Short communication: In vitro antimicrobial susceptibility of *Mycoplasma agalactiae* strains isolated from dairy goats

A. Paterna, A. Sánchez,¹ A. Gómez-Martín, J. C. Corrales, C. De la Fe, A. Contreras, and J. Amores

Research Group on Ruminant Health, Animal Health Department, School of Veterinary Sciences, Regional Campus of International Excellence "Campus Mare Nostrum," University of Murcia, 30071 Murcia, Spain

ABSTRACT

This study examined the susceptibility to several antimicrobials of 28 isolates of *Mycoplasma agalactiae* obtained from goats in a region (southeastern Spain) where contagious agalactia is endemic. For each isolate, the minimum inhibitory concentration (MIC) against 12 antimicrobials of the quinolone, macrolide, aminoglycoside, and tetracycline families was determined. The antimicrobials with the lowest MIC were enrofloxacin, ciprofloxacin, tylosin, and doxycycline, all with MIC₉₀ (concentration at which growth of 90% of the isolates is inhibited) <1 µg/mL. Norfloxacin (a quinolone) showed a wide MIC range (0.1–12.8 µg/mL), suggesting a resistance mechanism toward this antimicrobial that was not elicited by enrofloxacin or ciprofloxacin (the other quinolones tested). Erythromycin showed the highest MIC₉₀ such that its use against *Mycoplasma agalactiae* is not recommended. Finally, *Mycoplasma agalactiae* isolates obtained from goat herds with clinical symptoms of contagious agalactia featured higher MIC₉₀ and MIC₅₀ (concentration at which growth of 50% of the isolates is inhibited) values for many of the antimicrobials compared with isolates from asymptomatic animals. The relationship between the extensive use of antimicrobials in herds with clinical contagious agalactia and variations in MIC requires further study.

Key words: *Mycoplasma agalactiae*, antimicrobial, minimum inhibitory concentration, contagious agalactia

Short Communication

Mycoplasma agalactiae is the main causative agent of contagious agalactia (CA), a syndrome that affects small ruminants and causes mastitis, arthritis, keratoconjunctivitis, pneumonia, and abortion. Contagious agalactia is distributed worldwide although it was traditionally reported to affect animals in southern Europe

(Bergonier et al., 1997). The most significant symptom in ruminants reared for dairy purposes is mammary gland infection, which may lead to the complete loss of milk production as well as loss of the mammary gland itself. The disease first presents as an explosive outbreak affecting most lactating females in a herd and compromising herd viability as a consequence of milk and animal losses. Moreover, in endemic areas, the infection tends to persist in a chronic form and it is possible to isolate *Mycoplasma agalactiae* from herds with or without recent history of CA; for example, from auricular carriers or bulk tank milk samples (BTM) from asymptomatic herds (Mercier et al., 2007; Contreras et al., 2008). In goat herds, a single clone of *Mycoplasma mycoides* ssp. *capri* has been described during a clinical outbreak of CA, and different strains of *M. mycoides* ssp. *capri* have been isolated from asymptomatic auricular carriers and BTM of herds in the absence of a recent mycoplasmosis episode (Tardy et al., 2007). In a recent study, *M. agalactiae* was isolated from clinical mastitis specimens and BTM in herds without clinical signs of CA in an endemic area (Amores et al., 2012). Further, the presence of different strains of *M. agalactiae* of different epidemiological origins has been reported in dairy goat farms in an endemic CA area (De la Fe et al., 2012). Besides losses in milk production, classic CA outbreaks or chronic infection in a herd generates serious economic losses arising from the management procedures that need to be implemented to control the disease.

The control of CA is complex and mainly based on antimicrobial treatment and preventive handling procedures. Because mycoplasmas lack a cell wall, they are not susceptible to the β-lactam class of antimicrobials. In addition, the chronic nature of CA infection in endemic areas has given rise to uncontrolled antimicrobial treatment, which could lead to the appearance of resistant strains (Ayling et al., 2000). The antimicrobial drug families known to be effective against mycoplasmas are macrolides, fluoroquinolones, tetracyclines, and aminoglycosides (Hirsh, 2000), macrolides and quinolones being widely used in endemic areas. However, some resistance to macrolides and fluoroquinolones has been observed and attributed to several gene mutations

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¹Corresponding author: asanlope@um.es

(Furneri et al., 2001; Lysnyansky et al., 2009). The diversity of genetic populations of *M. agalactiae* isolates from goats detected in endemic areas and the wide use of antimicrobials could lead to inefficient control therapy.

Considering the different clinical and epidemiological aspects of endemic CA, this study was designed to determine MIC of 12 antimicrobials against 28 *M. agalactiae* isolates. The isolates used in this study form part of our laboratory culture collection and were grouped according to the available information (clinical signs and anatomic origin). Samples were cultured in modified Hayflick broth, a medium specific for mycoplasmas, and characteristic mycoplasma colonies were visualized on agar. Single colonies were subcultured and the species identified by biochemical tests (tetrazolium reduction and film and spot production) and by PCR targeting the *polC* gene (Marenda et al., 2005). For the present purpose, isolates were selected according to epidemiological data. Eighteen of the isolates were obtained during 3 reported CA outbreaks from goats showing classic symptoms (mastitis, arthritis, or eye symptoms): 9 of these were selected from cultures of mastitic specimens, 2 from cultures of conjunctival swabs, 2 from cultures of swabs taken of the external auditory canal (**EAC**) in animals with mastitis, 2 from cultures of joint fluid, and 3 from cultures of the BTM of each herd. The remaining 10 *M. agalactiae* isolates were obtained from herds with no prior clinical CA outbreak: 6 from the BTM of 6 different herds, 3 from the EAC of 3 animals in 3 different herds, and 1 from a semen sample from a single buck. The isolates obtained from the EAC and semen were obtained after *M. agalactiae* had been detected in the BTM of the herds. A reference *M. agalactiae* strain (PG2, NCTC 10123) was also included.

The concentration range of the antimicrobials to be tested was determined by taking into account the active ingredient of each: streptomycin and erythromycin (1–128 µg/mL); doxycycline, ciprofloxacin, enrofloxacin, and tylosin (0.006–0.8 µg/mL); gentamicin, norfloxacin, spiramycin, kanamycin, spectinomycin, and neomycin (0.1–12.8 µg/mL). The MIC for each isolate and antimicrobial was determined according to the recommendations of Hannan (2000). In brief, a mycoplasma growth curve was prepared for each isolate. Cultures were grown in modified Hayflick medium supplemented with 1% phenol red in 96-well round-bottomed plates. The antimicrobials were added to achieve each of the pre-established final concentrations and a final concentration of the mycoplasma cultures of 10^5 to 10^3 color-changing units/mL. We also prepared positive controls lacking antibiotics and negative controls lacking mycoplasma isolates. The plates were then sealed

and incubated at 37°C until the positive control (antibiotic-free) changed color from red to yellow and the negative control (mycoplasma-free) remained red. The MIC was defined as the lowest concentration at which no bacterial growth (no color change) was observed. Depending on the isolate, the color change occurred after 24 to 72 h of incubation. For each group of isolates (from herds with clinical CA or asymptomatic herds), we determined the MIC range, MIC₅₀, and MIC₉₀ (the lowest concentration of antimicrobial at which growth of 50% or 90% of the isolates, respectively, is inhibited; Table 1).

Among the antimicrobials tested, enrofloxacin, ciprofloxacin, tylosin, and doxycycline showed the lowest MIC values, all with MIC₉₀ <1 µg/mL. Enrofloxacin had the lowest values (MIC₉₀ = 0.1 µg/mL) for both groups of isolates. In contrast, erythromycin had the highest MIC₉₀ for both groups, in agreement with previous reports (Antunes et al., 2008; de Garnica et al., 2013). High MIC of erythromycin has also been described for *Mycoplasma bovis* and *Mycoplasma hominis* (Furneri et al., 2001; Francoz et al., 2005). These mycoplasmas are closely related to *M. agalactiae* and also belong to the hominis group. In effect, *M. hominis* is naturally resistant to erythromycin because of a mutation in the central loop of 23S rRNA domain V. This mutation is also present in *Mycoplasma flocculare* and *Mycoplasma hyopneumoniae* (Furneri et al., 2001). These 2 mycoplasmas also belong to the hominis group, suggesting that the resistance to erythromycin observed in *M. agalactiae* could be natural resistance common to the entire hominis group. This should be considered when treating CA, because erythromycin is used to treat mammary infections but is inefficient against *M. agalactiae*. The following breakpoints have been described for the effectiveness of tylosin against *M. agalactiae*: ≤1 µg/mL susceptible, ≤2 µg/mL intermediate susceptible, and ≥4 µg/mL resistant (Hannan, 2000). In our study, no *M. agalactiae* isolate showed a MIC >4 µg/mL, indicating their susceptibility to this antimicrobial, in contrast to the results obtained for the other macrolides.

Most of the aminoglycosides examined showed high MIC₉₀, with streptomycin having the highest values (MIC₉₀ of 32 µg/mL). It has been reported that the presence of thymidine at position 912 (*Escherichia coli* numbering) of the 16S rRNA gene confers *E. coli* resistance to streptomycin (Cundliffe, 1990). This mutation is also present in mycoplasmas of the hominis group, in which *M. agalactiae* is included, and has been related to *M. agalactiae* resistance to streptomycin (Königsson et al., 2002).

Quinolones had lowest MIC values of antimicrobials against our *M. agalactiae* isolates, in accordance with

Table 1. Minimum inhibitory concentrations¹ (µg/mL) of antimicrobials recorded for *Mycoplasma agalactiae* isolates obtained from dairy goat herds with or without clinical signs of contagious agalactia (CA)

Antimicrobial agent	Isolates from herds with clinical signs of CA (n = 18)			Isolates from herds without clinical signs of CA (n = 10)			PG2
	MIC range	MIC ₅₀	MIC ₉₀	MIC range	MIC ₅₀	MIC ₉₀	MIC
Quinolones							
Norfloxacin	0.1–12.8	3.2	12.8	0.4–6.4	1.6	6.4	0.8
Ciprofloxacin	0.012–0.4	0.2	0.4	0.012–0.4	0.1	0.4	0.05
Enrofloxacin	0.025–0.8	0.05	0.1	0.012–0.8	0.1	0.1	0.012
Macrolides							
Spiramycin	0.1–12.8	1.6	12.8	0.2–12.8	0.8	12.8	0.2
Tylosin	0.05–0.8	0.4	0.8	0.025–0.8	0.4	0.8	0.05
Erythromycin	16–128	64	128	32–128	64	128	16
Aminoglycosides							
Neomycin	0.2–12.8	6.4	12.8	3.2–12.8	6.4	12.8	0.8
Kanamycin	0.8–12.8	6.4	12.8	1.6–12.8	3.2	6.4	0.8
Spectinomycin	0.4–12.8	3.2	6.4	0.8–6.4	1.6	6.4	0.8
Gentamicin	0.8–6.4	1.6	6.4	0.2–3.2	0.8	3.2	0.2
Streptomycin	2–64	8	32	2–16	4	8	2
Tetracyclines							
Doxycycline	0.025–0.8	0.05	0.8	0.025–0.2	0.05	0.1	0.025

¹MIC₅₀ and MIC₉₀ = lowest concentration at which growth of 50% and 90% of the isolates, respectively, is inhibited; PG2 = reference strain of *Mycoplasma agalactiae* (NCTC 10123).

in vitro susceptibilities reported for isolates from sheep (de Garnica et al., 2013). Enrofloxacin and ciprofloxacin showed the lowest MIC₉₀ for both groups of isolates and the MIC range was consistently <1 µg/mL. Hannan (2000) proposed a MIC breakpoint for enrofloxacin of ≤0.5 µg/mL to define susceptible isolates, ≤1 µg/mL for intermediately resistant isolates, and ≥2 µg/mL for resistant isolates. According to these breakpoints, 21 of the present isolates were resistant and 4 were intermediately resistant to norfloxacin. However, no isolate was resistant to ciprofloxacin and only 2 isolates showed intermediate resistance to enrofloxacin. The 3 quinolones tested here inhibit DNA gyrase or topoisomerase IV, blocking cell division. Quinolone resistance can arise because of point mutations that lead to amino acid substitutions in the quinolone resistance-determining region of DNA gyrase subunits *gyrA* and *gyrB* or topoisomerase IV subunits *parC* and *parE* (Lysnyansky et al., 2009). The different susceptibilities to ciprofloxacin, enrofloxacin, and norfloxacin detected here are in contrast to the findings of a study in which enrofloxacin and difloxacin were observed to show similar MIC patterns and the same MIC₅₀ for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* (Gerchman et al., 2008). Those authors attributed the latter to cross resistance in this family of antimicrobials (Gerchman et al., 2008). However, in a more recent study, a discrepancy was noted between the MIC of enrofloxacin and quinolone resistance-determining region mutations in *M. gallisepticum* (Lysnyansky et al., 2012). Thus, the difference detected in the susceptibility of our isolates to norfloxacin compared with the other quinolones

along with this recent discrepancy suggest that determining resistance to quinolones in mycoplasmas should not be based solely on the genes *parC* and *gyrA*. Other quinolone resistance mechanisms have been described in other bacteria (Hawkey, 2003) and further work is needed to establish whether these mechanisms also play a role in mycoplasmas.

The isolates from herds with clinical signs of CA had higher MIC₉₀ and MIC₅₀ for norfloxacin, kanamycin, streptomycin, and gentamicin than isolates from herds without clinical signs of disease (Table 1). Spectinomycin and spiramycin also showed higher MIC₅₀ in isolates from herds with clinical CA signs, suggesting lower susceptibility of these isolates to some antimicrobials. This finding could be related to the intense use of antimicrobials in these herds, likely inducing mycoplasmal resistance (Ayling et al., 2000), and should be confirmed in further studies. Despite the fact that most of the antimicrobials analyzed are not licensed in Spain to treat mastitis in goats, the majority are, in fact, licensed for veterinary use. In the absence of a specific product for goats, veterinarians can use them to control caprine mastitis by the intramammary (erythromycin) or intramuscular route. Future studies should address whether the genetic diversity seen in the geographical area of study (De la Fe et al., 2012) is related to antimicrobial susceptibility.

Our findings indicate the natural resistance of *M. agalactiae* to erythromycin and streptomycin, such that these antimicrobials are not recommended for treatment of *M. agalactiae* infections. Quinolones had the lowest MIC values against *M. agalactiae*, although

differences in MIC₅₀ and MIC₉₀ observed among the antimicrobials of this family suggest a different resistance mechanism in *M. agalactiae* than is reported for other mycoplasmas. Finally, *M. agalactiae* isolates from herds with clinical signs of CA showed higher MIC compared with isolates from herds with no symptoms. The relationship between MIC values and use of antimicrobials in an endemic area requires further study.

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