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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 therapy 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. Moreover, these mutations enable us to provide an interpretative breakpoint of antimicrobial resistance for Mcc at MIC 0.8 μg/ml.

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 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

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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 2–20). 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 °C 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) and the numbering of nucleotide or amino-acid positions is based on the *23S rRNA* encoding genes or L4/L22 proteins of *Escherichia coli* K-12 substrain MG1655 (NC_000913.3). **Supplementary table S1** shows the resulting DNA alignments and the accession numbers of the sequences that have been submitted to NCBI.

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	Ala89Asp
CK-Tyl-11	16	32	32	32	A2058G	A2058G	Ala89Asp
CK-Tyl-20	32	64	32	32	A2058G	A2058G	Ala89Asp
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	Ala89Asp
Cap24-Tyl-11	16	32	32	32	A2058G	A2058G	Ala89Asp
Cap24-Tyl-20	32	64	32	32	A2058G	A2058G	Ala89Asp
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.

3. Results

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

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