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Antimicrobial Resistance in *Mycoplasma* spp.

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ABSTRACT Mycoplasmas are intrinsically resistant to antimicrobials targeting the cell wall (fosfomycin, glycopeptides, or β-lactam antibiotics) and to sulfonamides, first-generation quinolones, trimethoprim, polymixins, and rifampicin. The antibiotics most frequently used to control mycoplasmal infections in animals are macrolides and tetracyclines. Lincosamides, fluoroquinolones, pleuromutilins, phenicols, and aminoglycosides can also be active. Standardization of methods used for determination of susceptibility levels is difficult since no quality control strains are available and because of species-specific growth requirements. Reduced susceptibility levels or resistances to several families of antimicrobials have been reported in field isolates of pathogenic *Mycoplasma* species of major veterinary interest: *M. gallisepticum* and *M. synoviae* in poultry; *M. hyopneumoniae*, *M. hyorhinis*, and *M. hyosynoviae* in swine; *M. bovis* in cattle; and *M. agalactiae* in small ruminants. The highest resistances are observed for macrolides, followed by tetracyclines. Most strains remain susceptible to fluoroquinolones. Pleuromutilins are the most effective antibiotics *in vitro*. Resistance frequencies vary according to the *Mycoplasma* species but also according to the countries or groups of animals from which the samples were taken. Point mutations in the target genes of different antimicrobials have been identified in resistant field isolates, *in vitro*-selected mutants, or strains reisolated after an experimental infection followed by one or several treatments: DNA-gyrase and topoisomerase IV for fluoroquinolones; 23S rRNA for macrolides, lincosamides, pleuromutilins, and amphenicols; 16S rRNAs for tetracyclines and aminoglycosides. Further work should be carried out to determine and harmonize specific breakpoints for animal mycoplasmas so that *in vitro* information can be used to provide advice on selection of *in vivo* treatments.

INTRODUCTION

Mycoplasmas belong to the phylum *Firmicutes* (Gram-positive bacteria with low G+C content), to the class *Mollicutes* (from Latin: *mollis*, soft; *cutis*, skin), to the order *Mycoplasmatales*, and to the family *Mycoplasmataceae*. They presumably evolved by degenerative evolution from Gram-positive bacteria and are phylogenetically most closely related to some clostridia. Mycoplasmas are the smallest self-replicating prokaryotes (diameter of approximately 0.2 to 0.3 μm) with the smallest genomes (500 to 1,000 genes). They are characterized by the lack of a cell wall. The mycoplasma cell contains the minimum set of organelles essential for growth and replication: a plasma membrane, ribosomes, and a genome consisting of a double-stranded circular DNA molecule (1). The mycoplasma genome is characterized by a low G+C content and by the use of the universal stop codon UGA as a tryptophan codon. As a result of their limited genetic information, mycoplasmas express a small number of cell proteins and lack many enzymatic activities and metabolic pathways (1). Their nutritional requirements are therefore complex, and they are dependent on their host for many nutrients. This phenomenon explains the great difficulty of *in vitro* cultivation of mycoplasmas, with complex media containing serum (as a source of fatty acids and cholesterol) and

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a metabolizable carbohydrate (as a source of energy, for example, glucose, arginine, or urea).

All mycoplasmas cultivated and identified so far are parasites of humans or animals (2–5), with a high degree of host and tissue specificity. The primary habitats of mycoplasmas are epithelial surfaces of the respiratory and urogenital tracts, serous membranes, and mammary glands in some animal species. Many *Mycoplasma* species are pathogens, causing various diseases and significant economic losses in livestock productions. Mycoplasmas have developed mechanisms to resist their hosts' immune systems: modulatory effects on the host immune system, a highly plastic set of variable surface proteins responsible for rapid changes in major surface protein antigens (6, 7) and invasion of nonphagocytic host cells (8–11). These mechanisms contribute to the persistence of mycoplasmas in their hosts and to the establishment of chronic infections. The main pathogenic species in humans and animals are listed in Table 1.

Since *in vitro* culture of mycoplasmas is difficult to achieve (only performed by specialized laboratories) because of their specific requirements and slow growth, diagnosis of mycoplasmal infections is usually based on serologic tests (enzyme-linked immunosorbent assay, rapid plate agglutination) or specific PCR tests.

Vaccination, when available, can be an effective way of reducing clinical signs and improving herd performances. However, vaccination provides only partial protection and does not prevent infection (12–15). Eradication programs have also been implemented for several *Mycoplasma* species such as *Mycoplasma gallisepticum* and *Mycoplasma meleagridis* in poultry (16). However, the use of antimicrobials can be necessary in case of outbreaks and to control infections of *Mycoplasma* species for which vaccines and control programs are not available.

Without a cell wall, mycoplasmas are unaffected by many antibiotics such as β-lactams, glycopeptides, and fosfomycin that target cell-wall synthesis. Mycoplasmas are also naturally resistant to rifampicin, polymixins, sulfonamides, first-generation quinolones such as nalidixic acid, and trimethoprim (17, 18). Resistance to rifampicin was found to be due to a natural mutation in the *rpoB* gene of the RNA polymerase β subunit, which prevents the antibiotic from binding to its target (19, 20). Resistance to polymyxins and sulfonamides/trimethoprim is due to the absence in mycoplasmas of lipopolysaccharides and folic acid synthesis, respectively, which are the initial targets of these antimicrobials (21, 22). The most active and widely used antimicrobial agents in animals against mycoplasmal infections are tetracyclines, macrolides, fluoroquinolones, and pleuromutilins (12, 23, 24).

Methods used for antibiotic susceptibility testing of pathogenic *Mycoplasma* species of major veterinary interest (Table 1) will be described in this article. Activities of antimicrobials and resistance mechanisms will also be reviewed.

IN VITRO DETERMINATION OF ANTIMICROBIAL ACTIVITY

The effectiveness on antimicrobials *in vivo* can be indirectly assessed by *in vitro* susceptibility testing to determine the MIC and minimum bactericidal concentration (MBC) of an antimicrobial agent toward *Mycoplasma* strains.

MIC Determination

Numerous studies of the MIC determination of different antimicrobial agents for animal mycoplasmas have been published (for review see 23–25). However, because of their slow growth, very small size of colonies, and

TABLE 1 Main pathogenic *Mycoplasma* species in humans and livestock animals[a]

Host	*Mycoplasma* species	Clinical signs or syndrome
Humans	*M. genitalium*	Urethritis, often associated with bacterial vaginosis and cervicitis
	M. hominis	Urogenital tract infections
	M. pneumoniae	Upper respiratory disease, bronchopneumonia
Cattle	*M. bovis*	Infectious enzootic bronchopneumonia, mastitis, arthritis, otitis
Chickens, turkeys	*M. gallisepticum*	Chronic respiratory disease, infectious sinusitis
	M. synoviae	Subclinical respiratory tract infections, infectious synovitis, eggshell apex abnormality syndrome in laying-hen flocks
Swine	*M. hyopneumoniae*	Enzootic pneumonia
	M. hyorhinis	Polyserositis, arthritis
	M. hyosynoviae	Arthritis, polyarthritis

[a]From references 67, 69, 84, 95, 97, 145–147.

complex growth medium requirements, standard procedures used to test the susceptibility of classic bacteria, such as the disk diffusion method, are not recommended for mycoplasmas. The lack of consensus procedures (several culture media and methods, different presentation of results; see Table 2 for examples) and quality control (QC) strains makes comparisons between studies difficult or impossible. Studies comparing several testing methods for the same strains underlined the importance of using standardized methods, especially for the titer of the strains tested and the time of reading (initial versus final MIC values) (25–29).

Recommendations for antimicrobial susceptibility testing of animal *Mycoplasma* species were proposed in 2000 by the International Research Programme on Comparative Mycoplasmology (IRPCM) (25). More recently, the Clinical and Laboratory Standards Institute (CLSI) established standardized antimicrobial

TABLE 2 Examples of methods used (culture media, methods and measurement, expression of results) for the determination of antimicrobial activities toward animal mycoplasmas[a]

Mycoplasma species	Culture medium (agar or broth)	Methods and measurement	Expression of results[b]
M. agalactiae	Eaton's medium Hayflick's type medium Mycoplasma medium with pyruvate PH medium PPLO culture medium	Agar dilution: colonies on agar Broth dilution: color changes (sodium pyruvate fermentation), growth in wells after centrifugation (inverted mirror) Etest method: intersection of the inhibition zone with the MIC scale Flow cytometry: cell counts at different times	MIC per strain, MIC distribution (range), MIC_{50} and/or MIC_{90} Growth curves (flow cytometry)
M. bovis	Eaton's medium Friis medium Hayflick's type medium *Mycoplasma* medium *M. bovis* medium PPLO broth	Agar dilution: colonies on agar Agar diffusion: inhibition diameters Broth dilution (prepared or Sensititre plates): color changes (glucose fermentation, sodium pyruvate fermentation, AlamarBlue reagent, redox reagent resazurin), growth in wells after centrifugation (inverted mirror) Etest method: intersection of the inhibition zone with the MIC scale Flow cytometry: cell counts at different times	Initial and/or final MIC MIC per strain, MIC distribution (range), MIC_{50} and/or MIC_{90} Growth curves (flow cytometry)
M. gallisepticum	FM4 medium Frey's medium Frey's modified medium Friis medium Hayflick's modified medium	Agar dilution: colonies on agar Broth dilution (prepared or Sensititre plates): color changes (glucose fermentation) Etest method: intersection of the inhibition zone with the MIC scale	Initial and/or final MIC MIC per strain, MIC distribution (range), MIC_{50} and/or MIC_{90} Means
M. hyopneumoniae	Difco Turkey Serum (D-TS) medium Friis medium Friis modified medium Hank's-lactalbumin medium Hayflick's type medium	Agar dilution: colonies on agar Agar diffusion: inhibition diameters Broth dilution (prepared or Sensititre plates): color changes (glucose fermentation) Flow cytometry: cell counts at different times Microtiter biphasic agar-broth medium: colonies on agar	Initial and/or final MIC MIC per strain, MIC distribution (range), MIC_{50} and/or MIC_{90} Growth curves (flow cytometry)
M. hyorhinis	Friis medium Friis modified medium Hayflick's type medium M medium	Agar dilution: colonies on agar Agar diffusion: inhibition diameters Broth dilution (prepared or Sensititre plates): color changes (glucose fermentation)	Initial and/or final MIC MIC per strain, MIC distribution (range), MIC_{50} and/or MIC_{90}
M. hyosynoviae	Friis medium with mucin Hayflick's type medium Arginin/mucin-enriched Hayflick's medium Modified Difco medium with arginine	Agar dilution: colonies on agar Broth dilution (prepared or Sensititre plates): color changes (arginine hydrolysis)	Initial and/or final MIC MIC per strain, MIC distribution (range), MIC_{50} and/or MIC_{90}
M. synoviae	FM4 medium Frey's medium Frey's modified medium Friis medium with NAD	Agar dilution: colonies on agar Broth dilution (prepared or Sensititre plates): color changes (glucose fermentation) Etest method: intersection of the inhibition zone with the MIC scale	Initial and/or final MIC MIC per strain, MIC distribution (range), MIC_{50} and/or MIC_{90}

[a]These examples were compiled from references 18, 29, 34, 36, 38–43, 47, 49, 56–59, 62–66, 73–76, 81, 86, 87, 93, 94, 98–100, 102, 107–112, 148, 149.
[b]MIC_{50}, MIC which inhibits 50% of the tested isolates; MIC_{90}: MIC which inhibits 90% of the isolates tested.

susceptibility testing guidelines to determine MICs for human mycoplasma pathogens (30). However, these guidelines cannot be used for all mycoplasmas because nutritional requirements, metabolic capacities, and fitness vary among species, as evidenced by an international multilaboratory collaborative study performed with human mycoplasmas (31): sufficient consensus of results necessary to generate 3- to 4-dilution QC ranges for some antimicrobial agents were not obtained, evidencing the difficulties generated by the fastidious nature of mycoplasmas. Such a collaborative study has not been conducted yet with animal mycoplasmas, and no veterinary reference strains well characterized for MICs are available and shared by laboratories for QC purposes. Recent studies performed with animal mycoplasmas therefore often take the IRPCM recommendations (32–35) or CLSI guidelines for human mycoplasmas (36–38) as a basis for MIC determinations, but with different media and controls.

Titration of strains is important since the inoculum concentration can influence MIC values obtained in broth or on agar medium (25, 30, 31). Because of their small size, titration of mycoplasmas cannot be performed by optical density determination like for classical bacteria. Titrations are performed in different broth or agar media, depending on the *Mycoplasma* species studied (Table 2). For broth titrations, series of 1:10 dilutions of cultures are performed in broth medium with a metabolic indicator (for example, phenol-red to detect pH changes due to glucose fermentation). Dilution of the last tube to show growth is taken as the number of color-changing units. For agar titrations, the number of colonies is determined by observation with a stereomicroscope. Titers are obtained after several days, depending on the growth of the *Mycoplasma* species being studied. According to IRPCM recommendations, strain dilutions should be performed to yield 10^3 to 10^5 color-changing units/ml in broth medium or 10^3 to 10^5 CFU/ml on agar medium, whereas CLSI recommends 10^4 to 10^5 CFU/ml for broth and agar assays. For broth dilution MIC testing, MIC is generally defined as the lowest antibiotic concentration that inhibits growth (usually detected by a color change of the medium) when growth is compared to the growth observed in the control without antibiotic (25, 30, 31). However, final MIC values (when strains are incubated for longer periods) are also reported in several studies performed in broth medium (29, 39, 40). For agar dilution MIC testing, strains are usually transferred onto agar via a replicator, and MIC is generally defined as the lowest antibiotic concentration that prevents colony formation (visualized under a stereomicroscope) when colonies are observed on the antibiotic-free control plate (30, 31). However, MIC is sometimes defined as the concentration resulting in strong reduction (50% or more, depending on the studies performed) in colony number (25, 39, 41) or size (39, 42, 43).

No QC reference strains are currently available for MIC assays with animal mycoplasmas, whereas Waites and collaborators (31) published values for QC reference strains of human mycoplasmas. It is therefore important to repeat MIC determination assays several times on separate occasions and to include, when available, one or several strains already tested, with known MIC values for the antibiotics studied, to validate the results obtained.

The absence of interpretation criteria (breakpoint concentrations) for mycoplasmas makes it difficult to evaluate the likely *in vivo* therapeutic efficacy from MIC data established *in vitro*. MIC values are often compared to breakpoints given for classical bacteria (44, 45) or to breakpoints suggested by Hannan and collaborators (25) or Ter Laak and collaborators (39).

Finally, it should be noted that, because of their fastidious nature, only a few laboratories are able to isolate animal mycoplasmas (especially slow-growing ones such as *Mycoplasma hyopneumoniae* and *Mycoplasma hyosynoviae* in pigs and *Mycoplasma synoviae* in poultry, for example), and susceptibility testing methods have to be carried out over several weeks (from titration to MIC determinations). Susceptibility testing of mycoplasmas is therefore not performed as routine monitoring like it is for classical bacteria, and studies are often performed with strains from one *Mycoplasma* species from one country.

MBC Determination

Antibiotics are commonly classified into bactericidal and bacteriostatic agents based on their antimicrobial action: bacteriostatic agents prevent the growth of bacteria, and bactericidal agents kill bacteria. Determination of MBC is performed to know if an antibiotic has more a bacteriostatic or a bactericidal activity toward bacteria. MBC is defined as the lowest antibiotic concentration that kills ≥99.9% of the cells. If the MBC value is close to the MIC value, the antimicrobial agent has a bactericidal effect, and if the MBC value is significantly higher than the MIC value, the antimicrobial agent has a bacteriostatic effect.

Very few studies determining minimum bactericidal (or mycoplasmacidal) concentrations against animal *Mycoplasma* species have been published, and no standardized

method has been described for veterinary or human mycoplasmas. Guidelines for performing bactericidal tests with classical bacteria were published in 1999 (46) but cannot be applied strictly to mycoplasmas because of their slow growth and medium requirements.

Several methods have been used: killing curves with *M. hyopneumoniae* (41), subcultures on agar at the same time as recording of initial MIC with *M. synoviae* (47), or antibiotic dilution for *Mycoplasma bovis* (48, 49) or *M. hyopneumoniae* (50). Two methods (dilution or filtration) were also described by Taylor-Robinson to remove antibiotics from the surviving mycoplasmas (51). Subculture on agar medium is the most widely used method for the determination of MBC for human mycoplasmas (52–54), but dilutions in broth medium are also described (55). Flow cytometric assessment of *in vitro* antimicrobial activity toward strains of *M. agalactiae* (56, 57), *M. bovis* (58), and *M. hyopneumoniae* (59) also provided information on the bactericidal or bacteriostatic activity of antibiotics.

Among the antibiotics tested, fluoroquinolones were shown to be mycoplasmacidal *in vitro* (41, 49, 54), whereas antibiotics of the tetracycline group and tiamulin were mycoplasmastatic (41, 47). Macrolides, lincosamides, and spectinomycin are usually classified as mycoplasmastatic antibiotics but showed a better mycoplasmacidal activity for *M. synoviae* in the Kleven and Anderson study (47) than did tetracycline antibiotics.

Finally, it should be noted that, due to their instability under *in vitro* conditions, the antimicrobial activity of some antibiotics may be underestimated during *in vitro* susceptibility tests. Moreover, the stability of several antimicrobials is known to be affected *in vitro* by light, composition of the medium, temperature, and pH (60, 61). This degradation can be associated with an increase of the MIC and MBC values, which may be clinically significant for slow-growing bacteria such as mycoplasmas. Due to the very slow growth of mycoplasmas, the time required for the determination of MICs can vary from 1 day to 1 week. These longer incubation times can lead to degradation or loss of activity of some antibiotics *in vitro* and thus lead to an underestimation of the actual activity of these antibiotics. Results of *in vitro* assays should therefore always be taken with precaution because they do not always reflect the *in vivo* action of antimicrobial agents. Host-linked factors also contribute to success or failure of a treatment on *in vitro* susceptible bacteria (pH values and cation concentrations in different body compartments, differences between intracellular and extracellular antibiotic concentrations, etc.).

IN VITRO ACTIVITIES OF ANTIBIOTICS AGAINST MYCOPLASMAS OF VETERINARY ORIGIN

Mycoplasmas are intrinsically resistant to all antimicrobials targeting the cell wall, such as fosfomycin, glycopeptides, or β-lactam antibiotics (23). Several studies evidenced high MICs for β-lactam antibiotics in several *Mycoplasma* species (18, 39, 42, 58, 62–65). Mycoplasmas are also intrinsically resistant to sulfonamides (18, 21, 22), first-generation quinolones such as nalidixic acid (41, 50), trimethoprim (18, 66), polymixins, and rifampicin (19–22).

The antibiotics most frequently used to control *Mycoplasma* infections in animals are macrolides and tetracyclines. Other antimicrobial agents—lincosamides, fluoroquinolones, pleuromutilins, phenicols, and aminoglycosides—can also be active against mycoplasmas.

Ribosomes are targets for most of these classes of antimicrobials. Macrolides, lincosamides, and pleuromutilins inhibit protein synthesis by binding to the peptidyl transferase component of the 50S subunit of ribosomes. Tetracyclines also inhibit protein synthesis in the ribosome by binding to the 30S ribosomal subunit. Florfenicol binds to the 50S ribosomal subunit, inhibiting the peptidation reaction and the translation of bacterial mRNA, whereas aminoglycosides disturb peptide elongation at the 30S ribosomal subunit level, giving rise to inaccurate mRNA translation. Fluoroquinolones have affinity for DNA gyrase and topoisomerase IV and prevent DNA replication of bacteria.

Susceptibility profiles (ranges of MIC) of the main *Mycoplasma* species of veterinary interest are presented in Table 3 (avian mycoplasmas), Table 4 (porcine mycoplasmas), and Table 5 (ruminant mycoplasmas).

Poultry

Avian mycoplasmoses can cause significant economic losses on poultry farms. *M. gallisepticum* is responsible for chronic respiratory disease of chickens and infectious sinusitis of turkeys (67). *M. synoviae* causes subclinical respiratory tract infections and infectious synovitis (68) and is also responsible for the eggshell apex abnormality syndrome (69). *M. meleagridis* and *Mycoplasma iowae* are mainly observed in turkeys and may cause growth retardations and embryonic mortality (70, 71).

Several studies report *in vitro* susceptibility levels of *M. gallisepticum* field isolates (26, 29, 33, 41, 62, 72–80) (Table 3). Tiamulin MICs are consistently lower than those for other antimicrobial agents tested *in vitro*, even if strains with reduced susceptibility were found in old (before 2000) and recent (after 2000) studies (33,

TABLE 3 MIC values (range in μg/ml) for various antimicrobials against avian *Mycoplasma* species (*M. gallisepticum* and *M. synoviae*)

	M. gallisepticum		*M. synoviae*	
Antimicrobials[b]	Old strains[a]	Recent strains[a]	Old strains[a]	Recent strains[a]
Tetracyclines:				
Tetracycline	0.08–0.64	ND	1–2	ND
Oxytetracycline	0.05–0.5	≤0.03–4	0.1–>100	0.39–3.12
Doxycycline	ND[d]	≤0.03–0.79	ND	ND
Chlortetracycline	ND	0.2–32	1–2	0.32–>12.5
Macrolides:				
Erythromycin	0.02–>80	≤0.03–>64	>40	32–>128
Tylosin	0.0025–10	≤0.03–5	0.025–10	≤0.006–2
Tilmicosin	ND	≤0.03–32	ND	0.03–>8
Josamycin	ND	0.2–>50	ND	ND
Spiramycin	0.5–>20	ND	ND	ND
Tylvalosin	ND	ND	ND	≤0.006–0.012
Lincosamides:				
Lincomycin	1.25–40	0.1–12.5	1–2	0.125–8
Pleuromutilins:				
Tiamulin	0.0005–1	≤0.03–2	0.1–1	0.012–0.12
Fluoroquinolones:				
Flumequine	2.5–10	ND	5–50	ND
Enrofloxacin	0.005–1[c]	≤0.03–10	0.1–10[c]	0.03–8
Danofloxacin	0.01–0.5	ND	0.1–0.5	ND
Amphenicols:				
Florfenicol	ND	0.125–4	ND	ND
Aminoglycosides:				
Spectinomycin	0.5–10	≤0.03–2	0.5–2	ND
Gentamicin	≥10–≥50	1–32	1	ND

[a]Data were compiled from studies performed on old strains (before 2000) (26, 41, 47, 62, 72–75) and more recent strains (2000 to 2016) (29, 32, 33, 76–82). Several methods were used to determine these MIC values.

[b]Antimicrobial family and antibiotics belonging to this family.

[c]For enrofloxacin, one study (29) compared isolates from 1997 to 2003 and from 2005 to 2006; results obtained for isolates from 1997 to 2003 are classified as old strains in this table.

[d]ND, no data found.

74). Most *M. gallisepticum* isolates are also susceptible to tetracycline antibiotics (Table 3), with lower MIC values for oxytetracycline and doxycycline than for chlortetracycline (77, 79). *M. gallisepticum* is not intrinsically resistant to 14-membered ring macrolides such as erythromycin, and most strains are susceptible to macrolides (Table 3). However, high MIC levels of erythromycin, tylosin, and tilmicosin were evidenced in strains isolated before and after 2000 in several countries (26, 33, 62, 72, 74, 75, 78, 79). For example, Gerchman and collaborators (78) reported that acquired resistance to tylosin and tilmicosin was present in 50% of *M. gallisepticum* strains isolated in Israel from 1997 to 2010. An increase in MIC levels was also reported for enrofloxacin, especially in studies comparing old and recent isolates (29, 79), even if most strains remained susceptible to this fluoroquinolone antimicrobial *in vitro*, with low MIC values (29, 74, 77, 79, 80). A marked decrease in susceptibility to fluoroquinolones was evidenced in field strains of *M. gallisepticum* in Israel (29), and 72% of the strains isolated since 2006 showed acquired resistance to enrofloxacin and macrolides (78). Only one study reported florfenicol MIC determination for *M. gallisepticum* isolates and showed good activity, with MICs ranging from 0.125 to 4 μg/ml (79).

Several studies report *in vitro* susceptibility levels of *M. synoviae* field isolates (29, 32, 41, 47, 74–76, 81, 82) (Table 3). *M. synoviae* was shown to be intrinsically resistant to 14-membered ring macrolides such as erythromycin (47, 82), and recent studies (published after 2000) evidenced strains with reduced susceptibility to other macrolides and lincosamides (82). In several studies, *M. synoviae* was found to be intrinsically less susceptible to fluoroquinolones than *M. gallisepticum* (29, 83) and resistant to flumequine (75). Recent *M. synoviae* strains (isolated between 2009 and 2012) with decreased susceptibility to enrofloxacin (MIC ranging from 1 to 16 μg/ml) were found in Italy, Austria, and Israel (32), and several strains isolated between

TABLE 4 MIC values (range in μg/ml) for various antimicrobials against swine *Mycoplasma* species (*M. hyopneumoniae*, *M. hyorhinis*, and *M. hyosynoviae*)

	M. hyopneumoniae		*M. hyorhinis*		*M. hyosynoviae*	
Antimicrobials[b]	Old strains[a]	Recent strains[a]	Old strains[a]	Recent strains[a]	Old strains[a]	Recent strains[a]
Tetracyclines:						
Tetracycline	0.025–1	ND[c]	≤0.03–0.5	≤0.5–2	0.01–10	ND
Oxytetracycline	0.025–2	0.03–12.5	0.025–10	0.1–6.3	0.1–10	0.5–>4
Doxycycline	≤0.03–1	0.03–6.25	≤0.03–0.5	ND	ND	ND
Chlortetracycline	0.12–50	3.12–100	0.12–8	0.2–12.5	ND	0.5–>4
Macrolides:						
Erythromycin	16–>16	6.25–>400	>16	>16	ND	ND
Tylosin	≤0.006–6.25	0.008–16	≤0.03–25	0.4–100	0.025–>10	≤0.25–1
Tilmicosin	ND	≤0.25–>16	ND	≤0.25–8	ND	≤2–32
Josamycin	≤0.006–0.2	0.1–>12.5	0.2–50	0.2–50	ND	ND
Spiramycin	0.06–0.5	0.03–25	≤0.03–4	ND	ND	ND
Tylvalosin	ND	0.016–0.06	ND	ND	ND	ND
Tulathromycin	≤0.004–0.125	ND	ND	ND	ND	1–≥32
Lincosamides:						
Lincomycin	0.025–1.56	≤0.025–>12.5	0.06–200	≤0.25–50	0.03–1	ND
Clindamycin	0.12–0.25	ND	0.06–1	ND	ND	≤0.12–0.25
Pleuromutilins:						
Tiamulin	≤0.006–0.3	≤0.01–0.125	0.025–0.78	0.2–1.56	0.0025–0.1	≤0.25
Valnemulin	0.00025–0.001	0.08	ND	ND	0.0001–0.00025	ND
Fluoroquinolones:						
Flumequine	0.25–1	0.25–>16	2.5–25	ND	5–50	ND
Enrofloxacin	0.0025–0.1	0.015–25	≤0.03–2	0.06–4	0.05–0.5	0.12–0.5
Danofloxacin	0.01–0.05	ND	0.25–1	ND	0.1–0.5	0.25–0.5
Amphenicols:						
Florfenicol	ND	ND	ND	ND	ND	0.25–4
Chloramphenicol	0.5–2	0.5–4	0.5–4	ND	ND	ND
Thiamphenicol	ND	ND	0.2–12.5	1.56–12.5	ND	ND
Aminoglycosides:						
Spectinomycin	0.5–6.5	0.06–2	0.12–4	≤1–8	ND	4
Gentamicin	0.1–2.5	≤0.125–1	ND	1–4	0.25–0.5	0.5

[a]Data were compiled from studies on old strains (isolated before 2000) (39–42, 63, 75, 86, 87, 89, 90, 93, 94) and more recent strains (2000 to 2016) (18, 43, 50, 66, 91–93). Several methods were used to determine these MIC values.
[b]Antimicrobial family and antibiotics belonging to this family.
[c]ND, no data found.

1996 and 2008 in Israel already showed decreased susceptibility to this antibiotic (29). Tetracycline antimicrobials and tiamulin showed a relatively good *in vitro* activity against *M. synoviae* strains (Table 3), but strains with higher tetracycline MICs have been reported (75, 81).

Very few MIC determination studies have been performed with *M. meleagridis* and *M. iowae* strains. Two studies reported MIC values of enrofloxacin, tylosin, and tiamulin for very few strains (mainly reference strains of *M. iowae* and *M. meleagridis*) (41, 74), whereas a third study reported values of several antimicrobials for 19 strain of *M. iowae* (75). All the antibiotics tested showed a good activity against most strains. Danofloxacin, enrofloxacin, and tiamulin were the most effective antibiotics *in vitro*, whereas higher MIC values were observed for flumequine and tylosin (75).

Swine

Pathogenic swine mycoplasmas are considered to play an important role in pig production. *M. hyopneumoniae* is one of the primary pathogens associated with the porcine respiratory disease complex, one of the most common and economically important diseases for swine producers worldwide (84). *M. hyopneumoniae* is the etiological agent of enzootic pneumonia in swine, a chronic respiratory disease characterized by high morbidity and low mortality rates. Polyserositis and arthritis, induced by *Mycoplasma hyorhinis* and *M. hyosynoviae*, generally affect growing pigs (84). *Mycoplasma flocculare* is isolated in the swine respiratory tract and is genetically close to *M. hyopneumoniae*. Its role is still unclear, and it is often considered a commensal bacterium (85).

Several studies reported *in vitro* susceptibility levels of *M. hyopneumoniae* field isolates (39, 41, 50, 63, 75,

TABLE 5 MIC values (range in µg/ml) for various antimicrobials against ruminant *Mycoplasma* species (*M. bovis* and *M. agalactiae*)

	M. bovis		*M. agalactiae*	
Antimicrobials[b]	Old strains[a]	Recent strains[a]	Old strains[a]	Recent strains[a]
Tetracyclines:				
Tetracycline	0.05–1	0.05–>256	ND	0.125–32
Oxytetracycline	0.1–128	0.05–>256	0.1–4	0.06–16
Doxycycline	ND[c]	0.023–8	ND	0.008–1
Chlortetracycline	3.12–100	0.25–>32	ND	0.125–8
Macrolides:				
Erythromycin	50–>100	1–>512	ND	6–>256
Tylosin	0.025–>100	0.125–>256	0.1–1	0.03–12.8
Tilmicosin	1–>128	0.5–>1024	0.12–1	0.12–64
Spiramycin	0.39–>100	ND	ND	0.125–4
Gamithromycin	32–>128	128–>128	ND	4–32
Tulathromycin	1–64	0.25–>1024	ND	1–8
Azythromycin	ND	0.25–>256	ND	ND
Lincosamides:				
Lincomycin	0.39–3.12	0.06–>256	ND	0.125–4
Clindamycin	ND	≤0.03–>256	ND	≤0.12
Pleuromutilins:				
Tiamulin	0.05–1	ND	0.05–0.25	0.125–0.5
Valnemulin	ND	≤0.03	ND	ND
Fluoroquinolones:				
Flumequine	10–100	ND	ND	>128
Enrofloxacin	0.05–1	≤0.03–32	0.05–1	0.06–1.6
Danofloxacin	0.125–2.5	0.08–32	0.05–2.5	0.25–0.5
Marbofloxacin	0.25–1	0.25–>32	ND	0.1–12.8
Amphenicols:				
Chloramphenicol	6.25–25	0.25–32	ND	1–8
Florfenicol	1–64	0.06–32	1–8	2–8
Aminoglycosides:				
Spectinomycin	1–>128	0.38–>256	1–8	0.25–8
Gentamicin	ND	2.8	ND	0.5–16

[a]Data were compiled from studies performed on old strains (before 2000) (36, 38, 41, 49, 75, 87, 101) and more recent strains (2000 to 2016) (34, 36, 38, 58, 64, 65, 98–100, 102–105, 107–114). Several methods were used to determine these MIC values.
[b]Antimicrobial family and antibiotics belonging to this family.
[c]ND, No data found.

86–93) (Table 4). *M. hyopneumoniae* was shown to be intrinsically resistant to erythromycin (39, 50, 63, 93) but usually susceptible to 16-membered ring macrolides such as tylosin and tilmicosin (Table 4). However, strains with reduced susceptibility or resistance to macrolides and lincomycin were evidenced in studies performed in Belgium, Spain, and Thailand between 2004 and 2014 (50, 91, 93). Comparison between *M. hyopneumoniae* strains isolated from 1970 to 1981 and 1989 to 1990 in Japan and from 1997 to 1998 and 2006 to 2011 in Thailand suggested a decrease in chlortetracycline susceptibility (88, 93). As already seen for avian mycoplasmas, MIC values of chlortetracycline were higher than values for oxytetracycline and doxycycline, and most strains remained susceptible to these antibiotics (Table 4). Pleuromutilins (tiamulin and valnemulin) were the most active antimicrobials *in vitro*, with MIC values not higher than 0.3 µg/ml. Most studied strains were also susceptible to fluoroquinolones, but strains with reduced susceptibility or resistance were isolated in Thailand and Belgium after 2000 (91, 93).

Like *M. hyopneumoniae*, *M. hyorhinis* is intrinsically resistant to erythromycin (39, 66). Even if macrolides and lincosamides still had good *in vitro* activity against most strains, several studies evidenced the selection of resistant strains (39, 42, 43, 66, 75). Strains of *M. hyorhinis* with resistance to 16-membered macrolides and lincomycin four times higher than 10 years before were isolated in Japan (43), and two strains were resistant to all macrolides and lincomycin. However, this resistance reverted to susceptibility by serial *in vitro* subcultures without antibiotics. Fluoroquinolone MIC values were higher for *M. hyorhinis* than for *M. hyopneumoniae* (Table 4), and isolates with reduced sus-

ceptibility were evidenced in studies performed with strains isolated before and after 2000 (39, 42, 43, 66, 75). Tiamulin remained one of the most active antimicrobials *in vitro* against *M. hyorhinis*, but MIC values for recent strains (isolated after 2000) were 10 times higher than for old strains (isolated before 2000) (Table 4).

Several studies published before 2000 and one recent study (2012) report *in vitro* susceptibility levels of *M. hyosynoviae* field isolates (18, 40, 41, 75, 87, 89, 94) (Table 4). Resistance to macrolide antibiotics was evidenced in strains isolated before 2000: 2 of 54 old Japanese strains of *M. hyosynoviae* isolated between 1980 and 1995 showed resistance to all 14- and 16-membered macrolide antibiotics tested (94). Reduced susceptibility or resistance to tylosin was also evidenced for several Danish *M. hyosynoviae* strains isolated from 1995 to 1996 compared to older strains (1968 to 1971) (40). However, another study showed good *in vitro* activity of tylosin and clindamycin against U.S. strains isolated between 1997 and 2011 but higher MIC values for tilmicosin and tulathromycin (18). *M. hyosynoviae* strains isolated from 1994 to 1995 were less susceptible to tetracyclines than strains isolated from 1980 to 1984 (40). All strains of *M. hyosynoviae* were susceptible to tiamulin, valnemulin, gentamicin, enrofloxacin, and danofloxacin (Table 4).

Only a very limited number of reports are available on MIC values for *M. flocculare*. Two studies, published in 1991 and 1994, reported good *in vitro* activity of tetracyclines, lincosamides, tiamulin, enrofloxacin, and spectinomycin (39, 87). *M. flocculare* strains were resistant to erythromycin but susceptible to tylosin and spiramycin (Table 4).

Cattle and Other Ruminants

In cattle, *M. bovis* causes respiratory disease, mastitis, arthritis, and otitis (95). This *Mycoplasma* species is frequently implicated in cases of bovine respiratory disease in calves raised in feedlots (96). *Mycoplasma agalactiae* is the causative agent of contagious agalactia, a serious disease of sheep and goats, affecting mammary glands, joints, and eyes and causing severe economic losses (97).

Several studies reported *in vitro* susceptibility levels of *M. bovis* field isolates to several antimicrobials (34, 36, 41, 49, 58, 64, 65, 75, 87, 98–105) (Table 5). All *M. bovis* strains were found to be resistant to erythromycin (64, 99, 106), suggesting an intrinsic resistance to the 14-membered ring macrolides. Resistance to tetracyclines and macrolides was already reported in strains isolated before 2000 (36, 49, 75), but resistant isolates were more frequently found in recent isolates (34, 36, 100, 102, 103) (Table 5). In France, an overall decrease in antimicrobial susceptibility was evidenced for *M. bovis* isolates by comparison between old (1978 to 1979) and recent (2010 to 2012) strains isolated from cattle (36): susceptibility of *M. bovis* decreased significantly for eight antimicrobials from the tetracycline, fluoroquinolone, aminoglycoside, and macrolide families. This led to a high prevalence of multiresistant strains of *M. bovis* in France (36): 100% of the *M. bovis* isolates tested harbored a reduced susceptibility or resistance to eight antimicrobials. However, no high-level resistance to fluoroquinolones was evidenced in recent French isolates of *M. bovis*: 2- to 4-fold increases of the MIC levels of fluoroquinolones were evidenced in most of these strains, suggesting an ongoing shift of French isolates toward a low-level resistance phenotype (36). Resistant strains were also found in other countries. Strains with acquired resistance to spectinomycin, clindamycin, tetracycline, and azithromycin were found in Canada between 2001 and 2003 (99), enrofloxacin being the most effective antibiotic, with MICs of ≤0.5 μg/ml. Recent Chinese isolates (2011 to 2013) were susceptible or had medium sensitivity to enrofloxacin and doxycycline but were frequently resistant to macrolides (103). All recent Japanese strains of *M. bovis* isolated from milk samples were susceptible to fluoroquinolones, but several strains were resistant to kanamycin (aminoglycoside), oxytetracycline, and macrolides (102).

Fluoroquinolone-resistant strains were isolated in Europe (105), with marbofloxacin MICs ranging from 0.5 to 4 μg/ml. *M. bovis* strains isolated between 2008 and 2014 in the Netherlands also harbored high MIC values for several antimicrobial agents (34). In this study, fluoroquinolones appeared to be the most efficacious in inhibiting *M. bovis* growth *in vitro*, followed by tulathromycin and oxytetracycline. However, strains with reduced susceptibility or resistance were observed for these antibiotics. The highest MIC values were obtained for macrolides. For tulathromycin, MIC_{50} (MIC inhibiting 50% of the strains studied) for respiratory isolates was higher than for isolates from mastitis or arthritis (34), which can probably be explained by the frequent use of this antibiotic to treat respiratory infections and the absence of registration for mastitis or arthritis. Similarly, a significant difference in the susceptibility levels between quarter milk and lung isolates was found for spectinomycin (58), showing that the sample source can have an effect on antimicrobial activity profiles. Moreover, Gerchman and collaborators showed that local strains (isolated from cattle in Israel)

were significantly more resistant to macrolides than strains from imported animals but were more susceptible to fluoroquinolones and spectinomycin (100). All these results also showed that the frequency of resistance in *M. bovis* isolates varies considerably from one country to another and that resistance can be observed for all the families of antimicrobials tested (tetracyclines, macrolides, fluoroquinolones, lincosamides, amphenicols, and aminoglycosides) except pleuromutilins (Table 5).

Old and recent studies reporting *in vitro* susceptibility levels of *M. agalactiae* field isolates showed that antimicrobial susceptibility profiles for this *Mycoplasma* species were different from antimicrobial susceptibility profiles of *M. bovis* field isolates (38, 75, 107–114) (Table 5). Even if resistance to macrolides and tetracyclines was evidenced in recent studies (38, 110), levels and frequencies of resistance were lower. Most strains remained susceptible or intermediate for fluoroquinolones and lincosamides (Table 5). One Spanish study found a wide MIC range for marbofloxacin (0.1 to 12.8 μg/ml) compared to other fluoroquinolones (114). Poumarat and collaborators, comparing old (1980 to 1990) and recent (2008 to 2012) strains from ovine or caprine origin, showed that a moderate shift toward higher MICs (two to four times higher) was observed for most of the antimicrobials tested, whereas the increase was more marked in ovine isolates but was restricted to macrolides (38): ovine isolates were shown to remain mainly susceptible over time. The authors hypothesized that this difference between caprine and ovine isolates could be due to different antimicrobial uses. Similarly, Paterna and collaborators found higher MIC values for several antimicrobials with *M. agalactiae* isolates from goat herds with clinical symptoms than from asymptomatic animals (114).

MYCOPLASMA RESISTANCE TO ANTIMICROBIALS

Several studies showed that resistance to antibiotics could be selected *in vitro* by several passages in subinhibitory concentrations of various antibiotics such as macrolides, fluoroquinolones, tetracyclines, or pleuromutilins (43, 115, 116). The rate of selection of resistant mutants appeared to be dependent on both the *Mycoplasma* species and the antibiotic (or family of antibiotics) used to select these mutants. Macrolide-resistant mutants were rapidly selected in *M. gallisepticum*, *M. synoviae*, *M. iowae*, and *M. bovis*, whereas more *in vitro* passages in the presence of subinhibitoty concentrations of antibiotics were necessary for fluoroquinolones, tetracyclines, and pleuromutilins (115–117). High MIC levels for tylosin were also reported in *M. hyopneumoniae* within five to seven *in vitro* passages whereas only a slight increase of MIC for oxytetracycline and no significant increase in MIC of valnemulin or tiamulin for two strains of *M. hyopneumoniae* were evidenced after 10 *in vitro* passages (89). This progressive increase in the level of resistance of strains to some antibiotics suggests a progressive selection of resistance mechanisms, such as point mutations, in different sites or target genes, whereas a rapid increase suggests the selection of a single mechanism conferring a high level of resistance.

Selection of mutants with reduced susceptibility or resistance to antimicrobials has also been reported after *in vivo* fluoroquinolone treatments of hens experimentally infected with *M. synoviae* (83) or *M. hyopneumoniae*-infected pigs (118). Links between field usage of antimicrobials and development of resistance were also evidenced: for example, according to Khalil and collaborators (119), the shift of *M. bovis* strains toward resistance to oxytetracycline happened earlier than for macrolides, which is in accordance with the earlier marketing authorization date for tetracycline and its earlier use in field conditions than macrolides.

Several recent studies described resistance mechanisms of animal *Mycoplasma* species in clinical isolates or in mutants obtained *in vitro*. Since mycoplasmas do not harbor plasmids, most resistance mechanisms described in mycoplasmas are point mutations in their chromosome, and few mechanisms are associated with a transposon.

Macrolides

Macrolide and lincosamide antibiotics are chemically distinct but share a similar mode of action. Bacteria become resistant to macrolide and lincosamide antibiotics (i) through target-site modification by methylation or mutation that prevents the binding of the antibiotic to its ribosomal target, (ii) through efflux of the antibiotic, and (iii) by drug inactivation. Modification of the ribosomal target confers broad-spectrum resistance to macrolides and lincosamides, whereas efflux and drug inactivation affect only some of these molecules (120).

Macrolides bind within the tunnel of the 50S ribosomal subunit and interact mainly with the A2058 nucleotide of the 23S rRNA (domain V), with an additional interaction with and around the G748 nucleotide (23S rRNA, domain II) and with the surface of proteins L4 and L22 (121, 122). Most of the point mutations

described in *Mycoplasma* isolates harboring decreased susceptibility or resistance to macrolides are described at these positions or nearby (Table 6).

Several point mutations in domain V of the 23S rRNA gene were evidenced in macrolide-resistant *M. gallisepticum* isolates from Egypt (33), China (117), and Israel (78): the G2057A, A2058G, and A2059G substitutions were shown to be implicated in reduced susceptibility or resistance to macrolide antibiotics (Table 6).

Reduced susceptibility or resistance to macrolides or lincosamides in *M. synoviae* was also correlated with the presence of several amino acid substitutions in the 23S rRNA alleles (82) (Table 6). *M. synoviae* has an intrinsic resistance to 14-membered macrolides such as erythromycin, correlated with a G2057A substitution in the 23S rRNAs in all strains (82). The presence of point mutations A2058G and A2059G was correlated with a significant decrease in susceptibility to tylosin, tilmicosin, and lincomycin. A nucleotide substitution G748A in domain II was also evidenced: its presence in one or both 23S rRNA alleles may be responsible for a slight increase in MICs to macrolides, but no correlation between the presence of G748A and decreased susceptibility to lincomycin was found. Mutations G64E and Q90K/H were identified in the L4 and L22 proteins, respectively, but their impact on decreased susceptibility to macrolides and lincomycin was not clear (82).

M. hyopneumoniae has an intrinsic resistance to 14-membered macrolides due to a G2057A transition in their 23S rRNA (92). An additional, acquired A2058G point mutation was found in the 23S rRNA of a field strain resistant to 16-membered macrolides such as tylosin and to lincosamides (Table 6).

M. hyorhinis has an intrinsic resistance to 14-membered macrolides such as erythromycin or oleandomycin, but most strains remained susceptible to tylosin and tilmicosin (42, 94). Mutants of *M. hyorhinis* that were resistant to macrolides/lincosamides were selected *in vitro* by serial passages in subinhibitory concentrations of tylosin or lincomycin (43). The same A2059G mutation was found in mutants selected in tylosin and in field strains. Other mutations were evidenced in domains II and V of 23S rRNA of the mutant selected in lincomycin: addition of an adenine at pentameric adenine sequence in domain II, G2597U, and C2611 in domain V. After 11 tylosin passages of this lincomycin-resistant mutant, another point mutation at position A2062G was detected (Table 6).

In *M. bovis*, the presence of any of the point mutations G748A or C752T (domain II), A2058G, or A2059G/C (domain V) in one or both alleles of the 23S rRNA was correlated with decreased susceptibility to tylosin and tilmicosin (123). The A2058G substitution was also evidenced in Chinese macrolide-resistant clinical isolates (103). However combination of mutations in the two domains seems to be necessary to achieve higher MICs (123). Point mutations in domain II may play a more critical role in acquired resistance to tilmicosin than tylosin, suggesting that there may be differences in the way these two macrolides interact within the binding site (122, 124). Sulyok and collaborators suggested that mutations in domain II (position 748 and insertion after nucleotide C752) were necessary to achieve tilmicosin and tylosin MICs of ≥128 and ≤32 μg/ml, respectively, whereas an additional mutation in domain V (positions 2059, 2060, 2063, and 2067) was needed to reach highly elevated tylosin (MIC, ≥128 μg/ml) and lincomycin (MIC, ≥64 μg/ml) MICs (116). Several mutations in L4 and L22 proteins were evidenced in *M. bovis* isolates (Table 6), but their contribution to increased MIC levels was difficult to establish since other point mutations were often present in the same isolates in domain II of both *rrl* alleles (123).

Two substitutions in protein L22 (Ser89-Leu and Gln90-Lys/His) were evidenced in clinical isolates of *M. agalactiae* with reduced susceptibility to macrolides, whereas a mutation A2058G in domain V of the 23S rRNA gene was involved in a higher level of resistance (111) (Table 6). The substitutions Ser89-Leu and Gln90-Lys were also observed in protein L22 of *in vitro*-selected mutants. The A2058G substitution was not observed in mutants, but the mutation A2059G in both alleles led to a high level of resistance to macrolides (MIC, >128 μg/ml for tylosin and tilmicosin) and lincosamides (MIC, 6.4 μg/ml for lincomycin and clindamycin). Selection in lincomycin led to the selection of a C2611T substitution in both alleles of domain V, with an increase of MIC values for macrolides (3- to 10-fold) and lincosamides (2-fold) when associated with the A2059G substitution in one allele (Table 6).

Resistance to macrolides can also be the result of methylation of key nucleotides in domains II and/or V in bacteria (120). Methylation of DNA is an epigenetic modification (thus reversible) which concerns cytosines associated with guanine, by adding a methyl-CH3 group on carbon 5. This chemical modification, ensured by DNA-methyltransferases, may cause inhibition of the expression of certain genes without changing the sequence. Methylation has been reported in mycoplasmas (125), and methyltransferases responsible for methylation have been described in several *Mycoplasma* species (126–128), but no methylated G748 or A2058 has been identified until now.

TABLE 6 Mutations in the 23S rRNA genes and in the ribosomal proteins L4 and L22 conferring resistance in animal *Mycoplasma* species

Mutations in[a]	*Mycoplasma* species (host species)	Impact on MIC values[b]	References
23S rRNA domain II			
G748A in one or both alleles (*rrl*3 and *rrl*4)	*M. synoviae* (chicken, turkey)	Increase for Ty (up to 16-fold) and Tm (up to 67-fold)	82
G748A in both alleles (*rrl*3 and *rrl*4)	*M. bovis* (cattle)	Increase for Ty (up to 64-fold) and Tm (up to 256-fold)	116, 119, 123
C752T in *rrl*4	*M. bovis* (cattle)	No clear impact	123
G954A in *rrl*3	*M. bovis* (cattle)	ND: no isolate with only this single mutation	119
23S rRNA domain V			
G2057A	*M. hyopneumoniae* (swine)	Intrinsic resistance to Ery	92
	M. synoviae (chicken, turkey)	Intrinsic resistance to Ery	82
	M. gallisepticum (chicken)	Increase for Ery (up to 128-fold)	33, 117
A2058G in one or both alleles (*rrl*3 and *rrl*4)	*M. synoviae* (chicken, turkey)	Significant increase for Ty (up to 67-fold), Tm (up to 267-fold) and Ln (up to 64-fold)	82
	M. gallisepticum (chicken, turkey)	Significant increase for Ery (up to 8,533-fold), Ty (up to 125-fold), Tm (up to 1,000-fold) and Ln (up to 128-fold)	33, 78, 117
	M. bovis (cattle)	Significant increase for Ty (up to 32-fold) and Tm (up to 512-fold)	103, 119, 123
	M. agalactiae (sheep and goats)	Significant increase for Ty (8- to 64-fold)	111
A2059G in one or both alleles (*rrl*3 and *rrl*4)	*M. synoviae* (chicken, turkey)	Significant increase for Ty (up to 67-fold), Tm (up to 267-fold), and Ln (up to 64-fold)	82
	M. gallisepticum (chicken, turkey)	Significant increase for Ery, Ty, Tm, and Ln	33, 78, 117
	M. bovis (cattle)	Significant increase for Ty, Tm, and Ln (up to 32-fold)	116, 123
	M. agalactiae (sheep and goats)	Significant increase for Ty (320-fold) and Ln (320-fold)	111
G2144A in *rrl*3	*M. bovis* (cattle)	No clear impact	119
C2152 in *rrl*4	*M. bovis* (cattle)	No clear impact	119
A2503U	*M. gallisepticum* (*in vitro*-selected mutants)	ND: mutation always described combined with A2058G or A2059G	117
G2526A	*M. bovis* (cattle)	No clear impact	119
C2611G	*M. gallisepticum* (chicken)	ND: no isolate with this single mutation	33
C2611T	*M. agalactiae* (sheep and goats)	Increase for Ty (3- to 10-fold) and Ln (2-fold) when associated with a A2059G substitution	111
L4 protein			
G64E	*M. synoviae* (chicken, turkey)	No clear impact	82
G185R/W	*M. bovis* (cattle)	No effect alone	119
G185A/L/R/V/W	*M. bovis* (cattle)	No clear impact alone	123
T186P	*M. bovis* (cattle)	No clear impact alone	123
L22 protein			
S89L	*M. agalactiae* (sheep and goats)	Slight increase for Ty (2- to 8-fold) and no impact for Ln	111
Q90K/H	*M. agalactiae* (sheep and goats)	Slight increase for Ty (2-fold) and Ln (2-fold)	111
	M. synoviae (chicken, turkey)	No clear impact	82
Q93K/H	*M. bovis* (cattle)	Increase for Ty (up to 8-fold) and Tm (up to 16-fold)	119
Q90H	*M. bovis* (cattle)	No clear impact	123

[a]*Escherichia coli* numbering.

[b]A quantitative impact is given into brackets when the MIC increase could be calculated (when the mutation was observed alone and/or when MIC values were available to compare isolates with or without this mutation). Ery, erythromycin; Ty, tylosin; Tm, tilmicosine; Ln, lincomycin; ND, not determined (or impact difficult to evaluate because several mutations were observed at the same time).

No efflux mechanism involved in macrolide resistance has been described so far in *Mycoplasma* species, but an *erm*B methylase gene and three subtypes of active efflux *msr* gene have been reported in a macrolide- and lincosamide-resistant *Ureaplasma urealyticum* strain, which belongs to the *Mycoplasmataceae* family (129).

Tetracyclines

Tetracyclines bind to the 30S ribosomal subunit. Their binding pocket is formed by an irregular minor grove of helix 34 (residues 1196 to 1200:1053 to 1056) in combination with residues 964 to 967 from the helix 31 stem-loop (130).

Decreased susceptibilities to tetracycline in *M. bovis* strains (MICs, ≥2 μg/ml) were associated with mutations at two (A965T and A967T/C) or three (A965T, A967T/C, and G1058A/C) positions of the two 16S rRNA-encoding genes (*rrs3* and *rrs4* alleles) (116, 131). Another study showed that for *M. bovis* resistance to oxytetracycline, a single A967T point mutation in one *rrs* allele of 16S rRNA had a minor impact on MIC values (119). Homozygote mutations in positions 965 and 967 of the *rrs* genes are necessary and sufficient to increase oxytetracycline MICs and to categorize such isolates as resistant. Other point mutations evidenced in these *rrs* genes in positions 1058, 1192, and 1199 did not further modify MIC values (119). Cross-resistance between tetracycline and spectinomycin was reported in tetracycline-resistant mutants obtained *in vitro* (116).

Fluoroquinolones

Fluoroquinolones kill dividing bacteria by inhibiting the topoisomerases II and IV, which are required for DNA replication (132). Resistance to fluoroquinolones in several *Mycoplasma* species is due to alterations in the quinolone resistance-determining regions (QRDR) of the *gyr*A and *gyr*B genes encoding DNA-gyrase and the *par*C and *par*E genes encoding topoisomerase IV. The targeting of either DNA-gyrase or topoisomerase IV as the primary target by fluoroquinolones varies with the bacterial species and specific fluoroquinolone (132). Alteration of the primary target site can be followed by secondary mutations in lower-affinity binding sites, and highly resistant organisms typically carry a combination of mutations within DNA-gyrase and topoisomerase IV (133, 134).

In *M. gallisepticum*, substitutions Ser83-Arg in GyrA and Ser80-Leu/Trp in ParC QRDR were shown to have the greatest impact on resistance to fluoroquinolones (133–136). Even if DNA-gyrase seemed to be the primary target of enrofloxacin in *M. gallisepticum*, several mutations in both DNA-gyrase and topoisomerase IV were needed to reach high-level resistance to fluoroquinolones in mutant strains selected *in vitro* (134). The position and the nature of the amino acid also influenced the resistance level (134).

Reduced susceptibility or resistance to enrofloxacin in *M. synoviae* was correlated with the presence of several amino acid substitutions in the ParC QRDR (32) (Table 7): 26/43 strains with MICs between 1 and 16 μg/ml harbored the Thr80-Ile. A Ser81-Pro was also evidenced in the ParC QRDR of *M. synoviae* isolates after an *in vivo* treatment with marbofloxacin of a hen experimentally infected with *M. synoviae* (83).

Mutations in the QRDR of ParC (Ser80-Phe and Asp84-Asn) were detected in *M. hyopneumoniae* strains with reduced susceptibility to marbofloxacin isolated from infected pigs after an *in vivo* marbofloxacin treatment (118). A Ser80-Tyr substitution was also evidenced in the ParC QRDR of five field strains isolated from pig herds in Belgium and harboring reduced susceptibility to flumequine and enrofloxacin (137), and an extra mutation, Ala83-Val, leading to further increase of the enrofloxacin MIC, was also evidenced in GyrA for one of these strains (Table 7).

For *M. bovis*, point mutations detected in the GyrA and ParC QRDR could be different according to the strain origin (country of isolation, field strains versus selected mutants) (Table 7). Results from Lysnyansky and collaborators' study of strains isolated in Israel suggested that a Ser83-Phe point mutation in GyrA is sufficient to reach an intermediate level of susceptibility to enrofloxacin (MICs between 0.5 and 2 μg/ml) but that an Asp84-Asn substitution in ParC is required for resistance (MIC, >2 μg/ml) (138). Japanese field isolates with fluoroquinolone MICs of ≤2 μg/ml harbored no QRDR mutations and no Ser83-Leu point mutation in GyrA, whereas resistant isolates (MICs, ≥4 μg/ml) had a Ser83-Leu mutation in GyrA and a Ser81-Pro mutation in ParC, or a Ser83-Phe substitution in GyrA and a Ser80-Ile mutation in ParC (139). Laboratory-derived fluoroquinolone-resistant mutants selected from two isolates with a Ser83-Leu mutation in GyrA had an amino acid substitution in ParC at the same position (Ser80-Ile or Ser81-Tyr) as fluoroquinolone-resistant isolates, suggesting that a substitution in ParC at position Ser80 or Ser81 is important in fluoroquinolone resistance in *M. bovis* isolates (139). No mutations in the GyrA and ParC QRDR regions of recent French *M. bovis* strains (2009 to 2014) were evidenced to explain the slight loss of susceptibility to fluoroquinolones compared to old strains (1978 to 1983) (37). The only recurrent mutation

TABLE 7 mutations in DNA-gyrase (GyrA and GyrB) and topoisomerase IV (ParC and ParE) associated with fluoroquinolone resistance in animal *Mycoplasma* species

Mutations in[a]	*Mycoplasma* species (host species for clinical isolates)	Origin of strains[b]	Impact on MIC values for enrofloxacin[c]	References
GyrA				
Thr58-Ile	*M. gallisepticum* (chicken, turkey)	CI	ND	136
His59-Tyr	*M. gallisepticum* (chicken, turkey)	CI	ND	136
Gly81-Ala	*M. gallisepticum*	M	4-fold increase	134
Asp82-Asn	*M. bovis*	M	ND	116
Ser83-Ile	*M. gallisepticum* (chicken, turkey)	M, CI	2-fold increase	80, 133–136
Ser83-Asn	*M. gallisepticum* (chicken, turkey)	M, CI	2-fold increase	80, 133, 134
Ser83-Arg	*M. gallisepticum*	M	32-fold increase	134
Ser83-Phe	*M. bovis* (cattle)	M, CI	32-fold increase	37, 116, 138
Ser83-Tyr	*M. bovis*	M	No impact	37, 116
Ala83-Val	*M. hyopneumoniae* (swine)	CI	>2-fold increase	137
Ala84-Pro	*M. gallisepticum*	M	2-fold increase	134
Glu87-Gly	*M. gallisepticum*	M	ND	134
Glu87-Gly/Lys/Val	*M. bovis*	M	No impact	37, 116
Glu87-Lys	*M. gallisepticum* (chicken, turkey)	M, CI	ND	80, 134, 136
Asn87-Ser/Lys	*M. synoviae* (chicken, turkey)	CI	4-fold increase	32
Asn87-Lys	*M. synoviae* (chicken, turkey)	CI	No impact	32
GyrB				
Val320-Ala	*M. bovis* (cattle)	CI	ND	116
Asp362-Asn	*M. bovis* (cattle)	M, CI	Slight increase (up to 2-fold)	37
Ser401-Tyr	*M. synoviae* (chicken, turkey)	CI	ND	32
Ser402-Asn	*M. synoviae* (chicken, turkey)	CI	ND	32
Ile423-Asn	*M. bovis*	M	ND	116
Asn424-Lys	*M. agalactiae*	M	8-fold increase	112
Asp426Asn	*M. gallisepticum* (chicken, turkey)	M, CI	2-fold increase	134, 136
Asp437-Asn	*M. gallisepticum* (chicken, turkey)	CI	ND	136
Asn464-Asp	*M. gallisepticum*	M	4-fold increase	134
Glu465-Lys	*M. gallisepticum*	M	No impact	134
Glu465-Gly	*M. gallisepticum*	M	2-fold increase	134
ParC				
Ala64-Ser	*M. gallisepticum*	M	4-fold increase	134
Gly78-Cys	*M. bovis*	M	ND	116
	M. agalactiae	M	2- to 8-fold increase	112
Asp79-Asn	*M. synoviae* (chicken, turkey)	CI	2-fold increase	32
	M. agalactiae	M	ND	112
Ser80-Leu	*M. gallisepticum* (chicken, turkey)	M, CI	8-fold increase	80, 101, 134–136
Ser80-Trp	*M. gallisepticum* (chicken)	M, CI	16-fold increase	134, 135
Ser80-Ile	*M. bovis* (cattle)	M, CI	2- to 8-fold increase	37, 116
Ser80-Phe	*M. hyopneumoniae* (swine)	EI	8-fold increase	118
Ser80-Tyr	*M. hyopneumoniae* (swine)	CI	8-fold increase	137
Thr80-Ile	*M. agalactiae*	M, CI	4- to 8-fold increase	112
Thr80-Ala/Ile	*M. synoviae* (chicken, turkey)	CI	2 to 8-fold increase	32
Ser81-Pro	*M. gallisepticum*	M	2 to 4-fold increase	134
	M. synoviae (chicken, turkey)	CI, EI	2 to 4-fold increase	32, 83
Glu84-Gly	*M. gallisepticum*	M	4-fold increase	134
Glu84-Gln	*M. gallisepticum*	M	2-fold increase	134
Glu84-Lys	*M. gallisepticum*	M	4-fold increase	134

(continued)

TABLE 7 mutations in DNA-gyrase (GyrA and GyrB) and topoisomerase IV (ParC and ParE) associated with fluoroquinolone resistance in animal *Mycoplasma* species *(continued)*

Mutations in[a]	*Mycoplasma* species (host species for clinical isolates)	Origin of strains[b]	Impact on MIC values for enrofloxacin[c]	References
Asp84-Asn	*M. bovis* (cattle)	M, CI	2-fold increase	37, 116, 138
	M. agalactiae (sheep and goats)	M, CI	2- to 8-fold increase	112
	M. synoviae (chicken, turkey)	CI	4-fold increase	32
	M. hyopneumoniae (swine)	EI	8-fold increase	118
Asp84-Tyr	*M. agalactiae* (sheep and goats)	M, CI	4- to 8-fold increase	112
Asp84-Tyr/Gly	*M. bovis* (cattle)	M, CI	ND	37
Thr98-Arg	*M. bovis*	M	ND	37
ParE				
Asp420-Asn	*M. gallisepticum*	M	2-fold increase	134
	M. synoviae (chicken, turkey)	CI	ND	32
Asp420-Lys	*M. gallisepticum*	M	ND	133
Gly429-Ser	*M. agalactiae*	M	ND	112
Glu459-Lys	*M. agalactiae*	M	8-fold increase	112
Ser463-Leu	*M. gallisepticum*	M	4-fold increase	134
Cys467-Phe	*M. gallisepticum*	M	ND	134

[a]Genes and amino acid substitutions (*Escherichia coli* numbering).
[b]M, mutants after *in vitro* selection, CI, clinical isolates, EI, experimental infection.
[c]A quantitative impact is given when the MIC increase could be calculated (when the mutation was observed alone and/or when MIC values were available to compare isolates or mutants with or without this mutation). ND, not determined (or impact difficult to evaluate because several mutations were observed at the same time).

that was present in all recent strains and absent from old ones was Asp362-Asn in the GyrB QRDR. However, alterations in GyrB have rarely been associated with a loss of susceptibility to fluoroquinolones, except in *M. gallisepticum*, where the Asp362-Asn substitution was detected in mutants selected *in vitro* (133) (Table 7). The most frequently observed substitutions in fluoroquinolone-resistant *M. bovis* clones selected *in vitro* from French clinical isolates were Ser83-Phe in GyrA and Asp84-Asn/Tyr in ParC, leading to 8- to 16-fold increases in the MICs. The Ser83-Phe in GyrA and Ser80-Ile in ParC combination of mutations was observed less frequently (only 3 of 72 selected clones) and was associated with 16- to 128-fold increases in the MICs (37). This combination of mutations was observed for Japanese and Chinese clinical isolates of *M. bovis* (139, 140).

In vitro resistance selection studies clearly confirmed the existence of hot spots for mutations conferring high resistance levels and the cumulative effects of mutations in GyrA and ParC on the MICs in several *Mycoplasma* species (37, 133, 139). Moreover, Khalil and collaborators showed that different clinical isolates, with different initial MICs and different genetic subtypes, were not equal in their ability to gain resistance to fluoroquinolones *in vitro*: some isolates were more likely to rapidly accumulate mutations in their QRDRs under selective pressure *in vitro* and hence to become resistant (37). Sulyok and collaborators showed that *in vitro*-selected fluoroquinolone-resistant mutants of *M. bovis* remained resistant after serial passages in antibiotic-free medium (116).

For *M. agalactiae*, point mutations were detected in the ParC QRDR of strains isolated between 2013 and 2015 (112): Asp83-Asn/Lys or Thr80-Ile point mutations resulted in 2- to 8-fold increases in MICs of fluoroquinolones (Table 7). Other mutations were evidenced in GyrB (position 424), ParC (positions 78, 79, 80, and 84), and ParE (positions 429 and 459) in mutants selected *in vitro* (112). The *parC* gene was the first gene harboring point mutations in isolates or mutants with reduced susceptibility to fluoroquinolones, suggesting that it could be the primary target of fluoroquinolones for *M. agalactiae*.

Target mutations are the main mechanisms conferring resistance to fluoroquinolones in *Mycoplasma* species. However, the active efflux mechanism is an alternative mechanism in mycoplasmas that could lead to acquired resistance to fluoroquinolones and explain a moderate shift in susceptibility. It has been described for *Mycoplasma hominis*, a human urogenital mycoplasma that belongs to the same phylogenetic group as *M. bovis*, and was linked to the overexpression of genes *md1* and *md2*, encoding multidrug resistance ATP-binding cassette transporters (141). In another ruminant mycoplasma, *Mycoplasma mycoides* subsp. *capri*, orthovanadate, an inhibitor of ATP-binding cassette efflux pumps, was able to induce a 2-fold decrease of the MICs of three fluoroquinolones in both clinical and *in vitro* mutants,

suggesting the contribution of an efflux mechanism to the overall resistance patterns of isolates (142). Since the moderate increase of the MICs observed between the recent (2009 to 2012) and old (1978 to 1983) *M. bovis* populations could be a consequence of an efflux system, which usually confers low levels of resistance, this efflux hypothesis was explored in a set of isolates with reduced susceptibility to fluoroquinolones, without success (37).

Other Antibiotics

Pleuromutilin antibiotics inhibit protein synthesis by binding to the bacterial 50S ribosomal subunit at the peptidyl transferase center, therefore inhibiting the peptide bond formation (143). Point mutations in the 23S rRNA gene and L3 protein are associated with decreased susceptibility to pleuromutilins (tiamulin or valnemulin) in several bacterial species. No mutation in protein L3 was evidenced in pleuromutilin-resistant mutants of *M. gallisepticum* selected *in vitro* (144). However, several point mutations were found in *rrn*A and/or *rrn*B alleles of domain V of the 23S rRNA gene at positions 2058, 2059, 2061, 2447, and 2503. Although a single mutation could cause an increase of tiamulin and valnemulin MICs, combinations of two or three mutations were necessary to produce high-level resistance (144). All pleuromutilin-resistant mutants exhibited cross-resistance to lincomycin, chloramphenicol, and florfenicol. Mutants with the A2058G or the A2059G mutation showed cross-resistance to macrolides (erythromycin, tilmicosin, and tylosin). In another study, all mutants selected *in vitro* for resistance to tiamulin showed cross-resistance to florfenicol and elevated lincomycin MICs (116). Substitutions C2035A, A2060G, G2062T, and C2500A were found in pleuromutilin-resistant mutants; these positions are closely associated with the pleuromutilin binding sites on the 23S rRNA genes.

Resistance to florfenicol was shown to be associated with a G2062T or a A2063T substitution in at least one allele of the 23S rRNA genes. In addition, a substitution G2506A showed cross-resistance with tiamulin (116).

Hungarian field strains of *M. bovis* with high spectinomycin MICs (≥256 μg/ml) and mutants selected *in vitro* in the presence of subinhibitory concentrations of spectinomycin harbored a single mutation: C1192A for field isolates and C1192T in mutants (116).

CONCLUSIONS

Several recent studies have shown a significant decrease in the susceptibility of animal mycoplasmas to several families of antibiotics. Some strains of *M. bovis* show currently high *in vitro* MIC levels for several antibiotics usually used to treat these infections *in vivo*. The highest resistances of the main veterinary *Mycoplasma* species are observed for macrolides, followed by tetracyclines. Although resistant strains have been described for fluoroquinolones, most strains remain susceptible to this family of antibiotics. Pleuromutilins are the most effective antibiotics *in vitro*. However, due to different usage practices of antimicrobials, frequencies of resistance can vary considerably from one country to another but also within a country between isolates from different origins (e.g., mastitis versus respiratory disease). It is therefore important to perform antimicrobial susceptibility testing periodically, on a regional basis, to monitor levels of susceptibility to several antibiotics for rational *in vivo* treatment strategies. The development of next-generation sequencing techniques in recent years has made it easier to study the resistance mechanisms of mycoplasmas to antibiotics and could rapidly detect mutations that have a significant impact on the resistance of mycoplasma species to antimicrobial agents, avoiding the long and tedious steps of *in vitro* culture. Further work should be carried out to determine breakpoints for veterinary mycoplasmas, based on molecular mutations, so that *in vitro* information can be used to provide advice for a prudent and targeted use of antimicrobials that are likely to be effective *in vivo*, to limit the development of antimicrobial resistance. However, the true measure of the effectiveness of an antimicrobial is its *in vivo* activity against mycoplasmas as well as other bacteria which are often associated with mycoplasmas and which often contribute to a more severe expression of the disease.

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