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In Vitro Susceptibilities of *Mycoplasma putrefaciens* Field Isolates[▽]

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MICs were determined for 15 antimicrobial agents against 37 *Mycoplasma putrefaciens* isolates. The most effective antimicrobial drug classes were the fluoroquinolones, the tetracyclines, the lincosamide lincomycin, and the macrolides. The susceptibility profile of the isolates correlated with the geographic origin. This is the first report of decreased susceptibility to the macrolides, lincomycin, and the tetracyclines in *M. putrefaciens* strains.

Mycoplasma putrefaciens is one of the etiologic agents of contagious agalactia (CA), a disease recognized by the World Organization for Animal Health (13). This syndrome affects the mammary glands, joints, and eyes and occasionally causes respiratory disease in sheep and goats. CA is a global disease and occurs in most countries in the Mediterranean basin (5).

M. putrefaciens is widespread in western France (12) and has been the mycoplasma species isolated most often in small ruminants in Jordan (3). In Spain, it was found to be associated with an outbreak of polyarthritis in kids (16), and in the United States, this organism was responsible for a serious outbreak of mastitis that required the destruction of nearly 700 goats (7). An important characteristic of this organism is its ability to induce mastitis when as few as 50 mycoplasma cells are inoculated into lactating goats by intramammary means (1). Although the treatment of mycoplasma infections is discouraged as it may induce a chronic carrier state, the lack of a commercial vaccine against *M. putrefaciens* encourages attempts to control the disease by chemotherapy (5).

This study evaluated the antimicrobial susceptibilities of *M. putrefaciens* isolates from two countries where they are endemic. Nineteen *M. putrefaciens* isolates were from clinical samples and bulk milk collected during routine screening of goats in France, and eighteen isolates were collected from a study of sheep and goats in Jordan (3). The reference strain KS1 was obtained from the National Collection of Type Cultures (NCTC). The Jordanian isolates had been previously tested against five of the antimicrobials included in this study (2).

Each of the antimicrobial agents was obtained as a pure substance, with the exception of tylosin, which was in a saline solution. The antimicrobials tested were nalidixic acid, norfloxacin, ciprofloxacin, enrofloxacin, doxycycline, spiramycin, spectinomycin, and lincomycin, all obtained from Sigma (MO); the agents chloramphenicol, oxytetracycline, chlortetracycline, erythromycin, streptomycin, gentamicin, and tylosin were obtained from Serva (Heidelberg, Germany). Although chloramphenicol and nalidixic acid are no longer used in veterinary clinical practice in Spain, they are useful for phenotypic characterization of possible resistance mechanisms, since they have efflux systems and mutation sites that are similar to those of the tetracyclines and fluoroquinolones (6).

MICs of these 15 antimicrobials were determined, taking into account the active ingredient of each antimicrobial agent, against the *M. putrefaciens* field isolates and the reference strain KS1. The MIC tests were carried out in standard 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark). Serial twofold dilutions were made in PH medium supplemented with 1% mannose and 0.2% phenol red to a final volume of 100 μl, as described previously (9). They were inoculated with 100 μl of *M. putrefaciens* culture grown aerobically for 24 h at 37°C in modified PH medium which had been diluted to give a final concentration of 2×10^3 to 2×10^5 color-changing units (CCU)/ml. The antimicrobial concentrations tested ranged from 512 to 4 μg/ml for nalidixic acid; 128 to 1 μg/ml for gentamicin, spectinomycin, and streptomycin; 4 to 0.03 μg/ml for fluoroquinolones, oxytetracycline, chlortetracycline, gentamicin, spectinomycin, chloramphenicol, and lincomycin; and 1 to 0.008 μg/ml for tylosin, spiramycin, erythromycin, and doxycycline. A control with no antimicrobial agent was also included. The plates were sealed and incubated aerobically at 37°C. The MIC was defined as the lowest concentration in which there was no bacterial growth, as evidenced by a lack of pH color change at the time the drug-free growth control showed a color change. This change of color was evident after 24 h of incubation. Each strain was tested in dupli-

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TABLE 1. MIC_{50}s, MIC_{90}s, and MIC ranges of the antimicrobial agents

Antimicrobial agent[a]	MIC (μg/ml) against *M. putrefaciens*						MIC (μg/ml) against reference strain KS1
	French isolates (n = 19)			Jordanian isolates (n = 18)			
	50%	90%	Range	50%	90%	Range	
NAL	>256	>256	>256–>256	>256	>256	>256–>256	512
NOR	2	4	0.5–4	2	2	2–2	2
CIP	0.250	0.5	0.06–0.5	0.250	0.250	0.125–0.250	0.250
ENR	0.125	0.250	0.125–0.250	0.125	0.125	0.06–0.125	0.125
CHL	4	4	2–4	2	4	2–4	4
CTE	8	16	2–16	4	4	4–4	4
OXY	1	4	0.250–8	0.5	0.5	0.5–1	0.5
DOX	0.250	2	0.125–2	0.125	0.250	0.125–0.250	0.03
ERY	0.03	0.125	0.008–2	0.03	0.03	0.03–0.03	0.03
TYL	0.03	0.06	0.015–2	0.015	0.015	0.015–0.015	0.06
SPI	0.250	0.5	0.06–2	0.125	0.250	0.125–0.250	0.250
GEN	64	64	16–64	32	32	32–64	64
STR	32	64	8–64	32	32	4–32	64
SPT	16	16	8–32	16	32	16–32	32
LIN	1	2	0.250–16	0.5	1	0.5–1	1

[a] NAL, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin; ENR, enrofloxacin; CHL, chloramphenicol; CTE, chlortetracycline; OXY, oxytetracycline; DOX, doxycycline; ERY, erythromycin; TYL, tylosin; SPI, spiramycin; GEN, gentamicin; STR, streptomycin; SPT, spectinomycin; LIN, lincomycin.

cate, at least twice for each antimicrobial on different days, with freshly prepared antimicrobial solutions for every test.

The MIC_{50}s and MIC_{90}s and MIC ranges obtained for the antimicrobials tested with the field strains and the reference strain are provided in Table 1. The duplicates tested all gave results within one dilution, and the highest MIC is given in Table 1.

Erythromycin and tylosin were the most effective agents against both the French and the Jordanian isolates, with MIC_{90} values of 0.06 μg/ml, followed by ciprofloxacin, spiramycin, and lincomycin, all with MIC_{90} values equal to or less than 1 μg/ml, which correlated with a previous study (2). One isolate had reduced susceptibility to the macrolides and lincomycin. Twelve isolates were less susceptible to tetracyclines than the others, with tetracycline MICs decreased by between two and four dilutions. One of these isolates was less susceptible to erythromycin than the others.

Tetracycline resistance has been previously described for other mycoplasma species, and it is known to occur naturally due to the presence of the streptococcal tetracycline *tetM* determinant in *M. hominis* (15). 16S rRNA mutations have been described in in vitro-selected mutant strains *of M. hominis* and *M. pneumoniae* (S. Dégrange, P. Gonzalez, A. Charron, H. Renaudin, C. Bébéar, and C. M. Bébéar, 16S rRNA mutations associated with tetracycline resistance in *Mycoplasma pneumoniae* and *Mycoplasma hominis*, presented at the 15th Congress of the International Organization for Mycoplasmology, Athens, GA, 2004). Although macrolides and lincosamides belong to different antimicrobial classes, they share the same antimicrobial mechanism, interacting with the 23S rRNA of the 50S ribosomal subunit and blocking protein synthesis (10). Simultaneous resistance to macrolides and lincosamides has been associated with 23S rRNA in several mycoplasma species (14, 17) but has never been described for *M. putrefaciens*. Resistance to 14-membered macrolides with susceptibility to 16-membered macrolides and lincomycin is known in other species, such as *M. hominis* (8). Although other mycoplasma species are known to be susceptible to aminoglycosides (9, 18), higher-than-expected MICs were seen for all isolates in this study. It has been previously suggested that mycoplasmas belonging to the *Spiroplasma* sp. cluster could have an intrinsic resistance mechanism to aminoglycosides (4). As for other mycoplasma species (4, 11), nalidixic acid had a limited antimicrobial effect on *M. putrefaciens*, which gave the highest MIC_{90} value.

With the exception of their susceptibilities to tylosin and erythromycin, the 18 Jordanian isolates were three dilutions more susceptible than previously reported (2), while the remaining isolates were more susceptible by no more than one dilution. This may be explained by differences in methodologies such as the incubation period and the inoculum size, which are known to affect MIC determination with other mycoplasma species (9). A previous study used a higher final inoculum concentration (5×10^6 CFU per ml, compared with 10^3 to 10^5 CCU per ml used in this study). The isolates with higher MICs were of French origin and were isolated from goats. The 2 isolates with higher MICs to the macrolides, lincomycin, and the tetracyclines were isolated from mastitic milk, while the 12 isolates with the highest MIC to tetracycline were from mastitic or normal milk samples. No high MICs were obtained for isolates from nasal swabs. In France, the macrolides, especially tylosin, are the agents most commonly used for treating CA, followed by the tetracyclines (P. Mercier, personal communication). Most of the isolates with higher MICs were from clinical samples, which may be due to previous exposure to any of these antimicrobial classes, with selection for possible resistant subpopulations.

In general, the MIC_{90}s and the MIC ranges were higher for most of the antimicrobials tested in the French isolates than those in the Jordanian isolates by one dilution. Larger differences were observed for the tetracyclines and macrolides, with higher MIC_{90}s and MIC ranges in French isolates. Curiously, the spectinomycin MIC_{90} was higher in the Jordanian isolates. These are interesting results that may reflect differences in the antimicrobial therapy measures between the two countries.

Although in vitro susceptibilities do not always relate to in

vivo susceptibilities, the results of this study led us to the conclusion that macrolides, tetracyclines, fluoroquinolones, and lincomycin are valid choices when selecting treatments for *M. putrefaciens* infections. To the authors' knowledge, this is the first report of increased MICs that indicate decreased susceptibility to the tetracyclines, macrolides, and lincomycin by *M. putrefaciens* isolates. The susceptibility profiles also correlate with the geographic origin of the isolates. Clinicians selecting antimicrobial treatments for *M. putrefaciens* should consider the potential of therapeutic failure due to acquired antimicrobial resistance.

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