Manuscript Details

Manuscript number	VETMIC_2017_1083_R1
Title	23S rRNA and L22 ribosomal protein are involved in the acquisition of macrolide and lincosamide resistance in Mycoplasma capricolum subsp. capricolum
Article type	Short Communication

Abstract

Mycoplasma capricolum subsp. capricolum (Mcc) is one of the causative agents of contagious agalactia, and antimicrobial treatment is the most commonly applied measure to treat outbreaks of this disease. Macrolides and lincosamides bind specifically to nucleotides at domains II and V of the 23S rRNA gene. Furthermore, rplD and rplV genes encode ribosomal proteins L4 and L22, which are also implicated in the macrolide binding site. The aim of this work was to study the relationship between these genes and the acquisition of macrolide and lincosamide resistance in Mcc. For this purpose, in vitro selected resistant mutants and field isolates were studied. This study demonstrates the appearance of DNA point mutations at the 23S rRNA encoding genes (A2058G, A2059G and A2062C) and rplV gene (Ala89Asp) in association to high minimum inhibitory concentration values. Hence, it proves the importance of 23S rRNA domain V and ribosomal protein L22 as molecular mechanisms responsible for the acquisition of macrolide and lincosamide resistance in both field isolates and in vitro selected mutants. Furthermore, these mutations enable us to provide an interpretative breakpoint of antimicrobial resistance for Mcc at MIC 0.8 µg/ml.

Keywords	contagious agalactia, antimicrobial resistance, macrolides, rplV gene
Manuscript category	Bacteria

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The Veterinary Microbiology Editorial Team

MS: VETMIC_2017_1083

Title: *23S rRNA* and L22 ribosomal protein are involved in the acquisition of macrolide and lincosamide resistance in *Mycoplasma capricolum* subsp. *capricolum*

Dear Editor,

We send the revised version of our manuscript **VETMIC_2017_1083.** We have followed the comments of the reviewers and hereby we enclose the point-by-point response to their concerns (prefaced by "AU"). We are very grateful for the reviewer's help and suggestions, as they have allowed improving the manuscript.

Best regards

Christian de la Fe

RESPONSE TO REVIEWERS

MS: VETMIC_2017_1083

Reviewer 1

1) Two recently published significant papers are not cited in the manuscript. I suggest the citation of these publications and discussion of your results in relation of these papers:

- Sulyok et al: Mutations Associated with Decreased Susceptibility to Seven Antimicrobial Families in Field and Laboratory-Derived Mycoplasma bovis Strains, Antimicrobial Agents and Chemotherapy 2017

- Sulyok et al: Development of molecular methods for the rapid detection of antibiotic susceptibility of Mycoplasma bovis, Veterinary Microbiology, 2018

AU: We agree. These references have been added in text and in the reference list (L266-274), together with the discussion of our results in relation with them (L41-42; 157-161; 167).

2) In line 88: Please describe what is the PH medium.

AU: This medium is based on PPLO broth. The sentence has been changed in order to clarify this concept (L88-89).

3) In line 93: I suggest to use the term „initial MIC" as well.

AU: We agree that with this term the sentence is clearer. Thus, it has been added (L94).

4) Table 2: Please correct „seg" to „sec".

AU: This mistake in Table 2 has been corrected.

Reviewer 2

1. The Discussion too narrowly focused on molecular aspects. The authors should discuss their findings in a wider context.

a) What are the implications in the field?

b) What recommendations on treatment of mycoplasma infections in small ruminants can be given to vets?

c) Although this is not an epidemiological study, what is the authors' perception of the general or regional dissemination/prevalence of resistant strains in Spanish flocks?

AU: We understand that although molecular aspects were the main aim of this work, the points remarked by the reviewer are also important. Therefore, we have addressed them in a new paragraph (L184-193).

2. The Results section requires some introductory remarks on aim(s) and subject(s) studied. The strains examined should be mentioned, and some of the info given in paragraph 2.1 can be transferred to this section.

AU: Introductory phrases including aims and subjects of this study have been added in the Results section. However, paragraph 2.1 stays as before as we think that all the information given is relevant for the Material & Methods section (L110-112; 120-122).

3. In Mat & Meths, a paragraph on DNA sequencing and analysis is missing. These points should be explained:

AU: A new paragraph at the M&M section has been added in which all these points are answered (L100-107):

a) Was the sequencing done in the authors' lab or by a company?

AU: The sequencing was done at the molecular biology service of the University of Murcia.

b) Have any sequences determined in this study been submitted to NCBI or ENA?

AU: Relevant sequences of field isolates and in vitro selected mutants of CK and Cap24 were submitted to NCBI. Accession numbers are provided in the new Supplementary table S1.

c) It should be mentioned which sequence(s) were used as reference (i.e. non-resistant strain).

AU: As now is described in the manuscript, numbering is based on E. coli K-12 positions and sequences of the type strain of Mcc (CK) were used as a reference of non-resistant strain.

d) Which software was used to handle the sequence alignments?

AU: MEGA 6.0 (Tamura et al., 2013).

e) For proper documentation, the sequence alignments revealing relevant mutations should be presented as supplemental material.

AU: We agree. Therefore, we have included the alignments of the hotspot mutation genomic regions in the new Supplementary table S1.

4. Language, typos:

a) The sentence in lines 38-45 is rather complex and too long. Please, rephrase.

AU: This sentence has been rephrased in order to make it more understandable (L38-41; 42-45).

b) Line 147: replace "strain" with "strains"

AU: The word has been corrected (L166).

c) Line 163: replace "by" with "to

AU: This word has been changed (L181).

1 **Short communication**

2

3 ***23S rRNA*** **and L22 ribosomal protein are involved in the acquisition of macrolide and**
4 **lincosamide resistance in *Mycoplasma capricolum* subsp. *capricolum***

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16

17 **Abstract**

18 *Mycoplasma capricolum* subsp. *capricolum* (Mcc) is one of the causative agents of contagious
19 agalactia, and antimicrobial treatment is the most commonly applied measure to treat
20 outbreaks of this disease. Macrolides and lincosamides bind specifically to nucleotides at
21 domains II and V of the *23S rRNA*. Furthermore, *rplD* and *rplV* genes encode ribosomal
22 proteins L4 and L22, which are also implicated in the macrolide binding site. The aim of this
23 work was to study the relationship between mutations in these genes and the acquisition of
24 macrolide and lincosamide resistance in Mcc. For this purpose, *in vitro* selected resistant
25 mutants and field isolates were studied. This study demonstrates the appearance of DNA point
26 mutations at the *23S rRNA* encoding genes (A2058G, A2059G and A2062C) and *rplV* gene
27 (Ala89Asp) in association to high minimum inhibitory concentration values. Hence, it proves
28 the importance of alterations in *23S rRNA* domain V and ribosomal protein L22 as molecular
29 mechanisms responsible for the acquisition of macrolide and lincosamide resistance in both
30 field isolates and *in vitro* selected mutants. Furthermore, these mutations enable us to provide
31 an interpretative breakpoint of antimicrobial resistance for Mcc at MIC 0.8 µg/ml.

32

33 **Keywords:** contagious agalactia, antimicrobial resistance, macrolides, *rplV* gene

34 ## 1. Introduction

35 Antimicrobials, especially macrolides and lincosamides, are one of the most commonly used
36 treatments against mycoplasmoses. Both antimicrobials share the same mechanism of action,
37 as they obstruct protein synthesis by binding specifically to nucleotides of the *23S rRNA*,
38 interacting with domains II (*hairpin 35*) and V at the 50S ribosomal subunit. Moreover, L4
39 and L22 proteins, which are encoded by *rplD* and *rplV* genes, respectively, are also implicated
40 in the ribosomal macrolide binding site (Waites et al., 2014). Previous reports on different
41 mycoplasma species such as *M. gallisepticum*, *M. synoviae* and *M. bovis* have demonstrated
42 the effect of point mutations in the *23S rRNA* encoding genes on the acquisition of macrolide
43 and lincosamide resistance (Gerchman et al., 2011; Lysnyansky et al., 2015; Sulyok et al.,
44 2017). On the other hand, variations in ribosomal proteins appeared in combination with
45 alterations at the *23S rRNA* when isolates reached very high minimum inhibitory
46 concentration (MIC) values (Khalil et al., 2017; Lerner et al., 2014) and thus, have been
47 scarcely described (Pereyre et al., 2006; Prats-van der Ham et al., 2017).

48 *Mycoplasma capricolum* subsp. *capricolum* (Mcc) is one of the etiologic agents of contagious
49 agalactia (CA) and it is usually associated to severe outbreaks of this disease in goat herds
50 (De la Fe et al., 2007). Prior reports have demonstrated the inefficacy of 14-membered
51 macrolides against this mycoplasma species (Tatay-Dualde et al., 2017). Therefore, 16-
52 membered macrolides, such as tylosin, and lincosamides, are used against this pathogen
53 nowadays. However, recent studies on other CA-causing mycoplasma species have
54 demonstrated a decrease in macrolide susceptibility in current field isolates (Poumarat et al.,
55 2016; Prats-van der Ham et al., 2017). More specifically in Mcc, prior works have shown that
56 close to the 20% of the contemporary field strains are resistant to tylosin (Tatay-Dualde et al.,
57 2017). In this sense, the lack of resistance breakpoints complicates the interpretation of *in*
58 *vitro* antimicrobial susceptibility tests. Therefore, some authors have proposed the use of

59 point mutations in the *23S rRNA* gene to establish molecular breakpoints for the minimal
60 inhibitory concentrations (MIC) values in other mycoplasma species (Gerchman et al., 2011).
61 However, there are no previous studies addressing molecular resistance mechanisms of
62 macrolide and lincosamide resistance in Mcc. Hence, the aim of this work was to analyse the
63 partial sequences of the *23S rRNA*, L4 and L22 encoding genes in order to study their
64 relationship with the acquisition of *in vitro* resistance to macrolides and lincosamides and
65 their connection with different MIC values of Mcc field isolates, determining which
66 molecular mechanisms are involved in the macrolide and lincosamide resistance of Mcc.

67

68 **2. Material and methods**

69 *2.1. Mycoplasma isolates*

70 Resistant mutants of the reference strain California Kid (CK, NCTC 10154) and a field isolate
71 of Mcc (Cap24) were selected *in vitro*. Additionally, 14 field isolates with different MIC
72 values for macrolides (tylosin and tilmicosin) and licosamides (clindamycin and lincomycin)
73 were also studied. Isolates were mainly retrieved from mastitic milk samples of different
74 farms from the Canary Islands, although some were isolated from auricular swabs (n=3) and
75 from farms of Murcia (n=2) and Andalusia (n=2)**.**

76 *2.2. Selection of resistant mutants*

77 The *in vitro* selection of resistant mutants was performed by 20 serial dilution passages at
78 subinhibitory concentrations of tylosin and tilmicosin, following a previously described
79 protocol (Antunes et al., 2015). Briefly, an initial minimum inhibitory concentration (MIC)
80 test was performed and the highest concentration at which the strain grew was subsequently
81 cultured at the same antimicrobial concentration (step 1). Afterwards, another MIC analysis

82 was performed and, in the same way, the highest antimicrobial concentration showing growth
83 was picked to be cultured in this following concentration (steps 2 to 20). When a decrease in
84 antimicrobial susceptibility between steps was observed, a MIC analysis was performed with
85 tylosin, tilmicosin, clindamycin and lincomycin. Besides, the partial sequences of *23S rRNA,*
86 *rplD* and *rplV* genes were then studied. This process is detailed in **Table 1**.

87 *2.3. Minimum inhibitory concentration tests*

88 The inhibitory effect of the studied antimicrobials was evaluated by the minimum inhibitory
89 concentration (MIC) technique, as previously described (Hannan, 2000). Microtitre plates
90 were used to perform this method. 150 µl of PPLO broth supplemented with 18% (v/v) heat-
91 inactivated horse serum, 1% (v/v) of 50% fresh yeast extract and 0.4% (w/v) DNA, with
92 0.007% of phenol red, 25.6 µl of each antimicrobial dilution and the inocula at a
93 concentration of 10^3 – 10^5 CFU/ml were added to each well. Moreover, two wells were used
94 as positive (without antimicrobial) and negative (without neither antimicrobial nor inocula)
95 controls. Plates were incubated at 37 ºC and they were read when the positive control showed
96 a change of colour due to acidification of the medium. Initial MICs of tylosin, tilmicosin,
97 clindamycin and lincomycin were assessed for field isolates and between each step of the *in*
98 *vitro* selection of resistant mutants study.

99 *2.4. Molecular analysis*

100 Novel PCR protocols were designed using PRIMER3 software (Koressaar and Remm, 2007)
101 in order to analyse partial sequences of *23S rRNA* (domains II and V), *rplD* and *rplV* genes.
102 PCR conditions and sequencing primers are shown in **Table 2**. PCR products were sequenced
103 at the molecular biology service of the University of Murcia. The obtained sequences were
104 compared to those of the Mcc type strain CK (NC_000913.3), which was used as a non-
105 resistant reference. Sequence analyses were conducted using MEGA6 (Tamura et al., 2013)

106 and the numbering of nucleotide or amino-acid positions is based on the *23S rRNA* encoding
107 genes or L4/L22 proteins of *Escherichia coli* K-12 substrain MG1655 (NC_000913.3).
108 **Supplementary table S1** shows the resulting DNA alignments and the accession numbers of
109 the sequences that have been submitted to NCBI.

110

111 **3. Results**

112 Two susceptible Mcc strains (CK and Cap24) were selected by serial passages at
113 subinhibitory concentrations of tylosin and tilmicosin in order to assess which DNA
114 alterations are related to the acquisition of macrolides resistance. **Table 1** summarizes MICs
115 and sequencing results of the obtained *in vitro* selected mutants**.** DNA changes were found in
116 domain V of the *23S rRNA* encoding genes (A2058G) of both mutant populations. This
117 transversion appeared always in both alleles from macrolide MIC values of 0.8 – 1.6 μg/ml
118 and lincosamide MIC values of 6.4 – 12.8 μg/ml. Furthermore, predicted amino acid changes
119 were observed in the L22 protein (Ala89Asp) from MIC values of 8 μg/ml and 16 μg/ml for
120 macrolides and lincosamides, respectively. No alterations were observed either in domain II
121 of the *23S rRNA* or in the predicted amino acid sequence of ribosomal protein L4.

122 Moreover, the *23S rRNA*, L4 and L22 encoding genes of 14 Mcc field isolates with
123 macrolides and lincosamides MIC values ranging from 0.025 to >128 μg/ml were also studied
124 so as to correlate decreases in their susceptibility with DNA mutations. **Table 3** synthesizes
125 their MICs and sequencing results. Mutations were observed in domain V of the *23S rRNA*
126 encoding genes but in different positions as in the *in vitro* study (A2059G and A2062G).
127 A2059G mutations appeared from macrolide MIC values of 0.8 μg/ml, but they did not affect
128 both alleles until MICs of 12.8 μg/ml were reached. On the other hand, predicted amino acid

129 changes in L22 protein (Ala89Asp) were detected in all field isolates with MIC values over
130 0.8 μg/ml for macrolides and 0.8 – 3.2 μg/ml for lincosamides.

131

132 **4. Discussion**

133 The analysis of partial sequences of the *23S rRNA*, L4 and L22 encoding genes of *in vitro*
134 selected mutants and field isolates of Mcc demonstrated the association between point
135 mutations and the acquisition of macrolide and lincosamide resistance in this mycoplasma
136 species.

137 As for *23S rRNA*, point mutations were detected in the *in vitro* as well as the field isolates
138 study, although they appeared at different positions. Mutation A2058G is one of the changes
139 most commonly associated to macrolide and lincosamide resistance in different mycoplasma
140 species (Gerchman et al., 2011; Lerner et al., 2014; Lysnyansky et al., 2015). Nonetheless,
141 differently from previous studies in which mutations in both alleles were associated with
142 higher MIC values (Lysnyansky and Ayling, 2016), our *in vitro* selected resistant strains
143 showed this DNA change in both *23S rRNA* alleles from the lowest MIC value at which
144 mutations started to appear (0.8 μg/ml). Thus, our *in vitro* study highlights the importance of
145 this mutation in the acquisition of macrolide resistance in Mcc from lower MIC values than in
146 other mycoplasma species (Lerner et al., 2014; Lysnyansky et al., 2015).

147 In addition, A2059G transition was observed in Mcc field isolates: it was detected in one
148 allele from MIC values of 0.8μg/ml for tylosin and tilmicosin, and in both *23S rRNA*
149 encoding alleles when MICs reached higher values (except for Cap22). This is in consistency
150 with previous studies on *M. bovis*, in which heterozygous mutations were related with
151 intermediate resistance whereas the highest MIC values were connected with mutations in

152 both alleles (Lysnyansky and Ayling, 2016). Moreover, Cap19 and Cap22, which were the
153 field isolates with the highest MIC values, also displayed a second mutation (A2062G), which
154 did not appear in the *in vitro* study. This combination of mutations A2059 and A2062 has
155 been previously described and related to antimicrobial treatment failures in *M. genitalium*
156 (Guschin et al., 2015). Thus, the outcomes of our *in vitro* selected mutants and field isolates
157 might explain treatment failures during outbreaks of CA caused by Mcc.

158 The present work demonstrated coherency between the *in vitro* assay and the analysis of Mcc
159 field isolates. Thus, our results demonstrate the acquisition of cross-resistance between
160 macrolides and lincosamides, as the MIC values for the studied antimicrobials increased
161 similarly in *in vitro* selected mutants and in the field isolates. This has been previously
162 reported in other mycoplasma species such as *M. agalactiae* and *M. bovis* (Prats-van der Ham
163 et al., 2017; Sulyok et al., 2017). Although *23S rRNA* mutations were found in different
164 positions, 2058, 2059 and 2062 are part of the peptidyltranferase loop of domain V of *23S*
165 *rRNA* where macrolides and lincosamides bind specifically (Waites et al., 2014). Therefore,
166 mutations of these positions should be considered as the same molecular resistance
167 mechanism. Moreover, the study of this genetic area could be interesting for a rapid detection
168 of resistant field strains, as has been described for other mycoplasma species including *M.*
169 *genitalium* and *M.bovis* (Gosse et al., 2016; Sulyok et al., 2018).

170 Regarding the study of ribosomal proteins, prior works on other mycoplasma species were not
171 able to correlate them with changes in antimicrobial susceptibility, as they appeared in
172 combination with *23S rRNA* mutations (Khalil et al., 2017; Lerner et al., 2014; Lysnyansky et
173 al., 2015) although in some cases, point mutations associated to an increase in MIC values
174 were reported in *M. pneumoniae* and *M. hominis* (Matsuoka et al., 2004; Pereyre et al., 2006),
175 and previous studies on *M. agalactiae*, which is also a CA-causing mycoplasma, remarked the
176 importance of ribosomal protein L22 in the acquisition of macrolide resistance, as mutations

177 encoding changes in the protein sequence were associated with decreased susceptibility values
178 (Prats-van der Ham et al. 2017). Interestingly, both our *in vitro* and field isolates studies
179 showed predicted amino acid changes in L22 and in the same position as previously reported
180 (Matsuoka et al., 2004; Pereyre et al., 2004; Prats-van der Ham et al., 2017). Specifically,
181 substitution Ala89Asp appeared in strains selected with tylosin from MIC values of 8 µg/ml.
182 Besides, our field isolates showed the same variation from MICs ≥ 0.8 µg/ml. Thus, the
183 change of a neutral amino acid (Ala) to a negatively charged one (Asp) could result in protein
184 conformational changes affecting antimicrobial binding, highlighting the importance of this
185 protein in the acquisition of antimicrobial resistance.

186 Although this is not an epidemiological study, previous works have shown that close to 20%
187 of the contemporary field strains of Mcc are tylosin resistant (Tatay-Dualde et al., 2017),
188 which can be explained by the acquisition of *23S rRNA* and/or L22 mutations. This decrease
189 in susceptibility has also been reported in other CA-causing mycoplasmas, namely *M.*
190 *agalactiae* (Poumarat et al., 2016; Prats-van der Ham et al., 2017). Therefore, the presence of
191 these strains in the field may lead to treatment failures when macrolides or lincosamides are
192 selected to treat CA outbreaks. In this sense, determining the antimicrobial susceptibility
193 profile of these pathogens in order to select the most convenient therapy would be advisable.
194 Notwithstanding, the lack of MIC breakpoints complicates the interpretation of these studies
195 and, therefore, the antimicrobial choice. Based on our results, molecular resistance
196 breakpoints of tylosin and tilmicosin could be fixed for Mcc at 0.8 µg/mL, as also suggested
197 previously for *M. agalactiae* (Prats-van der Ham et al 2017) and similarly to other
198 mycoplasma species such as *M. gallisepticum* and *M. synoviae* (Gerchman et al., 2011;
199 Lysnyansky et al. 2015).

200

201 **5. Conclusions**

202 Alterations in domain V of the *23S rRNA* and ribosomal protein L22 are responsible for the
203 acquisition of macrolide and lincosamide resistance in Mcc, and their study provides rapid
204 information about antimicrobial susceptibility in field isolates. Moreover, Mcc field isolates
205 with macrolide MIC values over 0.8 μg/ml should be considered as resistant to this
206 antimicrobial group.

207

208 **6. Conflict of interest statement**

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

211

212 **7. Acknowledgements**

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217

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284

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Highlights

- Report of molecular mechanisms involved in macrolide and lincosamide resistance in *M. capricolum* subsp. *capricolum*.
- *23S rRNA* and *rplV* genes provide useful information about antimicrobial resistance and explain treatment failures.
- Macrolide susceptibility breakpoint of *M. capricolum* subsp. *capricolum* is fixed at 0.8 µg/ml.

Short communication

23S rRNA and L22 ribosomal protein are involved in the acquisition of macrolide and lincosamide resistance in *Mycoplasma capricolum* subsp. *capricolum*

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Abstract

Mycoplasma capricolum subsp. *capricolum* (Mcc) is one of the causative agents of contagious agalactia, and antimicrobial treatment is the most commonly applied measure to treat outbreaks of this disease. Macrolides and lincosamides bind specifically to nucleotides at domains II and V of the *23S rRNA*. Furthermore, *rplD* and *rplV* genes encode ribosomal proteins L4 and L22, which are also implicated in the macrolide binding site. The aim of this work was to study the relationship between mutations in these genes and the acquisition of macrolide and lincosamide resistance in Mcc. For this purpose, *in vitro* selected resistant mutants and field isolates were studied. This study demonstrates the appearance of DNA point mutations at the *23S rRNA* encoding genes (A2058G, A2059G and A2062C) and *rplV* gene (Ala89Asp) in association to high minimum inhibitory concentration values. Hence, it proves the importance of alterations in *23S rRNA* domain V and ribosomal protein L22 as molecular mechanisms responsible for the acquisition of macrolide and lincosamide resistance in both field isolates and *in vitro* selected mutants. Furthermore, these mutations enable us to provide an interpretative breakpoint of antimicrobial resistance for Mcc at MIC 0.8 µg/ml.

Keywords: contagious agalactia, antimicrobial resistance, macrolides, *rplV* gene

1. Introduction

Antimicrobials, especially macrolides and lincosamides, are one of the most commonly used treatments against mycoplasmoses. Both antimicrobials share the same mechanism of action, as they obstruct protein synthesis by binding specifically to nucleotides of the *23S rRNA*, interacting with domains II (*hairpin 35*) and V at the 50S ribosomal subunit. Moreover, L4 and L22 proteins, which are encoded by *rplD* and *rplV* genes, respectively, are also implicated in the ribosomal macrolide binding site (Waites et al., 2014). Previous reports on different mycoplasma species such as *M. gallisepticum*, *M. synoviae* and *M. bovis* have demonstrated the effect of point mutations in the *23S rRNA* encoding genes on the acquisition of macrolide and lincosamide resistance (Gerchman et al., 2011; Lysnyansky et al., 2015; Sulyok et al., 2017). On the other hand, variations in ribosomal proteins appeared in combination with alterations at the *23S rRNA* when isolates reached very high minimum inhibitory concentration (MIC) values (Khalil et al., 2017; Lerner et al., 2014) and thus, have been scarcely described (Pereyre et al., 2006; Prats-van der Ham et al., 2017).

Mycoplasma capricolum subsp. *capricolum* (Mcc) is one of the etiologic agents of contagious agalactia (CA) and it is usually associated to severe outbreaks of this disease in goat herds (De la Fe et al., 2007). Prior reports have demonstrated the inefficacy of 14-membered macrolides against this mycoplasma species (Tatay-Dualde et al., 2017). Therefore, 16-membered macrolides, such as tylosin, and lincosamides, are used against this pathogen nowadays. However, recent studies on other CA-causing mycoplasma species have demonstrated a decrease in macrolide susceptibility in current field isolates (Poumarat et al., 2016; Prats-van der Ham et al., 2017). More specifically in Mcc, prior works have shown that close to the 20% of the contemporary field strains are resistant to tylosin (Tatay-Dualde et al., 2017). In this sense, the lack of resistance breakpoints complicates the interpretation of *in vitro* antimicrobial susceptibility tests. Therefore, some authors have proposed the use of

point mutations in the *23S rRNA* gene to establish molecular breakpoints for the minimal inhibitory concentrations (MIC) values in other mycoplasma species (Gerchman et al., 2011). However, there are no previous studies addressing molecular resistance mechanisms of macrolide and lincosamide resistance in Mcc. Hence, the aim of this work was to analyse the partial sequences of the *23S rRNA*, L4 and L22 encoding genes in order to study their relationship with the acquisition of *in vitro* resistance to macrolides and lincosamides and their connection with different MIC values of Mcc field isolates, determining which molecular mechanisms are involved in the macrolide and lincosamide resistance of Mcc.

2. Material and methods

2.1. Mycoplasma isolates

Resistant mutants of the reference strain California Kid (CK, NCTC 10154) and a field isolate of Mcc (Cap24) were selected *in vitro*. Additionally, 14 field isolates with different MIC values for macrolides (tylosin and tilmicosin) and licosamides (clindamycin and lincomycin) were also studied. Isolates were mainly retrieved from mastitic milk samples of different farms from the Canary Islands, although some were isolated from auricular swabs (n=3) and from farms of Murcia (n=2) and Andalusia (n=2)**.**

2.2. Selection of resistant mutants

The *in vitro* selection of resistant mutants was performed by 20 serial dilution passages at subinhibitory concentrations of tylosin and tilmicosin, following a previously described protocol (Antunes et al., 2015). Briefly, an initial minimum inhibitory concentration (MIC) test was performed and the highest concentration at which the strain grew was subsequently cultured at the same antimicrobial concentration (step 1). Afterwards, another MIC analysis

was performed and, in the same way, the highest antimicrobial concentration showing growth was picked to be cultured in this following concentration (steps 2 to 20). When a decrease in antimicrobial susceptibility between steps was observed, a MIC analysis was performed with tylosin, tilmicosin, clindamycin and lincomycin. Besides, the partial sequences of *23S rRNA, rplD* and *rplV* genes were then studied. This process is detailed in **Table 1**.

2.3. Minimum inhibitory concentration tests

The inhibitory effect of the studied antimicrobials was evaluated by the minimum inhibitory concentration (MIC) technique, as previously described (Hannan, 2000). Microtitre plates were used to perform this method. 150 µl of PPLO broth supplemented with 18% (v/v) heat-inactivated horse serum, 1% (v/v) of 50% fresh yeast extract and 0.4% (w/v) DNA, with 0.007% of phenol red, 25.6 µl of each antimicrobial dilution and the inocula at a concentration of 10^3 – 10^5 CFU/ml were added to each well. Moreover, two wells were used as positive (without antimicrobial) and negative (without neither antimicrobial nor inocula) controls. Plates were incubated at 37 ºC and they were read when the positive control showed a change of colour due to acidification of the medium. Initial MICs of tylosin, tilmicosin, clindamycin and lincomycin were assessed for field isolates and between each step of the *in vitro* selection of resistant mutants study.

2.4. Molecular analysis

Novel PCR protocols were designed using PRIMER3 software (Koressaar and Remm, 2007) in order to analyse partial sequences of *23S rRNA* (domains II and V)*, rplD* and *rplV* genes. PCR conditions and sequencing primers are shown in **Table 2**. PCR products were sequenced at the molecular biology service of the University of Murcia. The obtained sequences were compared to those of the Mcc type strain CK (NC_000913.3), which was used as a non-resistant reference. Sequence analyses were conducted using MEGA6 (Tamura et al., 2013)

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299 106 and the numbering of nucleotide or amino-acid positions is based on the *23S rRNA* encoding
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301 107 genes or L4/L22 proteins of *Escherichia coli* K-12 substrain MG1655 (NC_000913.3).
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303 108 **Supplementary table S1** shows the resulting DNA alignments and the accession numbers of
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305 109 the sequences that have been submitted to NCBI.
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311 111 **3. Results**
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314 112 Two susceptible Mcc strains (CK and Cap24) were selected by serial passages at
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316 113 subinhibitory concentrations of tylosin and tilmicosin in order to assess which DNA
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318 114 alterations are related to the acquisition of macrolides resistance. **Table 1** summarizes MICs
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320 115 and sequencing results of the obtained *in vitro* selected mutants**.** DNA changes were found in
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322 116 domain V of the *23S rRNA* encoding genes (A2058G) of both mutant populations. This
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324 117 transversion appeared always in both alleles from macrolide MIC values of 0.8 – 1.6 µg/ml
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326 118 and lincosamide MIC values of 6.4 – 12.8 µg/ml. Furthermore, predicted amino acid changes
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328 119 were observed in the L22 protein (Ala89Asp) from MIC values of 8 µg/ml and 16 µg/ml for
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330 120 macrolides and lincosamides, respectively. No alterations were observed either in domain II
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332 121 of the *23S rRNA* or in the predicted amino acid sequence of ribosomal protein L4.
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336 122 Moreover, the *23S rRNA*, L4 and L22 encoding genes of 14 Mcc field isolates with
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338 123 macrolides and lincosamides MIC values ranging from 0.025 to >128 µg/ml were also studied
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340 124 so as to correlate decreases in their susceptibility with DNA mutations. **Table 3** synthesizes
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342 125 their MICs and sequencing results. Mutations were observed in domain V of the *23S rRNA*
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344 126 encoding genes but in different positions as in the *in vitro* study (A2059G and A2062G).
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346 127 A2059G mutations appeared from macrolide MIC values of 0.8 µg/ml, but they did not affect
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348 128 both alleles until MICs of 12.8 µg/ml were reached. On the other hand, predicted amino acid
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changes in L22 protein (Ala89Asp) were detected in all field isolates with MIC values over 0.8 µg/ml for macrolides and 0.8 – 3.2 µg/ml for lincosamides.

4. Discussion

The analysis of partial sequences of the *23S rRNA*, L4 and L22 encoding genes of *in vitro* selected mutants and field isolates of Mcc demonstrated the association between point mutations and the acquisition of macrolide and lincosamide resistance in this mycoplasma species.

As for *23S rRNA*, point mutations were detected in the *in vitro* as well as the field isolates study, although they appeared at different positions. Mutation A2058G is one of the changes most commonly associated to macrolide and lincosamide resistance in different mycoplasma species (Gerchman et al., 2011; Lerner et al., 2014; Lysnyansky et al., 2015). Nonetheless, differently from previous studies in which mutations in both alleles were associated with higher MIC values (Lysnyansky and Ayling, 2016), our *in vitro* selected resistant strains showed this DNA change in both *23S rRNA* alleles from the lowest MIC value at which mutations started to appear (0.8 µg/ml). Thus, our *in vitro* study highlights the importance of this mutation in the acquisition of macrolide resistance in Mcc from lower MIC values than in other mycoplasma species (Lerner et al., 2014; Lysnyansky et al., 2015).

In addition, A2059G transition was observed in Mcc field isolates: it was detected in one allele from MIC values of 0.8µg/ml for tylosin and tilmicosin, and in both *23S rRNA* encoding alleles when MICs reached higher values (except for Cap22). This is in consistency with previous studies on *M. bovis*, in which heterozygous mutations were related with intermediate resistance whereas the highest MIC values were connected with mutations in

both alleles (Lysnyansky and Ayling, 2016). Moreover, Cap19 and Cap22, which were the field isolates with the highest MIC values, also displayed a second mutation (A2062G), which did not appear in the *in vitro* study. This combination of mutations A2059 and A2062 has been previously described and related to antimicrobial treatment failures in *M. genitalium* (Guschin et al., 2015). Thus, the outcomes of our *in vitro* selected mutants and field isolates might explain treatment failures during outbreaks of CA caused by Mcc.

The present work demonstrated coherency between the *in vitro* assay and the analysis of Mcc field isolates. Thus, our results demonstrate the acquisition of cross-resistance between macrolides and lincosamides, as the MIC values for the studied antimicrobials increased similarly in *in vitro* selected mutants and in the field isolates. This has been previously reported in other mycoplasma species such as *M. agalactiae* and *M. bovis* (Prats-van der Ham et al., 2017; Sulyok et al., 2017). Although *23S rRNA* mutations were found in different positions, 2058, 2059 and 2062 are part of the peptidyltranferase loop of domain V of *23S rRNA* where macrolides and lincosamides bind specifically (Waites et al., 2014). Therefore, mutations of these positions should be considered as the same molecular resistance mechanism. Moreover, the study of this genetic area could be interesting for a rapid detection of resistant field strains, as has been described for other mycoplasma species including *M. genitalium* and *M.bovis* (Gosse et al., 2016; Sulyok et al., 2018).

Regarding the study of ribosomal proteins, prior works on other mycoplasma species were not able to correlate them with changes in antimicrobial susceptibility, as they appeared in combination with *23S rRNA* mutations (Khalil et al., 2017; Lerner et al., 2014; Lysnyansky et al., 2015) although in some cases, point mutations associated to an increase in MIC values were reported in *M. pneumoniae* and *M. hominis* (Matsuoka et al., 2004; Pereyre et al., 2006), and previous studies on *M. agalactiae*, which is also a CA-causing mycoplasma, remarked the importance of ribosomal protein L22 in the acquisition of macrolide resistance, as mutations

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476 177 encoding changes in the protein sequence were associated with decreased susceptibility values
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478 178 (Prats-van der Ham et al. 2017). Interestingly, both our *in vitro* and field isolates studies
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480 179 showed predicted amino acid changes in L22 and in the same position as previously reported
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482 180 (Matsuoka et al., 2004; Pereyre et al., 2004; Prats-van der Ham et al., 2017). Specifically,
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484 181 substitution Ala89Asp appeared in strains selected with tylosin from MIC values of 8 µg/ml.
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486 182 Besides, our field isolates showed the same variation from MICs ≥ 0.8 µg/ml. Thus, the
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488 183 change of a neutral amino acid (Ala) to a negatively charged one (Asp) could result in protein
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490 184 conformational changes affecting antimicrobial binding, highlighting the importance of this
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492 185 protein in the acquisition of antimicrobial resistance.
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496 186 Although this is not an epidemiological study, previous works have shown that close to 20%
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500 188 which can be explained by the acquisition of *23S rRNA* and/or L22 mutations. This decrease
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502 189 in susceptibility has also been reported in other CA-causing mycoplasmas, namely *M.*
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504 190 *agalactiae* (Poumarat et al., 2016; Prats-van der Ham et al., 2017). Therefore, the presence of
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506 191 these strains in the field may lead to treatment failures when macrolides or lincosamides are
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508 192 selected to treat CA outbreaks. In this sense, determining the antimicrobial susceptibility
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510 193 profile of these pathogens in order to select the most convenient therapy would be advisable.
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512 194 Notwithstanding, the lack of MIC breakpoints complicates the interpretation of these studies
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514 195 and, therefore, the antimicrobial choice. Based on our results, molecular resistance
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516 196 breakpoints of tylosin and tilmicosin could be fixed for Mcc at 0.8 µg/mL, as also suggested
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518 197 previously for *M. agalactiae* (Prats-van der Ham et al 2017) and similarly to other
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520 198 mycoplasma species such as *M. gallisepticum* and *M. synoviae* (Gerchman et al., 2011;
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5. Conclusions

Alterations in domain V of the *23S rRNA* and ribosomal protein L22 are responsible for the acquisition of macrolide and lincosamide resistance in Mcc, and their study provides rapid information about antimicrobial susceptibility in field isolates. Moreover, Mcc field isolates with macrolide MIC values over 0.8 µg/ml should be considered as resistant to this antimicrobial group.

6. 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.

7. Acknowledgements

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Table 1. Minimum inhibitory concentrations (MIC) and mutations in the *23S rRNA* gene and L22 protein resulting from *in vitro* selection with tylosin and tilmicosin

Strain-Ab-Passage	MIC (µg/ml)				Mutations[a]		
	Tyl[b]	Tlm[c]	Cli[d]	Lin[e]	*23S rRNA* A1 (nt)	*23S rRNA* A2 (nt)	L22 (aa)
CK (NCTC 10154)	0.01	0.01	0.01	0.2	A2058	A2058	Ala89
Tylosin							
CK-Tyl-6	0.2	0.2	0.1	0.4	-	-	-
CK-Tyl-9	1.6	1.6	12.8	12.8	A2058G	A2058G	-
CK-Tyl-10	8	8	16	16	A2058G	A2058G	Asp
CK-Tyl-11	16	32	32	32	A2058G	A2058G	Asp
CK-Tyl-20	32	64	32	32	A2058G	A2058G	Asp
Tilmicosin							
CK-Tlm-5	0.4	0.2	0.05	0.4	-	-	-
CK-Tlm-7	0.8	0.8	6.4	6.4	A2058G	A2058G	-
CK-Tlm-9	12.8	6.4	32	32	A2058G	A2058G	-
CK-Tlm-10	16	8	32	32	A2058G	A2058G	-
CK-Tlm-20	32	32	64	64	A2058G	A2058G	-
Cap24	0.1	0.025	0.1	0.4	A2058	A2058	Ala89
Tylosin							
Cap24-Tyl-6	0.2	0.2	0.1	0.4	-	-	-
Cap24-Tyl-9	1.6	1.6	12.8	12.8	A2058G	A2058G	-
Cap24-Tyl-10	8	8	16	16	A2058G	A2058G	Asp
Cap24-Tyl-11	16	32	32	32	A2058G	A2058G	Asp
Cap24-Tyl-20	32	64	32	32	A2058G	A2058G	Asp
Tilmicosin							
Cap24-Tlm-5	0.4	0.2	0.1	0.4	-	-	-
Cap24-Tlm-7	0.8	0.8	6.4	6.4	A2058G	A2058G	-
Cap24-Tlm-9	12.8	6.4	32	32	A2058G	A2058G	-
Cap24-Tlm-10	16	8	32	32	A2058G	A2058G	-
Cap24-Tlm-20	32	32	64	64	A2058G	A2058G	-

a: *E. coli* numbering positions

b: Tylosin

c: Tilmicosin

d: Clindamycin

e: Lincomycin

Table 2. Oligonucleotide sequences and PCR conditions applied for amplification and sequencing.

Target	Name	Primers 5'-3'	Protocol	PCR product size (bp)	Sequencing primers 5'-3'	Sequence size (bp)
23S rRNA allele 1 domain II	23S A1DII-F	TGCAAGCTGGTTTAGCATTG	93ºC 5 min. (93ºC 45 sec, 58.3ºC, 45 sec, 72ºC 2 min) x 30, 72 ºC 10 min	1850	GTACCGTGAGGGAAAGGTGA	839
	23S A1&2DII-R	GTCAAACGGCATGGAAGATT			GTCAAACGGCATGGAAGATT	
23S rRNA allele 1 domain V	23S A1DV-F	TCTGCTAAGTCGCAAGACGA	93ºC 5 min. (93ºC 45 sec, 57.5ºC, 45 sec, 72ºC 2min30 sec) x 30, 72 ºC 10 min	3150	TCTGCTAAGTCGCAAGACGA	882
	23S A1DV-R	TGCATTCACTTTCTCCTTTCTTT			CATCCATTCCGGTCCTCTC	
23S rRNA allele 2 domain II	23S A2DII-F	CGGTAGAGCAACTGGCTTTT	93ºC 5 min. (93ºC 45 sec, 58.3ºC, 45 sec, 72ºC 2 min) x 30, 72 ºC 10 min	1694	GTACCGTGAGGGAAAGGTGA	839
	23S A1&2DII-R	GTCAAACGGCATGGAAGATT			GTCAAACGGCATGGAAGATT	
23S rRNA allele 2 domain V	23S A2DV-F	TCTGCTAAGTCGCAAGACGA	93ºC 5 min. (93ºC 45 sec, 58.3ºC, 45 sec, 72ºC 2min30sec) x 30, 72 ºC 10 min	3100	TCTGCTAAGTCGCAAGACGA	882
	23S A2DV-R	TGTTCTAGCGGTTATTGGGATT			CATCCATTCCGGTCCTCTC	
rplD	rplD-F	CCCGTGCTGAAGTATCTGGA	93ºC 5min. (93ºC 30 sec, 57.6ºC 45 sec, 72ºC 30 sec) x 30, 72ºC 10 min	469	Same as PCR	-
	rplD-R	TGCGTATACCTCCTCAACTGC			Same as PCR	
rplV	rplV-F	TGGTGATACTTTTTGTCCCATTT	93ºC 5min. (93ºC 30 sec, 57.6ºC 45 sec, 72ºC 30 sec) x 30, 72ºC 10 min	437	Same as PCR	-
	rplV-R	AATTCGGTGGTCATGGTGAT			Same as PCR	

Table 3. Minimum inhibitory concentration (MIC) and *23S rRNA* and L22 changes in the studied *M. capricolum* subsp. *capricolum* field isolates.

Strain	MIC (µg/ml)				Mutations[a]			
	Tyl[b]	Tlm[c]	Cli[d]	Lin[e]	*23S rRNA* A1 (nt)		*23S rRNA* A2 (nt)	L22 (aa)
CK	0.01	0.01	0.01	0.2	A2059	A2062	A2059	Ala89
Cap1	0.05	0.025	0.1	0.8	-		-	-
Cap8	0.05	0.025	0.1	0.4	-		-	-
Cap 3	0.05	0.025	0.1	0.2	-	-	-	-
Cap4	0.05	0.025	0.1	0.8	-	-	-	-
Cap21	0.1	0.025	0.1	0.4	-	-	-	-
Cap24	0.1	0.025	0.1	0.4	-	-	-	-
Cap20	0.1	0.05	0.2	0.8	-	-	-	-
Cap23	0.8	0.8	12.8	12.8	A2059G	-	-	Asp
Cap17	1.6	0.4	0.8	3.2	-	-	-	Asp
Cap18	3.2	0.8	0.8	3.2	A2059G	-	-	Asp
Cap16	12.8	12.8	12.8	12.8	A2059G	-	A2059G	Asp
Cap25	16	16	16	12.8	A2059G	-	A2059G	Asp
Cap19	16	>128	12.8	12.8	A2059G	A2062C	A2059G	Asp
Cap22	64	128	1.6	6.4	A2059G	A2062C	-	Asp

a: *E. coli* numbering positions
b: Tylosin
c: Tilmicosin
d: Clindamycin
e: Lincomycin