et al., 2000;
172 Pioletti et al., 2001). The *rpsJ*-F primer used for PCR was also used to generate
173 sequences of the *rpsJ* gene. Sequencing was conducted by an external facility at
174 Beckman Coulter Genomics (Genewiz, UK). Sequence editing and alignment
175 construction were performed with MEGA 6.0 software. Sequences of the type strains,

with the following accession numbers: *M. agalactiae* PG2^T (NC_009497.1), *M. agalactiae* 5632 (NC_013948.1), *M. mycoides* subsp. capri PG3^T (NC_005364.2), *M. capricolum* subsp. *capricolum* CK^T (NC_007633.1) and *M. putrefaciens* KS1^T (NC_015946.1), were retrieved from GenBank databases. For convenience, nucleotide numbering refers to *Escherichia coli* K12 positions (NC_000913.3).

Statistical analysis

For statistical analysis, the MIC values were first converted to a continuous variable by calculating their Log2 values. These Log2(MIC) values were then used to compare different paired subpopulations of isolates (e.g. France versus Spain, recent versus old, etc.) using a Mann-Whitney test with the significance level set at 0.05, as previously described (Poumarat et al., 2016). These analyses were run using the EpiInfo software available at https://www.cdc.gov/epiinfo/index.html.

3. Results

3.1. Choice of isolates and subtyping

Selection of the isolates included in the study was based on current knowledge of CA epidemiology in Spain and France. Two sets were prepared to take all the etiological agents into account, in proportions mimicking those observed in the field. Hence, 60 isolates of *M. agalactiae* were enclosed in the first set because this is the main species isolated in Spain and in French sheep flocks. The other 60 isolates belonged to the three other CA-causing subspecies and were included in set 2 in proportions comparable to their frequencies of isolation in the two countries, *i.e.* 30 *M. mycoides* subsp. *capri*, 17 *M. capricolum* subsp. *capricolum* and 13 *M. putrefaciens*. All isolates originated from different outbreaks or herds. The two sets consisted of approximately equal proportions of isolates from France (n=59) and Spain (n=61) since our aim was to compare the

overall level of susceptibility to tetracycline in these two countries. Similar numbers of old isolates (collected up to 2010 included (n=64) and current ones isolated from 2011 onwards (n=56)) were studied to determine the evolution of MICs over time. Finally, a similar number of isolates collected from acute clinical cases and those circulating asymptomatically in herds were examined, *i.e.* mastitis isolates (n=49) and isolates recovered from BTM (n=50).

These isolates were then further subtyped to analyse the link between diversity and loss of susceptibility to antimicrobials, as described previously (Khalil et al., 2017; Poumarat et al., 2016). The 60 *M. agalactiae* isolates analysed presented 15 different VNTR profiles (**Supplementary table S1**) including 3 new VNTR17 and 1 new VNTR19 genotypes (shown in **Supplementary table S3**). The most frequent subtype was ST 3.1 (n=32, more than half of the isolates). This was found in France and Spain and included all the isolates from sheep (8 French and 6 Spanish isolates) and 18 of the 46 caprine isolates. Interestingly, the only French caprine isolate with this profile came from the same area as the ovine isolates (Western Pyrenees). The only other genotype shared between France and Spain was ST 0.1, but was only found in 3 goats (2 French and 1 Spanish). The other 13 subtypes were mainly found in France (10/13). Thus the *M. agalactiae* isolates population in France is more diverse than in Spain, where ST 3.1 is also predominant in goats. Analysis of the *fusA* sequences, in isolates from the *M. mycoides* cluster, revealed a clear split into different (sub)species in different branches of the tree (data not shown). However, no subgroup of isolates within a (sub) species was evidenced that could be correlated to the geographical origin, year of isolation or MIC values.

3.2. MICs distribution

226 The MIC results are detailed in **Supplementary table S1** and summarized in **Table 1**.
227 Firstly, the MIC values of oxytetracycline were higher than those of doxycycline,
228 whatever the isolate tested, with a range of 0.125-8 µg/ml versus ≤0.0625-2 µg/ml,
229 respectively ($p < 0.001$). Nonetheless similar evolutions over time of MIC were
230 observed in our isolates population for both antimicrobials, *i.e.* a maximum 5-fold
231 increase of the antimicrobial concentration for both oxytetracycline and doxycycline.
232 Secondly, the MIC values of oxytetracycline were higher for *M. agalactiae* than for
233 isolates from the *M. mycoides* cluster ($p < 0.001$), with MIC_{90} of 4 and 0.5 µg/ml,
234 respectively. In contrast, the MIC values of doxycycline were similar for both groups of
235 isolates.
236 Within the *M. mycoides* cluster, the highest MIC values of oxytetracycline were
237 obtained for *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum* isolates (4
238 µg/ml), while the MICs for *M. putrefaciens* never exceeded 0.5 µg/ml. *M. mycoides*
239 subsp. *capri* isolates showed the highest MIC_{90} at 2µg/ml, while the MIC_{50} and MIC_{90}
240 were equal for the other (sub)species isolates at only 0.5 µg/ml. No statistical tests were
241 run because of the small size of the samples in each category.
242 **Figure 1** shows the oxytetracycline MIC distribution of mycoplasma isolates of *M.*
243 *agalactiae* (Fig.1A) and species belonging to the *M. mycoides* cluster (Fig.1B). For each
244 panel, the MICs distributions were compared as a function of their year of collection
245 (after 2010 or up to 2010 included), their geographical origin (France versus Spain), the
246 sample from which they were isolated (BTM versus mastitic milk) and their genetic
247 subtypes (only for *M. agalactiae* isolates).
248 The distributions of oxytetracyline MICs for *M. agalactiae,* as compared to isolates
249 from the *M. mycoides* cluster, were more heterogeneous and mainly centred around 0.5
250 µg/ml (Fig. 1B). The homogeneous distribution of isolates from the *M. mycoides* cluster

had already been suggested by the equal MIC_{50} and MIC_{90} values obtained for oxytetracycline and doxycycline **(Table 1)**.

Within each (group of) species, no significant differences in distribution were found between old and recent isolates (*M. agalactiae* p=0.095; *M. mycoides* cluster p=0.677), or between French and Spanish isolates (*M. agalactiae* p=0.193; *M. mycoides* cluster p=0.087).

The MIC for *M. agalactiae* isolates retrieved from mastitic milk samples were significantly higher than those of strains isolated from bulk tank milk (p=0.044). This difference was not observed between isolates from the *M. mycoides* cluster. Significant differences were also found (p=0.025) between the MIC values of the predominant *M. agalactiae* subtype 3.1 and those of the other subtypes.

3.3. Association between 16S rRNA alterations and MICs

The *rrs* genes in a subset of 32 *M. agalactiae* isolates, composed of 15 isolates with oxytetracycline MICs ≥2 µg/ml and 17 more susceptible ones (MIC ≤0.5 µg/ml), were examined. Mutations detected in the 2 *rrs* operons of *M. agalactiae* isolates were compared with strain $PG2^T$ (**Table 2**). Mutations in the Tet-1 primary binding pocket of tetracycline were observed in only 7 isolates and only in helix 31(positions 965-967) as no mutation was detected in helix 34. These isolates were all from Spain and had MICs ≥2 and ≥0.25 µg/ml for oxytetracycline and doxycycline, respectively. None of the isolates from France showed any mutations in these 3 hotspots. Fifteen isolates had no mutations at all and 10 isolates had mutations in other, non-hotspot positions. Eight of these 10 isolates showed mutations in only one allele of the *rrs* genes (except Ag304) and had the same MIC range as isolates of the wild-type genotype (0.25 – 2 µg/ml of oxytetracycline and ≤0.0625-0.25 µg/ml of doxycycline). None of these non-hotspot

276 mutations was detected in more than two isolates suggesting they are neutral, weakly
277 selected mutations. Two French isolates, (F16156 and F16160), showed increased MICs
278 to oxytetracycline at 4 µg/ml with mutations affecting positions 458, 461 and 1272 of
279 the *rrs* genes, i.e. outside the main tetracycline binding pocket **(Table 2).**
280 The *rrs* genes were also analysed in a subpopulation of 24 isolates selected from
281 different (sub)species in the *M. mycoides* cluster, which had oxytetracycline MIC values
282 ranging from 0.125 to 4 µg/ml (**Table 2**). No mutations associated with increased MICs
283 were detected, although some isolates (LC32, LC54, F9545, F10621, F10685, F10751)
284 had similar MIC values to *M. agalactiae* isolates harbouring Tet-1 mutations.
285 Furthermore, many mutations were observed outside the Tet-1 binding site in *rrs* but
286 their presence was not correlated with higher MIC values.
287
288 4. Discussion
289 *No statistically-significant difference in susceptibility*
290 No statistically-significant increase in tetracyclines MICs after 2010 was apparent for
291 any of the mycoplasma (sub)species involved in CA in France and Spain. Doxycycline,
292 a 2nd generation tetracycline with improved structure-activity features, yielded lower
293 MICs than oxytetracycline, but the MIC amplitude of variation for both antimicrobials
294 was comparable, suggesting that evolution over time and resistance mechanisms were
295 similar. Although the MICs of oxytetracycline for *M. agalactiae* were slightly higher
296 than those obtained for isolates from the *M. mycoides* cluster, the maximum attained
297 (8µg/ml) was moderate. Indeed, as no clinical breakpoints specific to veterinary
298 mycoplasmas are available, only one isolate with an MIC of 8µg/ml (Ag316) would be
299 classified, according to the generic breakpoints for pathogenic bacteria of cattle (the

only ones available for domestic ruminants (CLSI, 2015)), as resistant for oxytetracycline (MICs≥8µg/ml).

The different susceptibilities of *M. agalactiae,* versus the *M. mycoides* cluster, could partly be explained by the highly susceptible 22% of *M. putrefaciens* isolates in the *M. mycoides* subgroup. Our data for *M. agalactiae* are consistent with those previously reported (Poumarat et al., 2016) that evidenced a loss of susceptibility to tetracycline between 1990 and 2000. In the present study no further significant loss was observed after 2010.

However, despite this overall reassuring picture, the highest MICs were observed for the most recent isolates, which implies a current (maybe slow) development of resistance to tetracyclines in the field. This slow and moderate evolution can be related to the nature of the underlying mechanisms: indeed the increase of MICs, after passages of *M. bovis in vitro,* was shown to be more rapid for spectinomycin (a single mutation being sufficient to switch to the resistant genotype) than for tetracycline (several mutations required) (Sulyok et al., 2017).

Interestingly, the susceptibilities of Spanish and French *M. agalactiae* and *M. mycoides* isolates did not differ significantly despite the apparent differences in the amount of tetracycline used in each country. Indeed, according to the 7th annual report of the European Surveillance of Veterinary Antimicrobial Consumption, tetracyclines sales for food-producing animals were 5 times higher in Spain than in France in 2015 (http://www.ema.europa.eu/docs/en_GB/document_library/Report/2017/10/WC500236750.pdf). Nevertheless, this value has to be interpreted with caution since sales provide only an indirect indication of the actual use of antimicrobials, especially in small ruminants (De Briyne et al., 2014).

Coherence between MICs and epidemiology

The observed evolution of susceptibility patterns is coherent with the epidemiological situation and relative importance of each CA etiological agent. In France, the highest MICs were observed for *M. mycoides* subsp. *capri,* which is most often isolated from CA clinical outbreaks (Chazel et al., 2010). In contrast, the *M. agalactiae* isolates with the highest MICs came from Spain, where this species is the main causative agent of CA (Ariza-Miguel et al., 2012); (De la Fe, personal communication).

M. agalactiae isolates from mastitis (symptomatic herds) also showed higher MICs than isolates from BTM (asymptomatic herds), maybe due to the fact that only sick animals are usually exposed to antibiotherapy. However, this difference in susceptibility might also be influenced by the host animal as the isolates retrieved from mastitis were mainly caprine. Although no statistical comparison could be done due to the very few ovine samples, the MICs were higher in caprine isolates both in this and a previous study (Poumarat et al., 2016). Results might be further biased by the huge diversity of caprine isolates (up to 14 subtypes), whereas all ovine isolates belonged to the predominant European subtype ST3.1 (Ariza-Miguel et al., 2012; Poumarat et al., 2016) associated with lower MICs (Figure 1A).

Mutations in rrs loci

The increased tetracyclines MICs in *M. bovis*, another ruminant mycoplasma that shares many phenotypic and genotypic traits with *M. agalactiae*, have been associated with mutations in the genes encoding 16S rRNA, especially at hotspot positions, 965 and 967 (Amram et al., 2015; Khalil et al., 2017; Sulyok et al., 2017). We evidenced mutations in one of these two positions in 6 *M. agalactiae* isolates with MICs of oxytetracycline >2µg/ml (that could be classified as intermediate or resistant according to the CLSI breakpoints for pathogenic bacteria of cattle) and one with an MIC of 2µg/ml (susceptible). These $A_{967}C$ or $A_{965}G$ mutations were observed in one or both *rrs* alleles

but never simultaneously in a single isolate (Table 2). This could explain why no MIC greater than 8µg/ml was ever attained in *M. agalactiae,* whereas all recent strains of *M. bovis* from France harbour both mutations in both alleles and have MICs ≥ 32µg/ml (Khalil et al., 2017). Khalil et al. also showed that *M. bovis* isolates collected before the year 2000 had only one hotspot mutation in a single *rrs* allele and MICs of approximately 4µg/ml.

Whether the binding capacity of oxytetracyclines, and hence their MICs, could be influenced by the nature of the mutations at positions 965 and 967, has yet to be demonstrated. In *M. agalactiae* the mutations at positions 965 and 967 are transversions ($A_{965}G$ and $A_{967}C$), whereas in *M. bovis* they are mainly transitions, *i.e.* $A_{965}T$ and $A_{967}T$, associated to higher MICs (Amram et al., 2015; Khalil et al., 2017). Furthermore, the role of the additional mutation at position 966 in our *M. agalactiae* isolates has yet to be explored. It has never before been described *in vivo* and has been associated with increased MICs in laboratory-derived mutants of *M. bovis* and *M. hominis* (Degrange et al., 2008; Sulyok et al., 2017).

Interestingly, two French isolates (L16160 and L16156) yielded MIC values of 4µg/ml in the absence of hotspot mutations in the Tet-1 domain but with 3 mutations elsewhere in *rrs* genes **(Table 2)**. These isolates belonged to the ST 0.1 genetic subtype whereas all isolates with 965 or 967 mutations were of ST3.1 subtype. Thus, whereas resistance in *M. bovis* is associated with a single subtype and a dominant genotype (Khalil et al., 2017), it seems that different mechanisms of resistance would be potentially expressed in the more variable *M. agalactiae* species.

Similarly, 6 strains from the *M. mycoides* cluster had MIC values ≥2 µg/ml but no mutations in the *rrs* hotspots when compared to the wild type genotype. This clearly suggests the existence of other yet not-described resistance mechanims.

375 *Other resistance mechanisms*

376 One such mechanism could result from mutations in genes that encode ribosomal
377 proteins. Mutations in the *rpsJ* gene, leading to amino acids changes in positions 53 to
378 60 of the 30S ribosomal subunit protein S10, have been associated with tetracycline
379 resistance in several bacteria (Grossman, 2016), but never described in mycoplasmas.
380 Our first analysis of the *rpsJ* genes in a subset of *M. agalactiae* and *M. mycoides* cluster
381 isolates, with different MIC values and different 16S rRNA genotypes, was hampered
382 by large differences in coding sequence between the type strains of the two groups. For
383 instance, the S10 protein sequence between residues 53 to 60 was RSVHINKK for *M.*
384 *agalactiae* PG2^T with an MIC for oxytetracyline of 1µg/ml but RAVHKYKD for both
385 *Mmc* PG3 and *Mcc* CK^T with MICs of 0.25 and 0.125µg/ml, respectively. Furthermore
386 these wildtype genotypes were very different from those of other bacteria species
387 (Grossman, 2016).

388 Only 1 of 4 *M. agalactiae* strains with MIC>2 µg/ml, had a predicted amino acid
389 change ($H_{56}Y$, *E. coli* numbering) and no mutation was found in the French isolates
390 with increased MICs but lacking *rrs* alterations in the Tet-1 binding site (L16160 and
391 L16156). Two mutations were detected in isolates of the *M. mycoides* cluster: $Y_{58}D$ for
392 *M. mycoides* subsp. *capri* and $H_{56}Y\_K_{57}I$ for *M. capricolum* subsp. *capricolum*.
393 However, although the $H_{56}Y\_K_{57}I$ mutation was found in the *M. capricolum* subsp.
394 *capricolum* strain which had the highest MIC (F10621), the $Y_{58}D$ alteration was absent
395 from the *M. mycoides* subsp. *capri* strain with the highest MIC (F10751, **Table 2**).
396 More investigations are therefore required to establish a clear link between mutations in
397 the *rpsJ* gene and tetracycline MICs in mycoplasmas.

398 Since tetracycline-specific ribosomal protection is usually associated with high MICs
399 and has never been described in mycoplasmas other than *M. hominis*, the most probable

400 other mechanism of resistance to tetracycline might result from efflux pumps, as
401 described in Gram+ and Gram- bacteria (Nguyen et al., 2014). Efflux-based
402 antibioresistance has only been reported for mycoplasmas in relation to quinolone
403 resistance in *M. mycoides* subsp. *capri* and *M. hominis* (Antunes et al., 2015; Raherison
404 et al., 2005). It would be worth exploring a possible tetracycline efflux by *M. agalactiae*
405 and (sub)species of the *M. mycoides* cluster to explain the different MIC levels that
406 were not due to hotspot mutations in *rrs* or amino acid modifications of protein S10.
407
408 **Conclusion**
409 Our results revealed a statistically non-significant loss of susceptibility to tetracylines in
410 recent years in mycoplasma (sub)species responsible for CA, whatever the origin of the
411 isolates and current use of this antimicrobial family (Spain versus France). Evolution of
412 the susceptibility patterns was coherent with the epidemiological situation and relative
413 importance of each CA etiological agent. The (sub)species most often isolated were
414 most likely to be less susceptible. No simple relationship was found between mutations
415 in 16S rRNA genes and increased MICs, thus suggesting the existence of other
416 resistance mechanisms. Preliminary analyses revealed considerable diversity in the
417 amino-acid sequences of ribosomal proteins that might influence the binding capacity of
418 tetracyclines. However, due to this diversity, no clear conclusions can be drawn
419 regarding their relationship with MICs and efflux seems to be the most probable
420 hypothesis.
421
422 **Conflict of interest statement**
423 None of the authors has any financial or personal relationships that could
424 inappropriately influence or bias the content of this paper.

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Tables and Figures Titles

Table 1. MIC values (reference strains), MIC ranges, MIC_{50} and MIC_{90} (field strains) (μg/ml) of tetracyclines for mycoplasma species involved in contagious agalactia.

Table 2. Mutations in *rrs1*/*rrs2* genes (in and outside the main binding site) and in the 30S ribosomal subunit protein S10 in relation to MIC values of 32 *M. agalactiae* and 13 *M. mycoides* cluster strains.

Supplementary Table S1. List of the 120 mycoplasma isolates included in the study and details about their origin, MICs, VNTR profile and *rrs1/rrs2/rpsJ* genotype when available.

Supplementary Table S2. Primers and PCR protocols developed in the present study.

Supplementary Table S3. VNTR designation and nucleotide sequences of new profiles and variants, never described in previous studies.

Figure 1. Distributions of oxytetracycline minimal inhibitory concentration (MIC) of *M. agalactiae* isolates (A) and isolates belonging to the *M. mycoides* cluster (B).

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Highlights

- No statistically-significant increase in tetracyclines MICs in recent years for Contagious Agalactia agents.
- Same MICs distributions in France and Spain despite differences in tetracyclines use.
- The subspecies most often isolated are more prone to have increased MICs
- Mutations in 16S rRNA genes cannot account for all observed increases in MICs

The moderate drift towards less tetracycline-susceptible isolates of contagious agalactia causative agents might result from different molecular mechanisms.

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Keywords: antibioresistance; tetracycline; mycoplasmas; diversity; contagious agalactia

Abstract

Contagious agalactia is a mycoplasmosis that affects small ruminants, is associated with loss of milk production and high morbidity rates, and is highly deleterious to dairy industries. The etiological agents are four mycoplasma (sub)species, of which the relative importance depends on the countries and the animal host. Tetracyclines are non-expensive, broad-spectrum antimicrobials and are often used to control mastitis in dairy herds. However, the *in vitro* efficiency of tetracyclines against each of the etiological agents of contagious agalactia has been poorly assessed.

The aims of this study were i) to compare the tetracycline susceptibilities of various field isolates, belonging to different mycoplasma (sub)species and subtypes, collected over the years from different clinical contexts in France or Spain, and ii) to investigate the molecular mechanisms behind the decreased susceptibility of some isolates to tetracyclines.

The Minimum Inhibitory Concentrations (MICs) of tetracyclines were determined *in vitro* on a set of 120 isolates. Statistical analyses were run to define the significance of any observed differences in MICs distribution. As mutations in the genes encoding the tetracycline targets (*rrs* loci) are most often associated with increased tetracycline MICs in animal mycoplasmas, these genes were sequenced.

The loss of susceptibility to tetracyclines after year 2010 is not significant and recent MICs are higher in *M. agalactiae*, especially isolates from ovine mastitis cases, than in other etiological agents of contagious agalactia. The observed increases in MICs were not always associated with mutations in the *rrs* alleles which suggests the existence of other resistance mechanisms yet to be deciphered.

1. Introduction

Contagious agalactia (CA) is a syndrome affecting small ruminants and has a substantial economic impact on dairy industries due to its reduction/suppression of milk production and high morbidity rates. The three main clinical signs associated with CA are mastitis, arthritis and keratoconjunctivitis, but others like pneumonia or septicaemia have also been reported in young animals (Agnello et al., 2012; Corrales et al., 2007; Gomez-Martin et al., 2013). CA is caused by four *Mycoplasma* (sub)species: *M. agalactiae*, *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum* and *M. putrefaciens* (Corrales et al., 2007). In France, *M. agalactiae* is primarily isolated from

sheep herds reared in the Western Pyrenees, where it causes subclinical to acute mastitis, but is more rarely isolated from goats, and usually with no or few clinical signs (Poumarat et al., 2016). In contrast, *M. agalactiae* is the main mycoplasma species isolated from both Spanish ovine and caprine herds with CA (Ariza-Miguel et al., 2012), (De la Fe, personal communication). *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum* and *M. putrefaciens* are phylogenetically related, as they belong or are related to the *M. mycoides* cluster (Manso-Silvan et al., 2007). These mycoplasmas usually infect goats and are very seldom isolated from sheep. *M. mycoides* subsp. *capri* is the main species isolated from French herds with clinical CA (Chazel et al., 2010) and also the predominant species in some areas of Spain such as the Canary Islands (De la Fe et al., 2005). Although *M. capricolum* subsp. *capricolum* and *M. putrefaciens* are less frequently isolated, their presence in goat herds has been reported in severe CA outbreaks (De la Fe et al., 2007; Giadinis et al., 2008; Gil et al., 1999; Mercier et al., 2000).

Herds in endemic areas, such as Spain and France, tend to be chronically affected with CA-causing mycoplasmas and clinical outbreaks occur only sporadically. Due to the poor efficacy of currently available vaccines and their inability to prevent disease transmission or infection (Agnone et al., 2013), antibiotherapy is often used to control CA (Gomez-Martin et al., 2013). Tetracyclines are broad-spectrum, low cost antimicrobials and amongst the main ones used in veterinary medicine (De Briyne et al., 2014). Although three generations of tetracyclines now exist (Grossman, 2016), only first generation products such as oxytetracycline are specifically available for small ruminants in Spain and France (AEMPS, 2017; Summary of antimicrobials available in France for animals, accessible at http://www.ircp.anmv.anses.fr). The *in vitro* activity of oxytetracycline against CA-causing mycoplasmas has been demonstrated in several

studies (Antunes et al., 2007; Paterna et al., 2013; Paterna et al., 2016; Tatay-Dualde et al., 2017).

Tetracyclines exert their bacteriostatic activity by binding preferentially to the 30S ribosomal subunit and interacting with a highly conserved 16S rRNA target to prevent binding of aminoacyl-tRNA to the ribosomal acceptor site and hence to stop bacterial protein synthesis (Nguyen et al., 2014). Crystallographic studies have revealed a primary tetracycline binding site (Tet-1), located in a pocket formed between helices h31 (nucleotide positions 964-967, *E. coli* numbering) and h34 (positions 1052-1056 and 1196-1200). Five other minor binding sites in 16S rRNA have also been described (Brodersen et al., 2000; Pioletti et al., 2001). In addition, the 30S ribosomal protein S10, encoded by the *rpsJ* gene, forms a loop projecting towards the aminoacyl-tRNA-binding site and may play a role in the interaction of tetracyclines and 16S rRNA (Brodersen et al., 2000). Resistance to tetracyclines has been linked to several mechanisms such as energy-dependent efflux, presence of ribosomal protection proteins, mutations in the 16S rRNA encoding genes (the so-called *rrs* genes), and enzymatic inactivation of the drug (Grossman, 2016; Nguyen et al., 2014). Antimicrobial efflux in mycoplasmas has only been described *in vitro,* in association with fluoroquinolone resistance (Antunes et al., 2015; Raherison et al., 2005). Moreover, high level tetracycline resistance associated with the presence of the *tetM* determinant, which encodes a protective protein conferring cross resistance to all tetracyclines, has so far been reported only in *M. hominis* and *Ureaplasma* spp. (Waites et al., 2014). No *tetM* determinants and/or derivatives have been found in other mycoplasma species, such as *M. bovis* (Amram et al., 2015; Sulyok et al., 2017). Target-based mutations in *rrs* genes conferring tetracycline resistance have been reported in *in vitro* selected mutants of *M. hominis*, *M. pneumoniae* and *M. bovis*

(Degrange et al., 2008; Sulyok et al., 2017) and in field isolates of *M. bovis* (Amram et al., 2015; Khalil et al., 2017; Sulyok et al., 2017). However, the molecular mechanisms responsible for tetracycline resistance in CA-causing mycoplasmas have not yet been elucidated.

The aim of the present study was to assess and compare the tetracycline susceptibilities of different field isolates, belonging to different mycoplasma (sub)species, and obtained from subclinical or clinical infections in France and Spain. The susceptibility patterns were analysed in relation to the time of collection, sample origin (nature and place of collection), and the molecular subtypes of the isolates. In addition, the molecular mechanisms responsible for the decreased susceptibility to tetracyclines of some isolates were investigated.

2. Material and methods

2.1. Mycoplasma isolates and subtyping

A total of 120 field isolates of CA-causing mycoplasmas, namely 60 *M. agalactiae*, 30 *M. mycoides* subsp. *capri*, 18 *M. capricolum* subsp. *capricolum* and 12 *M. putrefaciens,* were analysed **(Supplementary Table S1)**. Isolates were defined as old when collected up to 2010 included (n = 64), or current, when they were isolated from 2011 included onwards (n = 56). They were retrieved from different regions of France (n = 59) or Spain (n = 61), and from different samples including mainly bulk tank milk (BTM, n = 50) and mastitic milk (n = 49) but also auricular swabs (n = 6), joints (n =6) and lungs (n = 6).

All isolates were identified by dot immunoblotting on a filtration membrane (Poumarat et al., 1991). In the case of ambiguous antigenic identification, complementary tests were conducted, including a species-specific PCR assay for *M. agalactiae* (Marenda et

al., 2005) or a *fusA* PCR followed by sequence analysis for species in the *M. mycoides* cluster (Maigre et al., 2008).

In addition, the type strains of the four studied mycoplasma species, namely *M. agalactiae* PG2^T (NCTC 10123); *M. mycoides* subsp. *capri* PG3^T (NCTC 10137); *M. capricolum* subsp. *capricolum* California Kid^T (NCTC 10154); and *M. putrefaciens* KS1^T (NCTC 10155) were used as controls. The 5632 strain of *M. agalactiae,* that defines the other genetic extremity of the species, was also included (Nouvel et al., 2010).

M. agalactiae isolates were subtyped using a previously described multilocus variable-number tandem-repeat (VNTR) scheme which analyses VNTR17 and VNTR19, the most discriminating loci (Nouvel et al., 2012). The sizes of the PCR products were estimated using an automated capillary electrophoresis device from Qiagen (QIAxcel System) and compared with those of previously described profiles (De la Fe et al., 2012; Nouvel et al., 2012; Poumarat et al., 2016). PCR products corresponding to new profiles were further sequenced using an external facility at Beckman Coulter Genomics (Genewiz, United Kingdom).

Isolates belonging to the *M. mycoides* cluster were subtyped by analysing a 561bp-partial sequence of their *fusA* gene, as previously described (Maigre et al., 2008; Manso-Silvan et al., 2007).

Tetracycline susceptibility testing

The Minimum Inhibitory Concentrations (MIC) of oxytetracycline and doxycycline (both purchased from Sigma-Aldrich, France) were determined using the agar dilution method on modified PPLO agar, as previously described (Khalil et al., 2016; Poumarat et al., 2016). Briefly, 1 µl of each isolate and the reference strains, diluted to 10^4 – 10^5 CFU/ml, were inoculated onto agar plates containing two-fold dilutions of each

antimicrobial, ranging from 0.0625 to 128 µg/ml. Plates were incubated at 37°C in 5% CO_2 for 4 or 2 days for *M. agalactiae* and *M. mycoides* cluster isolates, respectively. The MIC was defined as the minimum concentration of antimicrobial at which no growth was observed. Analyses were repeated twice and, if the results were not consistent, a third repetition was performed. In case of 3 assays, the final MIC value was the mode of the 3 values **(Supplementary table S1)**.

PCR amplification and sequence analysis of 16S rRNA tetracycline binding sites

A selected subset of field isolates, showing high, intermediate or low MIC values, was used to study the 16S rRNA encoding genes. This subset included 32 *M. agalactiae* isolates and 24 isolates of different species belonging to the *M. mycoides* cluster **(Supplementary table S1).** All these mycoplasma (sub)species have two *rrs* alleles, hereafter designated *rrs1* and *rrs2*.

Genomic DNA was extracted from 2 ml of broth culture using the DNeasy Blood&Tissue kit from Qiagen. Novel sets of primers were designed to separately amplify the 2 *rrs* copies, based on long range PCRs using primers targeting flanking genes, as previously proposed (Khalil et al., 2017), and the *rpsJ* gene of the four studied mycoplasma species **(Supplementary table S2)**. The individual *rrs* copies were further amplified by performing a nested PCR using the universal primers U1 and U8 (Johansson et al., 1998) on each long range PCR product diluted to 1/10. Final PCR products were then sequenced using primers U7 and U3 (Johansson et al., 1998) to cover all the previously described tetracycline binding sites (Brodersen et al., 2000; Pioletti et al., 2001). The *rpsJ*-F primer used for PCR was also used to generate sequences of the *rpsJ* gene. Sequencing was conducted by an external facility at Beckman Coulter Genomics (Genewiz, UK). Sequence editing and alignment construction were performed with MEGA 6.0 software. Sequences of the type strains,

with the following accession numbers: *M. agalactiae* PG2^T (NC_009497.1), *M. agalactiae* 5632 (NC_013948.1), *M. mycoides* subsp. capri PG3^T (NC_005364.2), *M. capricolum* subsp. *capricolum* CK^T (NC_007633.1) and *M. putrefaciens* KS1^T (NC_015946.1), were retrieved from GenBank databases. For convenience, nucleotide numbering refers to *Escherichia coli* K12 positions (NC_000913.3).

Statistical analysis

For statistical analysis, the MIC values were first converted to a continuous variable by calculating their Log2 values. These Log2(MIC) values were then used to compare different paired subpopulations of isolates (e.g. France versus Spain, recent versus old, etc.) using a Mann-Whitney test with the significance level set at 0.05, as previously described (Poumarat et al., 2016). These analyses were run using the EpiInfo software available at https://www.cdc.gov/epiinfo/index.html.

3. Results

3.1. Choice of isolates and subtyping

Selection of the isolates included in the study was based on current knowledge of CA epidemiology in Spain and France. Two sets were prepared to take all the etiological agents into account, in proportions mimicking those observed in the field. Hence, 60 isolates of *M. agalactiae* were enclosed in the first set because this is the main species isolated in Spain and in French sheep flocks. The other 60 isolates belonged to the three other CA-causing subspecies and were included in set 2 in proportions comparable to their frequencies of isolation in the two countries, *i.e.* 30 *M. mycoides* subsp. *capri*, 17 *M. capricolum* subsp. *capricolum* and 13 *M. putrefaciens.* All isolates originated from different outbreaks or herds. The two sets consisted of approximately equal proportions of isolates from France (n=59) and Spain (n=61) since our aim was to compare the

overall level of susceptibility to tetracycline in these two countries. Similar numbers of old isolates (collected up to 2010 included (n=64) and current ones isolated from 2011 onwards (n=56)) were studied to determine the evolution of MICs over time. Finally, a similar number of isolates collected from acute clinical cases and those circulating asymptomatically in herds were examined, *i.e.* mastitis isolates (n=49) and isolates recovered from BTM (n=50).

These isolates were then further subtyped to analyse the link between diversity and loss of susceptibility to antimicrobials, as described previously (Khalil et al., 2017; Poumarat et al., 2016). The 60 *M. agalactiae* isolates analysed presented 15 different VNTR profiles (**Supplementary table S1**) including 3 new VNTR17 and 1 new VNTR19 genotypes (shown in **Supplementary table S3**). The most frequent subtype was ST 3.1 (n=32, more than half of the isolates). This was found in France and Spain and included all the isolates from sheep (8 French and 6 Spanish isolates) and 18 of the 46 caprine isolates. Interestingly, the only French caprine isolate with this profile came from the same area as the ovine isolates (Western Pyrenees). The only other genotype shared between France and Spain was ST 0.1, but was only found in 3 goats (2 French and 1 Spanish). The other 13 subtypes were mainly found in France (10/13). Thus the *M. agalactiae* isolates population in France is more diverse than in Spain, where ST 3.1 is also predominant in goats. Analysis of the *fusA* sequences, in isolates from the *M. mycoides* cluster, revealed a clear split into different (sub)species in different branches of the tree (data not shown). However, no subgroup of isolates within a (sub) species was evidenced that could be correlated to the geographical origin, year of isolation or MIC values.

3.2. MICs distribution

The MIC results are detailed in **Supplementary table S1** and summarized in **Table 1**. Firstly, the MIC values of oxytetracycline were higher than those of doxycycline, whatever the isolate tested, with a range of 0.125-8 µg/ml versus ≤0.0625-2 µg/ml, respectively ($p < 0.001$). Nonetheless similar evolutions over time of MIC were observed in our isolates population for both antimicrobials, *i.e.* a maximum 5-fold increase of the antimicrobial concentration for both oxytetracycline and doxycycline.

Secondly, the MIC values of oxytetracycline were higher for *M. agalactiae* than for isolates from the *M. mycoides* cluster ($p < 0.001$), with MIC_{90} of 4 and 0.5 µg/ml, respectively. In contrast, the MIC values of doxycycline were similar for both groups of isolates.

Within the *M. mycoides* cluster, the highest MIC values of oxytetracycline were obtained for *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum* isolates (4 µg/ml), while the MICs for *M. putrefaciens* never exceeded 0.5 µg/ml. *M. mycoides* subsp. *capri* isolates showed the highest MIC_{90} at 2µg/ml, while the MIC_{50} and MIC_{90} were equal for the other (sub)species isolates at only 0.5 µg/ml. No statistical tests were run because of the small size of the samples in each category.

Figure 1 shows the oxytetracycline MIC distribution of mycoplasma isolates of *M. agalactiae* (Fig.1A) and species belonging to the *M. mycoides* cluster (Fig.1B). For each panel, the MICs distributions were compared as a function of their year of collection (after 2010 or up to 2010 included), their geographical origin (France versus Spain), the sample from which they were isolated (BTM versus mastitic milk) and their genetic subtypes (only for *M. agalactiae* isolates).

The distributions of oxytetracyline MICs for *M. agalactiae,* as compared to isolates from the *M. mycoides* cluster, were more heterogeneous and mainly centred around 0.5 µg/ml (Fig. 1B). The homogeneous distribution of isolates from the *M. mycoides* cluster

had already been suggested by the equal MIC_{50} and MIC_{90} values obtained for oxytetracycline and doxycycline **(Table 1)**.

Within each (group of) species, no significant differences in distribution were found between old and recent isolates (*M. agalactiae* p=0.095; *M. mycoides* cluster p=0.677), or between French and Spanish isolates (*M. agalactiae* p=0.193; *M. mycoides* cluster p=0.087).

The MIC for *M. agalactiae* isolates retrieved from mastitic milk samples were significantly higher than those of strains isolated from bulk tank milk (p=0.044). This difference was not observed between isolates from the *M. mycoides* cluster. Significant differences were also found (p=0.025) between the MIC values of the predominant *M. agalactiae* subtype 3.1 and those of the other subtypes.

3.3. Association between 16S rRNA alterations and MICs

The *rrs* genes in a subset of 32 *M. agalactiae* isolates, composed of 15 isolates with oxytetracycline MICs ≥2 µg/ml and 17 more susceptible ones (MIC ≤0.5 µg/ml), were examined. Mutations detected in the 2 *rrs* operons of *M. agalactiae* isolates were compared with strain $PG2^T$ (**Table 2**). Mutations in the Tet-1 primary binding pocket of tetracycline were observed in only 7 isolates and only in helix 31(positions 965-967) as no mutation was detected in helix 34. These isolates were all from Spain and had MICs ≥2 and ≥0.25 µg/ml for oxytetracycline and doxycycline, respectively. None of the isolates from France showed any mutations in these 3 hotspots. Fifteen isolates had no mutations at all and 10 isolates had mutations in other, non-hotspot positions. Eight of these 10 isolates showed mutations in only one allele of the *rrs* genes (except Ag304) and had the same MIC range as isolates of the wild-type genotype (0.25 – 2 µg/ml of oxytetracycline and ≤0.0625-0.25 µg/ml of doxycycline). None of these non-hotspot

mutations was detected in more than two isolates suggesting they are neutral, weakly selected mutations. Two French isolates, (F16156 and F16160), showed increased MICs to oxytetracycline at 4 µg/ml with mutations affecting positions 458, 461 and 1272 of the *rrs* genes, i.e. outside the main tetracycline binding pocket **(Table 2).**

The *rrs* genes were also analysed in a subpopulation of 24 isolates selected from different (sub)species in the *M. mycoides* cluster, which had oxytetracycline MIC values ranging from 0.125 to 4 µg/ml (**Table 2**). No mutations associated with increased MICs were detected, although some isolates (LC32, LC54, F9545, F10621, F10685, F10751) had similar MIC values to *M. agalactiae* isolates harbouring Tet-1 mutations. Furthermore, many mutations were observed outside the Tet-1 binding site in *rrs* but their presence was not correlated with higher MIC values.

4. Discussion

No statistically-significant difference in susceptibility

No statistically-significant increase in tetracyclines MICs after 2010 was apparent for any of the mycoplasma (sub)species involved in CA in France and Spain. Doxycycline, a 2nd generation tetracycline with improved structure-activity features, yielded lower MICs than oxytetracycline, but the MIC amplitude of variation for both antimicrobials was comparable, suggesting that evolution over time and resistance mechanisms were similar. Although the MICs of oxytetracycline for *M. agalactiae* were slightly higher than those obtained for isolates from the *M. mycoides* cluster, the maximum attained (8µg/ml) was moderate. Indeed, as no clinical breakpoints specific to veterinary mycoplasmas are available, only one isolate with an MIC of 8µg/ml (Ag316) would be classified, according to the generic breakpoints for pathogenic bacteria of cattle (the

only ones available for domestic ruminants (CLSI, 2015)), as resistant for oxytetracycline (MICs≥8µg/ml).

The different susceptibilities of *M. agalactiae,* versus the *M. mycoides* cluster, could partly be explained by the highly susceptible 22% of *M. putrefaciens* isolates in the *M. mycoides* subgroup. Our data for *M. agalactiae* are consistent with those previously reported (Poumarat et al., 2016) that evidenced a loss of susceptibility to tetracycline between 1990 and 2000. In the present study no further significant loss was observed after 2010.

However, despite this overall reassuring picture, the highest MICs were observed for the most recent isolates, which implies a current (maybe slow) development of resistance to tetracyclines in the field. This slow and moderate evolution can be related to the nature of the underlying mechanisms: indeed the increase of MICs, after passages of *M. bovis in vitro,* was shown to be more rapid for spectinomycin (a single mutation being sufficient to switch to the resistant genotype) than for tetracycline (several mutations required) (Sulyok et al., 2017).

Interestingly, the susceptibilities of Spanish and French *M. agalactiae* and *M. mycoides* isolates did not differ significantly despite the apparent differences in the amount of tetracycline used in each country. Indeed, according to the 7th annual report of the European Surveillance of Veterinary Antimicrobial Consumption, tetracyclines sales for food-producing animals were 5 times higher in Spain than in France in 2015 (http://www.ema.europa.eu/docs/en_GB/document_library/Report/2017/10/WC500236750.pdf). Nevertheless, this value has to be interpreted with caution since sales provide only an indirect indication of the actual use of antimicrobials, especially in small ruminants (De Briyne et al., 2014).

Coherence between MICs and epidemiology

The observed evolution of susceptibility patterns is coherent with the epidemiological situation and relative importance of each CA etiological agent. In France, the highest MICs were observed for *M. mycoides* subsp. *capri,* which is most often isolated from CA clinical outbreaks (Chazel et al., 2010). In contrast, the *M. agalactiae* isolates with the highest MICs came from Spain, where this species is the main causative agent of CA (Ariza-Miguel et al., 2012); (De la Fe, personal communication).

M. agalactiae isolates from mastitis (symptomatic herds) also showed higher MICs than isolates from BTM (asymptomatic herds), maybe due to the fact that only sick animals are usually exposed to antibiotherapy. However, this difference in susceptibility might also be influenced by the host animal as the isolates retrieved from mastitis were mainly caprine. Although no statistical comparison could be done due to the very few ovine samples, the MICs were higher in caprine isolates both in this and a previous study (Poumarat et al., 2016). Results might be further biased by the huge diversity of caprine isolates (up to 14 subtypes), whereas all ovine isolates belonged to the predominant European subtype ST3.1 (Ariza-Miguel et al., 2012; Poumarat et al., 2016) associated with lower MICs (Figure 1A).

Mutations in rrs loci

The increased tetracyclines MICs in *M. bovis*, another ruminant mycoplasma that shares many phenotypic and genotypic traits with *M. agalactiae*, have been associated with mutations in the genes encoding 16S rRNA, especially at hotspot positions, 965 and 967 (Amram et al., 2015; Khalil et al., 2017; Sulyok et al., 2017). We evidenced mutations in one of these two positions in 6 *M. agalactiae* isolates with MICs of oxytetracycline >2µg/ml (that could be classified as intermediate or resistant according to the CLSI breakpoints for pathogenic bacteria of cattle) and one with an MIC of 2µg/ml (susceptible). These $A_{967}C$ or $A_{965}G$ mutations were observed in one or both *rrs* alleles

but never simultaneously in a single isolate (Table 2). This could explain why no MIC greater than 8µg/ml was ever attained in *M. agalactiae,* whereas all recent strains of *M. bovis* from France harbour both mutations in both alleles and have MICs ≥ 32µg/ml (Khalil et al., 2017). Khalil et al. also showed that *M. bovis* isolates collected before the year 2000 had only one hotspot mutation in a single *rrs* allele and MICs of approximately 4µg/ml.

Whether the binding capacity of oxytetracyclines, and hence their MICs, could be influenced by the nature of the mutations at positions 965 and 967, has yet to be demonstrated. In *M. agalactiae* the mutations at positions 965 and 967 are transversions ($A_{965}G$ and $A_{967}C$), whereas in *M. bovis* they are mainly transitions, *i.e.* $A_{965}T$ and $A_{967}T$, associated to higher MICs (Amram et al., 2015; Khalil et al., 2017). Furthermore, the role of the additional mutation at position 966 in our *M. agalactiae* isolates has yet to be explored. It has never before been described *in vivo* and has been associated with increased MICs in laboratory-derived mutants of *M. bovis* and *M. hominis* (Degrange et al., 2008; Sulyok et al., 2017).

Interestingly, two French isolates (L16160 and L16156) yielded MIC values of 4µg/ml in the absence of hotspot mutations in the Tet-1 domain but with 3 mutations elsewhere in *rrs* genes **(Table 2)**. These isolates belonged to the ST 0.1 genetic subtype whereas all isolates with 965 or 967 mutations were of ST3.1 subtype. Thus, whereas resistance in *M. bovis* is associated with a single subtype and a dominant genotype (Khalil et al., 2017), it seems that different mechanisms of resistance would be potentially expressed in the more variable *M. agalactiae* species.

Similarly, 6 strains from the *M. mycoides* cluster had MIC values ≥2 µg/ml but no mutations in the *rrs* hotspots when compared to the wild type genotype. This clearly suggests the existence of other yet not-described resistance mechanims.

Other resistance mechanisms

One such mechanism could result from mutations in genes that encode ribosomal proteins. Mutations in the *rpsJ* gene, leading to amino acids changes in positions 53 to 60 of the 30S ribosomal subunit protein S10, have been associated with tetracycline resistance in several bacteria (Grossman, 2016), but never described in mycoplasmas. Our first analysis of the *rpsJ* genes in a subset of *M. agalactiae* and *M. mycoides* cluster isolates, with different MIC values and different 16S rRNA genotypes, was hampered by large differences in coding sequence between the type strains of the two groups. For instance, the S10 protein sequence between residues 53 to 60 was RSVHINKK for *M. agalactiae* $PG2^T$ with an MIC for oxytetracyline of 1µg/ml but RAVHKYKD for both *Mmc* PG3 and *Mcc* CK^T with MICs of 0.25 and 0.125µg/ml, respectively. Furthermore these wildtype genotypes were very different from those of other bacteria species (Grossman, 2016).

Only 1 of 4 *M. agalactiae* strains with MIC>2 µg/ml, had a predicted amino acid change ($H_{56}Y$, *E. coli* numbering) and no mutation was found in the French isolates with increased MICs but lacking *rrs* alterations in the Tet-1 binding site (L16160 and L16156). Two mutations were detected in isolates of the *M. mycoides* cluster: $Y_{58}D$ for *M. mycoides* subsp. *capri* and $H_{56}Y\_K_{57}I$ for *M. capricolum* subsp. *capricolum*. However, although the $H_{56}Y\_K_{57}I$ mutation was found in the *M. capricolum* subsp. *capricolum* strain which had the highest MIC (F10621), the $Y_{58}D$ alteration was absent from the *M. mycoides* subsp. *capri* strain with the highest MIC (F10751, **Table 2**). More investigations are therefore required to establish a clear link between mutations in the *rpsJ* gene and tetracycline MICs in mycoplasmas.

Since tetracycline-specific ribosomal protection is usually associated with high MICs and has never been described in mycoplasmas other than *M. hominis*, the most probable

other mechanism of resistance to tetracycline might result from efflux pumps, as described in Gram+ and Gram- bacteria (Nguyen et al., 2014). Efflux-based antibioresistance has only been reported for mycoplasmas in relation to quinolone resistance in *M. mycoides* subsp. *capri* and *M. hominis* (Antunes et al., 2015; Raherison et al., 2005). It would be worth exploring a possible tetracycline efflux by *M. agalactiae* and (sub)species of the *M. mycoides* cluster to explain the different MIC levels that were not due to hotspot mutations in *rrs* or amino acid modifications of protein S10.

Conclusion

Our results revealed a statistically non-significant loss of susceptibility to tetracylines in recent years in mycoplasma (sub)species responsible for CA, whatever the origin of the isolates and current use of this antimicrobial family (Spain versus France). Evolution of the susceptibility patterns was coherent with the epidemiological situation and relative importance of each CA etiological agent. The (sub)species most often isolated were most likely to be less susceptible. No simple relationship was found between mutations in 16S rRNA genes and increased MICs, thus suggesting the existence of other resistance mechanisms. Preliminary analyses revealed considerable diversity in the amino-acid sequences of ribosomal proteins that might influence the binding capacity of tetracyclines. However, due to this diversity, no clear conclusions can be drawn regarding their relationship with MICs and efflux seems to be the most probable hypothesis.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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Tables and Figures Titles

Table 1. MIC values (reference strains), MIC ranges, MIC_{50} and MIC_{90} (field strains) (μg/ml) of tetracyclines for mycoplasma species involved in contagious agalactia.

Table 2. Mutations in *rrs1*/*rrs2* genes (in and outside the main binding site) and in the 30S ribosomal subunit protein S10 in relation to MIC values of 32 *M. agalactiae* and 13 *M. mycoides* cluster strains.

Supplementary Table S1. List of the 120 mycoplasma isolates included in the study and details about their origin, MICs, VNTR profile and *rrs1/rrs2/rpsJ* genotype when available.

Supplementary Table S2. Primers and PCR protocols developed in the present study.

Supplementary Table S3. VNTR designation and nucleotide sequences of new profiles and variants, never described in previous studies.

Figure 1. Distributions of oxytetracycline minimal inhibitory concentration (MIC) of *M. agalactiae* isolates (A) and isolates belonging to the *M. mycoides* cluster (B).

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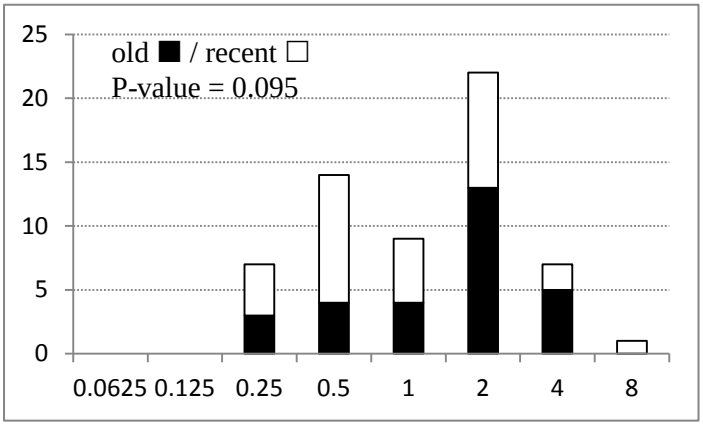
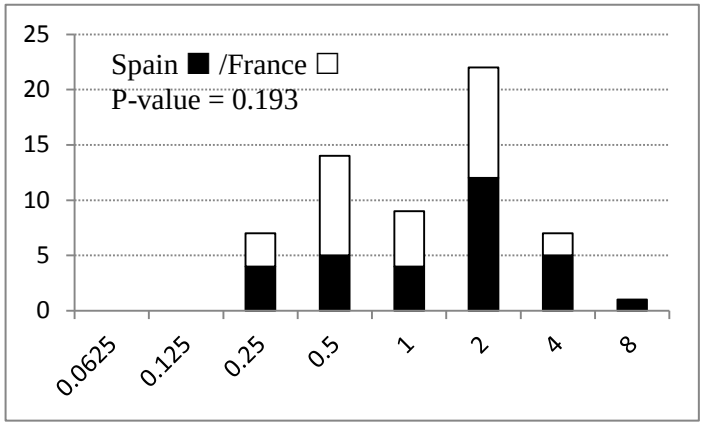
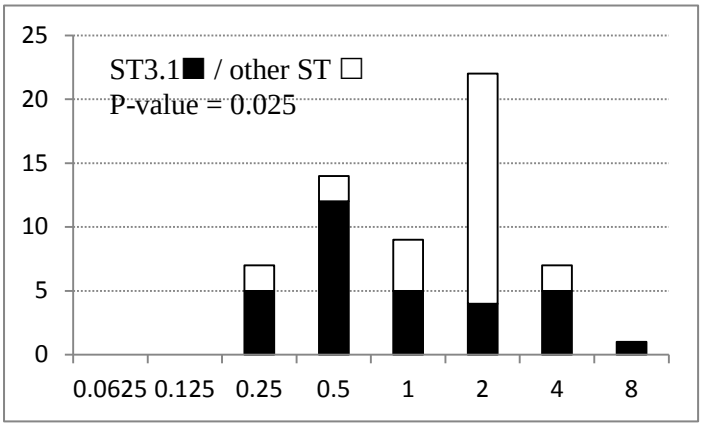
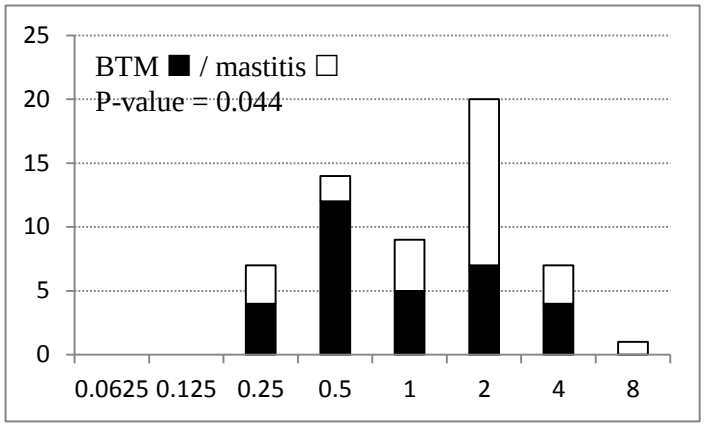
Figure 1. Distributions of oxytetracycline minimal inhibitory concentration (MIC) of *M. agalactiae* isolates (A) and isolates belonging to the *M. mycoides* cluster (B).

A: *M. agalactiae* isolates

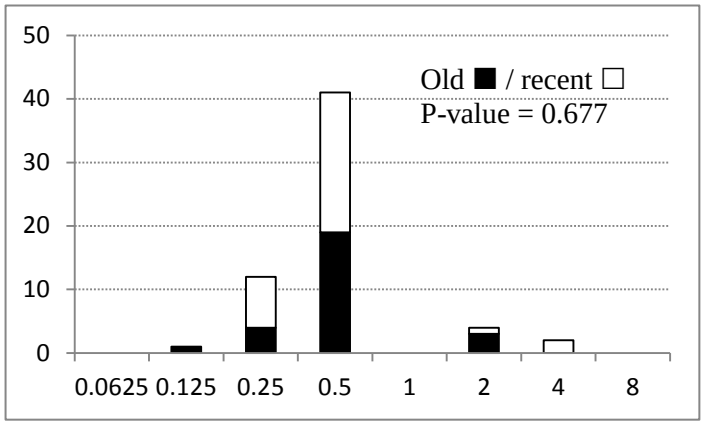


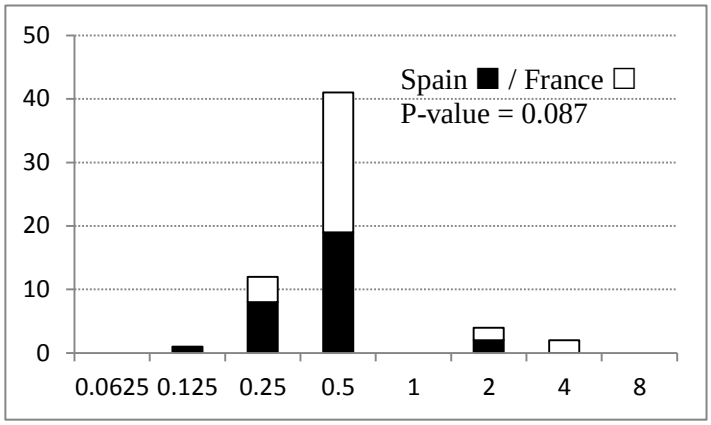


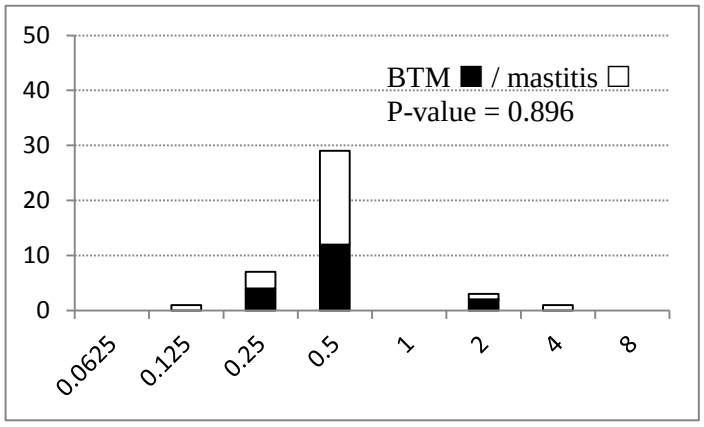




B: isolates belonging to the *M. mycoides* cluster







Isolates were grouped by their isolation year (≤2010, old or >2010, recent), country of origin, sample from which they were retrieved (Bulk Tank Milk, BTM versus mastitic milk) or genetic profile (ST3.1 versus other subtypes). X-axis, MICs in µg/ml; Y-axis, number of isolates for each MIC value.

Table 1. MIC values (reference strains), MIC ranges, MIC_{50} and MIC_{90} (field strains) (µg/ml) of tetracyclines for mycoplasma species involved in contagious agalactia.

Antimicrobials	Field strains[a] Range	MIC_{50}	MIC_{90}	Reference strains MIC	
M. agalactiae	(n = 60)			PG2^T	5632
OXY	0.25 – 8	1	4	1	2
DOX	0.0625 – 2	0.125	0.25	0.0625	0.25
***M. mycoides* cluster**	(n=60)				
OXY	0.125 – 4	0.5	0.5	-	
DOX	≤0.0625 – 1	0.125	0.25	-	
Details per (sub)species within the *M. mycoides* cluster					
M. mycoides* subsp. *capri	(n = 30)			PG3^T	
OXY	0.125 – 4	0.5	2	0.25	
DOX	≤0.0625 – 1	0.125	0.5	≤0.0625	
M. capricolum* subsp. *capricolum	(n = 18)			CK^T	
OXY	0.25 – 4	0.5	0.5	0.125	
DOX	≤0.0625 – 0.5	0.125	0.25	≤0.0625	
M. putrefaciens	(n = 12)			KS1^T	
OXY	0.5 – 0.5	0.5	0.5	0.5	
DOX	≤0.0625 – 0.25	0.125	0.125	0.125	

[a] the number of analysed strains is mentioned in brackets

OXY, oxytetracyline; DOX, doxycycline; CK, California Kid

Table 2. Mutations in *rrs1/rrs2* genes (in and outside the main tetracycline binding site) and in the 30S ribosomal subunit protein S10 in relation to MIC values of 32 *M. agalactiae* and 13 *M. mycoides* cluster strains

	Isolate no.	*rrs* mutations in Tet-1 (h31)[a]			*rrs* mutations outside Tet-1[a]	Mutations in the S10 protein (amino acids 53-60[a])	MIC (µg/ml)	
							OXY	DOX
M. agalactiae	Ag316	-	$G_{966}T^{1,2}$	$A_{967}C^{1,2}$	-	-	8	2
	Ag332, Ag335	$A_{965}G^{1,2}$	-	-	$G_{1184}A^{1,2}$	nd	4	0.5
	L16156, L16160	-	-	-	$C_{458}T^{1,2}$, $C_{461}T^{1,2}$, $T_{1272}C^{1}$	-	4	0.5
	Ag28	-	-	$A_{967}C^{2}$	$C_{320}T^{1}$	nd	4	0.25
	Ag30, Ag126	-	-	$A_{967}C^{2}$	$G_{691}A^{1}$	nd	4	0.25
	Ag33	-	-	$A_{967}C^{2}$	$G_{691}A^{1}$	nd	2	0.25
	Ag26, Ag123	-	-	-	$T_{723}C^{2}$	nd	2	0.25
	Ag304	-	-	-	$C_{376}T^{1}$, $C_{376}G^{2}$	nd	2	0.25
	L15241	-	-	-	$C_{369}T^{2}$, $G_{762}C^{2}$, $G_{1304}A^{2}$	nd	2	0.25
	Ag10	-	-	-	$C_{458}T^{2}$	nd	2	0.25
	L16157	-	-	-	$C_{458}T^{2}$, $T_{1272}C^{1}$	nd	2	0.25
	Ag305	-	-	-	-	$H_{56}Y$	2	0.25
	6 isolates[b]	-	-	-	-	nd	2	0.25
	L4908c	-	-	-	-	nd	1	≤ 0.625
	Ag241, Ag314, F8961, L15678	-	-	-	-	nd	0.5	≤ 0.625
	F10269	-	-	-	$C_{1087}T^{1}$	nd	0.5	≤ 0.625
	Ag292, L4211	-	-	-	-	nd	0.25	≤ 0.625
	Ag328	-	-	-	-	-	0.25	≤ 0.625
	L4258	-	-	-	$C_{320}T^{1}$	nd	0.25	≤ 0.625

M. mycoides cluster [c]	*Mmc* F10751	-	-	-	-	-	4	1
	Mmc F9545	-	-	-	-	$Y_{58}D$	4	0.5-1
	Mcc F10621	-	-	-	-	$H_{56}Y$, $K_{57}I$	2	0.5
	Mmc LC32	-	-	-	-	$Y_{58}D$	2	0.5
	Mmc LC54	-	-	-	$T_{212}A^{1}$	$Y_{58}D$	2	0.5
	Mmc F10685	-	-	-	$A_{1290}G^{1}$	$Y_{58}D$	2	0.5
	Mmc LC84	-	-	-	$C_{735}T^{2}$	nd	0.5	0.125
	Mcc Cap1	-	-	-	$G_{1007}C^{1}$	nd	0.5	0.125
	Mcc F10338	-	-	-	$A_{469}G^{1}$	nd	0.5	0.125
	Mput F8131	-	-	-	$A_{1000}G^{2}$, $C_{1100}T^{2}$	nd	0.5	0.125
	Mput Put13	-	-	-	$G_{976}A^{2}$, $C_{1257}T^{1,2}$	nd	0.5	0.125
	Mcc F3247	-	-	-	$C_{1328}T^{2}$	nd	0.25	0.125
	Mcc Cap7	-	-	-	-	-	0.25	0.0625

[a]*Escherichia coli* numbering. The first letter indicates the wild type genotype ($PG2^{T}$ for *M. agalactiae* isolates, *Mmc* $PG3^{T}$, *Mput* $KS1^{T}$ and *Mcc* CK^{T} for the *M. mycoides* cluster isolates), the number indicates the mutation position and the second letter indicates the substitution. "-" indicates the absence of mutation.

[b] Isolates names : Ag9, Ag271, L4054c, F10671, L15242, L16086.

[c] A total of 24 isolates were sequenced for the *rrs* genes, of which those with no mutation (n=13) are not listed here, except *Mmc*F10751 and *Mcc* Cap7 showing the highest and lowest MIC, respectively (see Supplementary Table S1).

[1,2] referring to *rrs1* and/or *rrs2*, respectively.

Mmc, *Mycoplasma mycoides* subsp. *capri*; *Mcc*, *Mycoplasma capricolum* subsp. *capricolum*; *Mput*, *Mycoplasma putrefaciens*; nd, not done; OXY, Oxytetracycline; DOX, Doxycycline.