Veterinary Microbiology 204 (2017) 188–193

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Antimicrobial susceptibility monitoring of *Mycoplasma hyopneumoniae* and *Mycoplasma bovis* isolated in Europe

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ARTICLE INFO

Keywords:
Antimicrobial susceptibility
Surveillance
Minimal inhibitory concentrations
Mycoplasma hyopneumoniae
Mycoplasma bovis

ABSTRACT

Mycoplasma hyopneumoniae in pigs and *Mycoplasma bovis* in cattle are major pathogens affecting livestock across Europe and are the focus of the MycoPath pan-European antimicrobial susceptibility monitoring programme. Fifty *M. hyopneumoniae* isolates from Belgium, Spain and the United Kingdom (UK), and 156 *M. bovis* isolates from France, Hungary, Spain and the UK that met specific criteria were tested for antimicrobial susceptibility in a central laboratory by using a microbroth dilution method. Specific isolate criteria included recovery from animals not recently treated with antimicrobials, isolates from different locations within each country and retaining only one isolate per farm. MIC_{50}/MIC_{90} values were 0.031/0.5, 0.031/0.5, 0.062/0.25, ≤0.001/0.004, 0.031/0.125, 0.25/0.5 and 0.062/0.25 mg/L for enrofloxacin, marbofloxacin, spiramycin, tulathromycin, tylosin, florfenicol and oxytetracycline respectively against *M. hyopneumoniae* and 0.25/4, 1/4, 4/16, > 64/ > 64, 32/ > 64, 2/4 and 4/64 mg/L, respectively against *M. bovis*. MIC_{50}/MIC_{90} values for tiamulin and valnemulin against *M. hyopneumoniae* were 0.016/0.062 and ≤0.001/ ≤0.001 mg/L respectively. The MIC_{50}/MIC_{90} values of danofloxacin and gamithromycin for *M. bovis* were 0.25/1 and > 64/ > 64 mg/L respectively. The highest MIC_{90} values for *M. hyopneumoniae* were found in the UK at 1.0 mg/L for enrofloxacin, marbofloxacin and florfenicol. In contrast, for *M. bovis* the lowest MIC_{90} value was 1.0 mg/L, but ranged to > 64 mg/L. Specific laboratory standards and clinical breakpoints for veterinary *Mycoplasma* species are required as no independently validated clinical breakpoints are specified for veterinary *Mycoplasma* species, which makes data interpretation and correlation to *in vivo* efficacy difficult.

1. Introduction

Mycoplasma species are responsible for causing many diseases that can affect animals or man with severe adverse impacts on health, welfare and economics. *Mycoplasma hyopneumoniae* in pigs and *Mycoplasma bovis* in cattle are major pathogens affecting livestock species across Europe and are the focus of the MycoPath pan-European antimicrobial susceptibility monitoring programme.

M. hyopneumoniae causes enzootic pneumonia in pigs which can be fatal, and can also reduce performance, and predispose pigs to secondary infections with bacteria including *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* (Marois et al., 2009) and *Streptococcus suis* (Maes et al., 2008). *M. hyopneumoniae* prolongs and potentiates the severity of porcine reproductive and respiratory syndrome virus

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http://dx.doi.org/10.1016/j.vetmic.2017.04.012
Received 4 February 2017; Received in revised form 7 April 2017; Accepted 12 April 2017
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(PRRSV) (Thacker et al., 1999) and porcine circovirus type 2 (PCV2) associated pneumonia in pigs (Opriessnig et al., 2004). Although several *M. hyopneumoniae* commercial vaccines are in use, antimicrobial treatment is also used to control infections.

M. bovis is a major cause of bovine respiratory disease (BRD), and also causes many other clinical conditions including mastitis and arthritis (Nicholas and Ayling, 2003). Although *M. bovis* can be the sole cause of BRD, it is often multifactorial, with secondary infections by other bacteria, including *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, *Trueperella pyogenes,* other *Mycoplasma* species; and viruses including bovine respiratory syncytial virus (BRSV), parainfluenza 3 (PI3), adenovirus, bovine viral diarrhoea virus (BVDV), and infectious bovine rhinotracheitis (IBR) (Taylor et al., 2010). No commercial vaccines for *M. bovis* are available in Europe, although some autogenous vaccines are produced, so antimicrobial treatment remains the only option for treating affected cattle.

Mycoplasma species lack a cell wall and are therefore refractory to all antimicrobials that target the cell wall (e.g., ß-lactams; Lysnyansky and Ayling, 2016). Hence, relatively few antimicrobials are effective or licensed for treating *Mycoplasma* infections. The antimicrobials included in this study belong to five chemical classes: the fluoroquinolones (enrofloxacin, marbofloxacin (and danofloxacin for *M. bovis*)); the macrolides (spiramycin, tulathromycin, tylosin (and gamithromycin for *M. bovis*)); the pleuromutilins (tiamulin and valnemulin, both just for *M. hyopneumoniae*); the amphenicols (florfenicol) and the tetracyclines (oxytetracycline). The fluoroquinolones have an affinity for the DNA gyrase and topoisomerase IV enzymes but also allow penetration of the bacterial outer membrane (Vicca et al., 2007). The main mode of action for the remaining antimicrobials is by inhibiting protein synthesis. The macrolides are thought to prevent peptidyl transferase from adding the growing peptide attached to tRNA to the next amino acid as well as inhibiting ribosomal translation (Stakenborg et al., 2005). Pleuromutilins bind to the 50S ribosomal subunit of bacteria and tiamulin and valnemulin are strong inhibitors of peptidyl transferase (Poulsen et al., 2001). Florfenicol also binds to the 50S ribosomal subunit inhibiting the peptidation reaction and the translation of bacterial mRNA. Tetracyclines are bacteriostatic antibiotics that bind irreversibly to receptors of the 30S bacterial ribosomal subunit and blocking an attachment of aminoacyl-tRNA to the acceptor site on the mRNA ribosome complex resulting in inhibition of bacterial protein synthesis (Chopra and Roberts, 2001; Bryskier, 2005).

The present study was conducted as part of the Centre Européen d'Etudes pour la Santé Animale (CEESA) monitoring programmes (de Jong et al., 2013). The MycoPath programme aims to create a pan-European collection of representative *Mycoplasma* pathogens isolated from clinical cases of diseased cattle and pigs not recently exposed to antimicrobials. Antimicrobial susceptibilities of *M. hyopneumoniae* and *M. bovis* recovered from three and four European countries respectively are presented here. Antimicrobial susceptibility testing for veterinary *Mycoplasma* species lack quality control standard strains, test methods and breakpoints, although guidelines have been published (Hannan, 2000). Standards for the *Mycoplasma* testing of significant clinical infections in humans (*Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Ureaplasma urealyticum*) have been published (CLSI, 2011; Waites et al., 2012). However, the growth requirements of these human *Mycoplasma* species differ from *M. hyopneumoniae* and *M. bovis*. So the growth media that is suitable for testing these human species cannot be applied to livestock species although the microbroth dilution methods used in this study essentially follow the CLSI guidelines.

2. Methodology

2.1. Bacterial collection

Mycoplasma isolates were obtained following specific criteria which included clinical signs, lack of antimicrobial treatment in the previous 15 days, samples from different locations within each participating country and only one isolate per farm. The participating national laboratories followed their standard *Mycoplasma* culture isolation and identification procedures (Nicholas and Baker, 1998) including PCR methods and 16S rRNA gene sequencing (Janda and Abbott, 2007). Isolates were stored at temperatures below −50 °C, before transfer to the central laboratory (Don Whitley Scientific, Shipley, UK) for antimicrobial susceptibility testing.

From 2010 to 2012, the Belgian, Spanish and UK national laboratories isolated *M. hyopneumoniae* from post-mortem sampling from pigs aged from three weeks to seven months with clinical signs of respiratory disease, or from slaughterhouse lung samples with pathology consistent with enzootic pneumonia if the pigs were from known infected herds. During the same period the French, Hungarian, Spanish and UK national laboratories obtained *M. bovis* cultures from cattle aged between three weeks and one year that had clinical signs of respiratory disease, including depression, hyperthermia, polypnea, dyspnea, cough or nasal discharge. Specimens included lung tissue and nasopharyngeal swabs.

2.2. Antimicrobial testing

All *Mycoplasma* isolates were transferred to the central laboratory for antimicrobial susceptibility testing. The isolates were checked for viability; with *M. hyopneumoniae* being cultured in Friis medium (Friis, 1975) and *M. bovis* in modified Hayflicks medium (Hayflick, 1965) with 5% Alamar Blue and 0.01% Nicotinamide Adenine Dinucleotide. These media were also used in the susceptibility testing. Each culture was grown and viable count determined by plating onto the appropriate agar medium so that cell density could be adjusted to 10^6 cfu/ml for the MIC test. Identity was confirmed for 25% of the isolates using PCR methods. *M. hyopneumoniae* identification was confirmed using a duplex PCR method that identified both *M. hyopneumoniae* and *M. hyorhinis* giving a 430 bp and 346 bp amplicon, respectively (Barate et al., 2012). *M. bovis* identification was confirmed using a PCR that targets the *vsp* genes (Tenk et al., 2006). *M. hyopneumoniae* (NCTC 10110/ATCC 25934) and *M. bovis* (NCTC 10131/ATCC 25523) strains were used as positive controls respectively for the PCRs and for the antimicrobial susceptibility testing.

Antimicrobial susceptibility testing used a microbroth dilution method to determine the minimal inhibitory concentrations (MICs). The antimicrobials were prepared using the CLSI recommended dilution method (CLSI, 2013) to give a final active concentration range from 0.001 to 64 mg/L. The stock solutions containing 1280 mg/L of each antimicrobial were prepared as follows. Tiamulin hydrogen fumarate (Novartis, Switzerland), valnemulin hydrogen chloride (Novartis, Switzerland) and oxytetracycline hydrochloride (Sigma-Aldrich, UK) were prepared in deionized water; danofloxacin (Sigma-Aldrich, UK), enrofloxacin (Bayer, Germany) and marbofloxacin (Sigma-Aldrich, UK) were prepared in half of the final volume of deionized water and then 1.0 M sodium hydroxide was added dropwise until dissolution occurred and then made to the correct final volume with deionized water; florfenicol (Sigma-Aldrich, UK), spiramycin (Sigma-Aldrich, UK) and tylosin tartrate (Sigma-Aldrich, UK) were dissolved in 95% ethanol before being made to the correct final volume with deionized water. Tulathromycin (Pfizer Inc., USA) was prepared as an equilibrated solution in accordance with the manufacturer's recommendations. This was achieved by adding 10 ml of 0.015 M citric acid solution to the tulathromycin which would give a final concentration of 1280 mg/L in 100 ml. The solution was checked to have a pH of 7.0 ± 0.2 and placed in a waterbath at 70 °C ± 2 °C for 90 min and shaken regularly. It was then cooled to 20 °C ± 2 °C and made to the final volume with deionized water, so the citric acid concentration was approximately 0.0015 M. Gamithromycin (Hovione, Ireland) was dissolved and made up to the final volume in 0.1 M phosphate buffer pH 6.0.

To determine the MICs for each isolate, 100 µl of the appropriate

antimicrobial solution was distributed into the conical wells of polystyrene microtitre plates, before 100 µl of culture which had been thawed and pre-incubated for 1 h was added to each well. This gave a range of antimicrobials from 0.001 to 64 mg/L with a final cell concentration of approximately 5×10^5 cfu/ml. A positive (growth) control well contained no antimicrobial with 100 µl of sterile medium in its place and a single well with 200 µl of sterile medium for each strain served as a negative uninoculated control. Immediately after inoculation, microtitre plates were placed in a humidified atmosphere and incubated at 35 °C ± 1 °C; for *M. hyopneumoniae* 2–12 days, for *M. bovis* 24 h. Plates were inspected daily. If no growth was evident in the positive control wells, plates were reincubated for a further 24 h. For each isolate, MIC results were read as soon as adequate growth was apparent in the positive control wells. All MIC plates were read against a white background to facilitate identification of colour changes in the medium. The colour changes were from red (no growth) to yellow (growth) for *M. hyopneumoniae* and from blue (no growth) to pink (growth) for *M. bovis*. The MIC of each antimicrobial was recorded as the lowest concentration that completely inhibited growth. For the test to be considered valid, it was necessary for a clear colour change to be visible in the positive control well and for the negative control well to remain unchanged. The reproducibility of the test was demonstrated by ensuring that the MIC results of the quality control strains did not vary by more than ± 1 doubling dilution of a central value. In cases where the MIC results obtained for an antimicrobial agent against one or more strains in a group deviated markedly from the MICs obtained against the majority of strains in that group, the MIC test was repeated twice. In such cases, the final MIC value was obtained on at least two separate occasions.

2.3. Data analysis

The MIC ranges, MIC distributions, MIC_{50} and MIC_{90} values were determined for each antimicrobial and *Mycoplasma* species, and for each country. Analyses also used the non-parametric Mann-Whitney-U-Test (Mann and Whitney, 1947) where a *P*-value of ≤0.05 was considered as a significant difference. The relevance of the differences between groups was quantified using as a corresponding effect size the Mann-Whitney (MW) superiority measure and its two-sided 95.0% confidence interval. The MW measure (0.0–1.0) gives the probability that a randomly selected case of the test group is better off than a randomly selected case of the comparator group. A MW estimator of 0.36 and 0.64 was used as a benchmark to indicate potentially significant differences, which was based on well-known benchmark values (Coldiz et al., 1988).

3. Results

3.1. Mycoplasma isolates

A total of 50 *M. hyopneumoniae* isolates associated with respiratory disease were submitted to the central laboratory for antimicrobial susceptibility testing, 16 from Belgium, 14 from Spain and 20 from the UK. Three of the UK isolates, fulfilling the inclusion criteria of our study, were obtained prior to 2010 and were additionally included. France submitted 43 *M. bovis* isolates, Hungary 37, Spain 37 and the UK 39, giving a total of 156 *M. bovis* strains. Nearly all *M. bovis* originated from cases of respiratory disease, with the exception of five isolates from vaginal swabs samples collected in Hungary that were associated with cases of abortion.

3.2. Antimicrobial susceptibilities for M. hyopneumoniae (Tables 1 and 2)

The *M. hyopneumoniae* MIC results for each country's isolates and for all isolates are given as MIC range, MIC_{50} and MIC_{90} in Table 1, including the MICs of the quality control isolate. Distribution of MIC values is presented in Table 2. For the fluoroquinolones, enrofloxacin and marbofloxacin, similar MIC values were determined ranging from 0.002 to 1 mg/L, with no significant differences between the three countries the isolates originated from. The macrolides and modified macrolides displayed $MIC_{50/90}$ values of 0.062/0.25 mg/L for spiramycin, ≤ 0.001/0.004 mg/L for tulathromycin and 0.031/0.125 mg/L for tylosin. Significantly higher MIC values were obtained for Belgium and the UK than for Spain for spiramycin and tylosin ($P \leq 0.05$). Low MIC ranges and $MIC_{50/90}$ values were found for both pleuromutilin antibiotics: MIC range of 0.002-0.125 mg/L and $MIC_{50/90}$ 0.016/0.062 mg/L for tiamulin and MIC range ≤0.001–0.002 mg/L and $MIC_{50/90}$ ≤0.001/ ≤ 0.001 mg/L for valnemulin. Similar valnemulin $MIC_{50/90}$ values were determined for all three countries but tiamulin MIC values were significantly higher for the UK when compared to those from Spain. Florfenicol MIC values ranged from 0.016 to 1 mg/L, with the maximum value of 1 mg/L observed in some isolates from the UK. Oxytetracycline showed the widest distribution in MIC values, ranging from ≤0.001 to 2 mg/L, with $MIC_{50/90}$ values of 0.031/0.25 (UK); 0.062/0.125 (Spain) and 0.062/0.5 mg/L (Belgium). The MIC distribution of the antimicrobials indicates a narrow distribution for valnemulin and tulathromycin in comparison to broader distribution for the other antimicrobials tested.

3.3. Antimicrobial susceptibilities for M. bovis (Tables 3 and 4)

The *M. bovis* results are given in Tables 3 and 4 and include the MIC values from the five Hungarian abortion cases which had comparable MIC values to those from respiratory cases. Similar MIC ranges were determined for danofloxacin (0.062– > 64 mg/L), enrofloxacin (0.125– > 64 mg/L) and marbofloxacin (0.25– > 64 mg/L) with $MIC_{50/90}$ values of 0.25/1, 0.25/4 and 1/4 mg/L, respectively. Only France and Spain had isolates with MIC at > 64 mg/L for danofloxacin, with Spain having the highest MIC_{90} value of 2 mg/L. The same Spanish and French isolates had MIC > 64 for enrofloxacin and marbofloxacin, which was significantly different from the UK. Spain also had the highest MIC_{90} values for marbofloxacin at 16 mg/L compared with 2 mg/L for the UK and Hungary and 1 mg/L for France. For all isolates the macrolide antimicrobial $MIC_{50/90}$ values were 4/16 mg/L for spiramycin; 32/ > 64 mg/L for tylosin; and > 64 mg/L for both gamithromycin and tulathromycin. When compared to French isolates, Hungary, Spain and partially UK had significantly lower MIC values for all four macrolide antimicrobials. The UK had the lowest MIC_{50} value for tulathromycin at 1 mg/L compared to > 64 mg/L for the other three countries. The florfenicol $MIC_{50/90}$ values of 2/4 mg/L were the same for all four countries, although France and Spain had one and two isolates respectively at > 64 mg/L. The MIC range for oxytetracycline was 0.25– > 64 mg/L with $MIC_{50/90}$ of 4/ > 64 mg/L, with Hungary, Spain and the UK isolates with MIC_{90} values of > 64 mg/L compared to France with an MIC_{90} of 16 mg/L.

4. Discussion

Although guidelines for testing veterinary *mycoplasmas* have been published (Hannan, 2000), different methods have been used historically in making comparison of published MIC results difficult. In this study, use of a single laboratory to perform all of the MIC testing ensured consistency in MIC values obtained for isolates received from the contributing laboratories of different EU countries and facilitates comparison of data among different countries. A notable observation in this study is the difference in the MIC values obtained for *M. hyopneumoniae* when compared to those of *M. bovis*. Comparison of the MIC_{50} values for all isolates highlights these differences: enrofloxacin and marbofloxacin both were 0.25 mg/L for *M. bovis* compared with 0.031 mg/L for *M. hyopneumoniae*; spiramycin, tylosin and tulathromycin were 4, > 64 and 32 mg/L respectively for *M. bovis* compared with 0.062, 0.031 and ≤0.001 mg/L for *M. hyopneumoniae*;

Table 1
Minimum inhibition concentration (MIC) values for nine antimicrobial agents against 50 *Mycoplasma hyopneumoniae* isolates. Total values and value for each of the three different countries. Control strain data is also given.

Country of Origin	MIC parameter	Result (mg/L)								
		Enrofloxacin	Marbofloxacin	Spiramycin	Tulathromycin	Tylosin	Tiamulin	Valnemulin	Florfenicol	Oxytetracycline
Belgium (16 isolates)	MIC Range	0.008–1	0.002–1	0.008–0.5	≤0.001–0.016	0.004–0.25	0.002–0.062	≤0.001–0.002	0.016–0.5	≤0.001–1
	MIC_{50}	0.016	0.031	0.062	0.002	0.031	0.016	≤0.001	0.25	0.062
	MIC_{90}	0.5	0.5	0.125	0.008	0.125	0.062	0.002	0.5	0.5
Spain (14 isolates)	MIC Range	0.016–0.5	0.016–0.5	0.016–0.125	≤0.001–0.004	0.008–0.031	0.004–0.031	≤0.001–0.002	0.031–0.5	0.016–0.25
	MIC_{50}	0.031	0.125	0.031	≤0.001	0.016	0.016	≤0.001	0.25	0.062
	MIC_{90}	0.5	0.5	0.062	0.004	0.031	0.016	≤0.001	0.5	0.125
United Kingdom (20 isolates)	MIC Range	0.008–1	0.016–1	0.008–0.5	≤0.001–0.016	0.008–0.5	0.008–0.125	≤0.001–0.002	0.016–1	≤0.001–2
	MIC_{50}	0.031	0.031	0.062	≤0.001	0.031	0.031	≤0.001	0.5	0.031
	MIC_{90}	1.0	1.0	0.25	0.004	0.25	0.062	≤0.001	1.0	0.25
All 50 isolates	MIC Range	0.008–1	0.002–1	0.008–0.5	≤0.001–0.016	0.004–0.5	0.002–0.125	≤0.001–0.002	0.016–1	≤0.001–2
	MIC_{50}	0.031	0.031	0.062	≤0.001	0.031	0.016	≤0.001	0.25	0.062
	MIC_{90}	0.5	0.5	0.25	0.004	0.125	0.062	≤0.001	0.5	0.25
Control NCTC 10110	MIC Range	0.016–0.031	0.031	0.062	≤0.001–0.002	0.016–0.031	0.008–0.031	≤0.001–0.002	0.5	0.125–0.25

Table 2
MIC distribution for nine antimicrobial agents against 50 *Mycoplasma hyopneumoniae* isolates from *Mycoplasma* infections in pigs.

Antimicrobial agent	MIC (mg/L)																MIC_{50} (mg/L)	MIC_{90} (mg/L)
	≤0.001	0.002	0.004	0.008	0.016	0.032	0.064	0.12	0.25	0.5	1	2	4	8	16	32	64	
Enrofloxacin				3	20	11	1	1	5	5	4							0.03	0.5
Marbofloxacin		1			14	18		2	5	6	4							0.03	0.5
Spiramycin				5	4	9	17	9	4	2								0.06	0.25
Tulathromycin	26	8	12	1	3													≤0.001	0.002
Tylosin			1	6	13	17	7	2	3	1								0.03	0.12
Tiamulin		1	1	12	14	14	6	2										0.016	0.06
Valnemulin	46	4																≤0.001	≤0.001
Florfenicol					3	2	4	5	16	16	4							0.25	0.5
Oxytetracycline	2	1	3	3	8	6	8	7	9	1	1	1						0.06	0.25

Table 3
Minimum inhibition concentration (MIC) values for nine antimicrobial agents against 156 *Mycoplasma bovis* isolates. Total values and value for each of the four different countries. Control strain data is also given.

Country of Origin	MIC parameter	Result (mg/L)								
		Danofloxacin	Enrofloxacin	Marbofloxacin	Gamithromycin	Spiramycin	Tulathromycin	Tylosin	Florfenicol	Oxytetracycline
France (43 isolates)	MIC Range	0.125– > 64	0.25– > 64	0.25– > 64	2– > 64	2– > 64	1– > 64	16– > 64	0.5– > 64	0.5– > 64
	MIC_{50}	0.25	0.25	1	> 64	8	> 64	64	2	4
	MIC_{90}	1	0.5	1	> 64	8	> 64	> 64	4	16
Hungary (37 isolates)	MIC Range	0.125–1	0.125–8	0.25–8	1– > 64	0.125– > 64	0.031– > 64	0.125– > 64	0.5–8	0.25– > 64
	MIC_{50}	0.5	0.5	1	> 64	1	> 64	1	2	4
	MIC_{90}	1	1	2	> 64	8	> 64	> 64	4	> 64
Spain (37 isolates)	MIC Range	0.062– > 64	0.125– > 64	0.25– > 64	2– > 64	0.125– > 64	0.062– > 64	0.5– > 64	0.5– > 64	1– > 64
	MIC_{50}	0.25	0.5	1	> 64	4	> 64	32	2	4
	MIC_{90}	2	16	16	> 64	16	> 64	> 64	4	> 64
United Kingdom (39 isolates)	MIC Range	0.062–8.0	0.125–8	0.5–8	1– > 64	0.125– > 64	0.062– > 64	0.25– > 64	0.5–8	0.25– > 64
	MIC_{50}	0.125	0.25	1	8	4	1	16	2	4
	MIC_{90}	1	2	2	> 64	16	> 64	> 64	4	> 64
All 156 isolates	MIC Range	0.062– > 64	0.125– > 64	0.25– > 64	1– > 64	0.125– > 64	0.031– > 64	0.125– > 64	0.5– > 64	0.25– > 64
	MIC_{50}	0.25	0.25	1	> 64	4	> 64	32	2	4
	MIC_{90}	1	4	4	> 64	16	> 64	> 64	4	> 64
Control NCTC 10131	MIC Range	0.125–0.25	0.125–0.25	0.5–1	2–4	0.25–0.5	0.125–0.25	0.125–0.25	1–4	0.25

florfenicol 2 mg/L, oxytetracyline 4 mg/L for *M. bovis* compared with 0.25 and 0.062 mg/L for *M. hyopneumoniae* respectively. The optimum growth media for each organism was used as described in the guidelines (Hannan, 2000); the only other minor difference between the tests was the growth indicator used. *M. hyopneumoniae* growth was detected by pH change resulting from the acid production by the fermentation of glucose, whereas Alamar Blue that detects respiration rather growth was used for *M. bovis* which could arguably be slightly more sensitive, but has been validated previously (Rosenbusch et al., 2005). These substantial differences in MIC values clearly indicate *M. bovis* has

Table 4
MIC distribution for nine antimicrobial agents against 156 *Mycoplasma bovis* isolates from *Mycoplasma* infections in cattle.

Antimicrobial agent	MIC (mg/L)																	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
	≤0.001	0.002	0.004	0.008	0.016	0.032	0.064	0.12	0.25	0.5	1	2	4	8	16	32	64	> 64	
Danofloxacin							3	49	52	21	23	7	1	1				3	0.25	1
Enrofloxacin								19	66	36	13	6	4	6	3			3	0.25	4
Marbofloxacin									6	47	75	9	6	7	3			3	1	4
Gamithromycin											5	17	11	9	2	2		110	> 64	> 64
Tulathromycin						1	14	7	4	7	9	3	1	1	1	1		107	> 64	> 64
Spiramycin								7	15	18	9	7	45	36	13		1	5	4	16
Tylosin								2	9	13	5	2	9	12	15	29	21	39	32	> 64
Florfenicol										6	29	69	46	3				3	2	4
Oxytetracycline									3	9	12	35	33	23	11			30	4	> 64

different *in vitro* antimicrobial susceptibility profiles to *M. hyopneumoniae*. Other studies on the antimicrobial susceptibility of *Mycoplasma* species usually refer to one species (Vicca et al., 2004; Tavío et al., 2014; Ayling et al., 2014). However, Hannan et al. (1997) examined the comparative susceptibilities of a wide range of *Mycoplasma* species, and have assessed similar susceptibility differences between *M. hyopneumoniae* (n = 20) and *M. bovis* (n = 20) for enrofloxacin, tylosin and oxytetracycline as in our study, but not for tiamulin.

Several factors could explain why differences in species and antimicrobial susceptibilities have occurred. It could be differences in the organism and their host affinities, their growth requirements with *M. hyopneumoniae* fermenting glucose and considered to be more fastidious, while *M. bovis* utilises pyruvate. Regardless, it is important to understand that *in vitro* MIC results do not necessarily correlate to the effectiveness of the antimicrobials *in vivo* and that interpretation of the MIC distributions is made difficult as veterinary *Mycoplasma* species do not have defined clinical breakpoints.

Several authors previously reported decreased susceptibility of *M. bovis* in Europe as reported by high MIC results (Ayling et al., 2000, 2014; Sulyok et al., 2014; Becker et al., 2015). All nine antimicrobials tested here had *M. bovis* isolates with MIC values of > 64 mg/L, which includes the fluoroquinolones and macrolide antimicrobials. It should be noted that the importance of correct buffering in the MIC test to avoid artificial MIC shifts (Godinho, 2008) was respected in our study. Whilst several reports demonstrated that *in vitro* testing does not necessarily relate to antimicrobial effectiveness in the field, especially for macrolides such as tulathromycin and especially in the absence of clinical breakpoints (Godinho et al., 2005; Bartram et al., 2016). Several research groups have demonstrated that high MIC values for *M. bovis* are associated with mutations in genes that are known to be associated with antimicrobial resistance in other bacteria (Sulyok et al., 2017). Lerner et al. (2014) reported that point mutations in the 23S rRNA alleles were associated with decreased susceptibility to the macrolides tylosin and tilmicosin. Amram et al. (2015) demonstrated that *M. bovis* isolates with tetracycline MIC ≥2 mg/L had mutations in the *rrs* alleles; Lysnyansky et al. (2009) reported that a change in the *gyrA* gene resulted in decreased susceptibility to fluoroquinolones and that a concurrent point mutation in the *par*C gene was required for fluoroquinolone resistance.

As a result, there is an urgent need for veterinary *Mycoplasma*-specific laboratory standards and clinical breakpoints for MIC data interpretation of *Mycoplasma* strains. As demonstrated by Lerner et al. (2014) genetic mutations are known to relate to antimicrobial resistance and they demonstrated that the number of mutations also related directly to MIC values, therefore the use of genetic mutation data may help determine interpretive criteria reducing the requirement for large studies in animals. Although the veterinary *Mycoplasma* species used in this study are not zoonotic, as with some other CEESA monitoring studies (de Jong et al., 2014; Moyaert et al., 2014; Morrissey et al., 2016), it is important to know the effectiveness of antimicrobials for food and companion animals to ensure minimal use of antimicrobials by using targeted and correct treatments. The information gained from *mycoplasma* antimicrobial susceptibility *in vitro* and *in vivo* studies will help target effective treatment, reduce the use of antimicrobials and therefore reduce the risk of developing antimicrobial resistance in these and other bacterial species that are present in these animals. The development of vaccines, possibly combined with antimicrobial therapies to obtain *M. bovis* free herds would be a future ideal to control disease caused by *M. bovis*.

Funding

The MycoPath project is a pan-European programme dedicated to the collection and to the monitoring of antimicrobial susceptibility of veterinary *mycoplasmas* from diseased food-producing animals not recently exposed to antimicrobials. MycoPath is an initiative of, and is coordinated by the Executive Animal Health Study Center (CEESA) in Brussels. It has been operational since 2010. CEESA's membership is composed of international pharmaceutical companies researching and producing veterinary medicinal products. This study was funded by Bayer Animal Health GmbH (Germany); CEVA Sante Animale (France); Merial (France); MSD Animal Health (The Netherlands); Novartis Animal Health Inc. (Switzerland); Vetoquinol S. A. (France); and Zoetis (Belgium).

Conflict of interest statement

None to declare.

Acknowledgements

MSc Marion Ocak is gratefully thanked for the performance of the statistical analyses. We thank Dr. I. Badiola (Centre de Recerca de Sanitat Animal, IRTA − CReSA, Bellaterra, Spain), Prof. M. Gyuranecz (Institute for Veterinary Medical Research, Budapest, Hungary) and Prof. L. Stipkovits (RT-Europe, Budapest, Hungary) for the provision of the isolates.

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