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New antimicrobial susceptibility data from monitoring of *Mycoplasma bovis* isolated in Europe

Ulrich Klein[a,b], Anno de Jong[a,c,*], Myriam Youala[a,d], Farid El Garch[a,e], Clelia Stevenin[a,f], Hilde Moyaert[a,g], Markus Rose[a,h], Salvatore Catania[i], Miklós Gyuranecz[j], Andrew Pridmore[k], Roger D. Ayling[l]

[a] MycoPath Study Group, c/o CEESA, 168 Av de Tervueren, 1150, Brussels, Belgium
[b] Elanco Animal Health, Basingstoke, UK
[c] Bayer Animal Health GmbH, Monheim, Germany
[d] Virbac, Carros, France
[e] Vetoquinol S.A, Lure, France
[f] Boehringer Ingelheim Vetmedica, Ingelheim, Germany
[g] Zoetis, Zaventem, Belgium
[h] MSD Animal Health Innovation GmbH, Schwabenheim, Germany
[i] Mycoplasma Unit, Istituto Zooprofilattico Sperimentale delle Venezie, Verona, Italy
[j] Institute for Veterinary Medical Research CAR-HAS, Budapest, Hungary
[k] Don Whitley Scientific, Bingley, UK
[l] Mycoplasma consultant, Sandhurst, Berkshire, UK

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ABSTRACT

Mycoplasma bovis is an important respiratory pathogen of cattle across Europe and is included in the MycoPath pan-European antimicrobial susceptibility monitoring programme. *M. bovis* strains (232) were isolated from cattle, not recently treated with antimicrobials, at diverse geographical locations in France, Great Britain, Hungary, Italy and Spain during 2014 to 2016. Only one isolate per farm and per outbreak was retained. For each isolate, the MICs of ten antimicrobials were determined in a central laboratory using a broth microdilution method with modified Eaton's medium and incubation at 35 °C ± 1 °C for 24 ± 6 h. MIC_{50}/MIC_{90} (mg/L) values for the 232 strains were: danofloxacin 0.25/1; enrofloxacin 0.5/8; marbofloxacin 1/4; gamithromycin > 64/ > 64; spiramycin 8/16; tilmicosin > 64/ > 64; tulathromycin > 64/ > 64; tylosin 64/ > 64; florfenicol 4/8; oxytetracycline 8/32. Minor between-country differences in the MIC_{90} values were observed for the fluoroquinolones, spiramycin and oxytetracycline, whilst the MIC values for the other compounds were similar. Spain and Italy had the higher MIC_{90} values for the fluoroquinolones. Compared with the 2010–2012 study (156 isolates) results are similar, with an overall MIC_{50} increase of at most one doubling dilution for enrofloxacin, spiramycin, tylosin, florfenicol and oxytetracycline. In contrast, the MIC_{90} value for oxytetracycline decreased from > 64 to 32 mg/L. Standardized laboratory methods and interpretive criteria for MIC testing of veterinary mycoplasmas are clearly needed; there are currently no clinical breakpoints available to facilitate data interpretation and correlation of MICs with *in vivo* efficacy.

1. Introduction

Mycoplasma bovis is increasingly recognised as a major pathogen of cattle and bison adversely affecting their health and welfare, with significant economic costs to the farming industry. In many countries around the world the infection is now endemic but where new infections have occurred in Finland and New Zealand substantial efforts and investments are being made to attempt to identify the source of infection (Haapala et al., 2018), to control and eliminate the pathogen (Ministry of Primary Industries, New Zealand, 2018). *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). Once a herd becomes infected options for control are limited, although a variety of commercial vaccines, including autogenous

* Corresponding author at: c/o CEESA, 168 Av de Tervueren, 1150, Brussels, Belgium.
E-mail address: anno.jong-bayer@kpnmail.nl (A. de Jong).

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preparations, are available in the United States (though little data exist to assess their effectiveness), they are not licensed for use outside the United States (Calcutt et al., 2018), so in Europe antimicrobials are used along with culling severely affected animals (Nicholas et al., 2016).

Mycoplasma species lack a cell wall and are therefore refractory to all antimicrobials that target the cell wall, *e.g.*, β-lactam antibiotics (Lysnyansky and Ayling, 2016). The antimicrobials included in this study are licensed for treating *Mycoplasma* infections and belong to four chemical classes: the fluoroquinolones (danofloxacin, enrofloxacin, marbofloxacin); the macrolides (gamithromycin, spiramycin, tilmicosin, tulathromycin and tylosin); the amphenicols (florfenicol) and the tetracyclines (oxytetracycline). Their modes of action are briefly described in Lysnyansky and Ayling (2016) and Klein et al. (2017). The fluoroquinolones generally have a mycoplasmacidal effect on *M. bovis*, while the other antimicrobials have an inhibitory, mycoplasmastatic effect, giving the host time to recover and develop its own immune response to the infection. Some antimicrobials also have anti-inflammatory attributes that help reduce the clinical signs caused by the infection.

Several of the other Centre Européen d`Etudes pour la Santé Animale (CEESA) monitoring studies (de Jong et al., 2014; Moyaert et al., 2014; de Jong et al., 2019) are on bacteria that may adversely impact human health. However the MycoPath programme, which includes this study on *M. bovis,* is indicative of the adverse impact of mycoplasma infections to the farming industry. The MycoPath programme aims to create a pan-European collection of representative *M. bovis* isolates from clinical cases of diseased cattle not recently exposed to antimicrobials. It is important to know if this pathogen is developing antimicrobial resistance, so that only effective antimicrobials are used for therapy thus ensuring minimal use of antimicrobials by using targeted and correct treatments. This is the second study (MycoPath II) to include *M. bovis* as part of the CEESA monitoring programmes (de Jong et al., 2013), so comparisons can be made with the previous study (MycoPath I; Klein et al., 2017).

Antimicrobial susceptibilities of *M. bovis* isolates recovered from five European countries during 2014 to 2016 are presented here. Testing was carried out at a central laboratory using culture media suitable for optimal growth of *M. bovis.* Although no standards for testing and interpretation of veterinary mycoplasma species are in place, standards for the *Mycoplasma* testing of significant clinical infections in humans have been published (CLSI, 2011; Waites et al., 2012). The broth microdilution method used in this study essentially followed the guidelines of CLSI (2011) and Hannan (2000).

2. Methodology

2.1. Mycoplasma bovis collection

From 2014 to 2016 the British, French, Hungarian, Italian and Spanish national laboratories isolated *M. bovis* following specific criteria. Only one isolate per farm per clinical episode in any three-month period was permitted. Isolates had to be from geographically spread areas within each country and from cattle that had no antimicrobial treatment in the previous 15 days before sampling. Cattle were aged between three weeks and 18 months and had clinical signs of respiratory disease, including depression, hyperthermia, polypnea, dyspnea, cough or nasal discharge. The participating national laboratories followed their standard *Mycoplasma* culture isolation and molecular identification procedures. Isolates were stored at a temperature of −80 °C, before transfer to the central laboratory (Don Whitley Scientific, Bingley, UK) on dry ice, or at ambient temperature as lyophilized cultures, together with a case report form for each isolate. At the central laboratory additional identity checks were performed on a random selection (approx. 5%) of the isolates using a PCR that targets the *vsp* genes (Tenk et al., 2006).

2.2. Antimicrobial testing

Antimicrobial susceptibility testing on all of the *M. bovis* isolates was carried out at the central laboratory. The isolates were checked for viability with *M. bovis* in modified Eaton's medium (Nicholas and Baker, 1998) with phenol red as an indicator and no antimicrobials. The same medium was also used in the susceptibility testing. *M. bovis* utilises pyruvate and its growth results in Eaton's medium changing to an orange colour that differentiates it from the glucose fermenting and arginine hydrolysing mycoplasma species, which provides an additional identification check. Each isolate was incubated in broth medium until a distinctive colour change was produced, then divided into aliquots and frozen without any additives at −80 °C. The viable count in one aliquot was determined by serial dilution and plating onto agar medium. During subsequent Minimal Inhibitory Concentration (MIC) tests, aliquots were thawed and diluted to achieve a cell density of 10^6 colony forming units (cfu) per mL for addition to the MIC plates. *Mycoplasma bovis* (NCTC 10,131/ATCC 25,523) strain was used as a positive control for both the PCR and the antimicrobial susceptibility testing.

Minimal inhibitory concentration determinations were performed using a broth microdilution method. Dilutions of antimicrobials were prepared using the CLSI recommended method (CLSI, 2013) to give a final active concentration range from 0.001 to 64 mg/L. For each antimicrobial, a stock solution containing 1280 mg/L of the active ingredient, was prepared as follows. Oxytetracycline hydrochloride (Sigma-Aldrich, UK) was prepared in deionized water; danofloxacin, enrofloxacin and marbofloxacin (all 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, spiramycin and tylosin tartrate (all 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 water bath 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 (Merial, France) was dissolved and made up to the final volume in 0.1 M phosphate buffer pH 6.0.

To determine the MIC 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 (thawed, pre-incubated for 1 h and then diluted as described above) 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. For each strain, 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 served as a negative uninoculated control. Immediately after inoculation, microtitre plates fitted with polystyrene lids were placed in a humidified atmosphere, and incubated at 35 °C ± 1 °C for 24 ± 6 h. Plates were inspected daily. If no growth was evident in the positive control wells, plates were re-incubated 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 orange (growth). 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 definite 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 strain were around a central, most frequently observed MIC value. In cases where the MIC results obtained for an antimicrobial agent against one or more strains of *M. bovis* deviated markedly from the MICs obtained against the majority of strains, the MIC test was repeated twice. In such cases, the final MIC value was obtained on at least two separate occasions.

3. Results

3.1. Mycoplasma isolates

A total of 232 *M. bovis* isolates all associated with respiratory disease were submitted to the central laboratory for antimicrobial susceptibility testing, 35 from France, 62 from Great Britain, 61 from Hungary, 56 from Italy and 18 from Spain. Two-hundred and twenty-eight isolates (98.3%) were covered from samples taken in 2015 and 2016. Most of the isolates were obtained from different farms, however four isolates each from Hungary and Italy, and three from Spain, were second samples taken from different farms in the respective countries when the clinical signs of infection flared up again at least three months after the original sampling.

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

The *M. bovis* results are given in mg/L as MIC range, MIC_{50} and MIC_{90} for each antimicrobial and for each country and combined in Table 1. The distribution of MIC values for all isolates is given in Table 2.

The fluoroquinolones danofloxacin, enrofloxacin and marbofloxacin had MIC_{50} values of 0.25, 0.5 and 1 mg/L respectively, the differences also being reflected in the observed ranges of 0.064–8, 0.125–32, and 0.25 to > 64 mg/L respectively, although at 8 mg/L enrofloxacin had the highest MIC_{90} value. All five countries had MIC_{50} values between 0.25 and 1 mg/L for the three fluoroquinolones. However minor between-country variations were apparent when the MIC_{90} values were compared. Italy (56 isolates) and Spain (18 isolates) had the higher values, notably for enrofloxacin at 16 and 32 mg/L respectively compared with 0.5–1 mg/L for the three other countries. Both Italy and Spain had MIC_{90} values for marbofloxacin at 8 mg/L compared with 1 mg/L for the other countries.

For all isolates the macrolide related antimicrobials $MIC_{50/90}$ values were 8/16 mg/L for spiramycin; 64/ > 64 mg/L for tylosin; > 64/ > 64 mg/L for gamithromycin, tilmicosin and tulathromycin. The lowest MIC_{50} values for spiramycin, tulathromycin and tylosin at 0.25, 1, and 8 mg/L respectively were observed in the British isolates. With the exception of Italy with a tylosin MIC_{50} at 32 mg/L; and France, Hungary, Italy and Spain with a spiramycin MIC_{50} at 16, 4, 32 and 16 mg/L respectively, all other macrolides MIC_{50} values were > 64 mg/L.

For florfenicol the MIC range was 0.5–32 mg/L with $MIC_{50/90}$ values of 4/8 mg/L. All countries florfenicol $MIC_{50/90}$ values were within one dilution of each other.

The MIC range for oxytetracycline was 0.25 - > 64 mg/L with $MIC_{50/90}$ values of 8/32 mg/L. Hungary had the lowest $MIC_{50/90}$ values at 4/8 mg/L and France the highest at 32/64 mg/L.

4. Discussion

Although guidelines for testing veterinary mycoplasmas have been published (Hannan, 2000), different methods have been used historically, making a comparison of published MIC results difficult. In this study, use of a single laboratory to perform all MIC testing ensured consistent methodology facilitating a comparison of isolates from the five different EU countries. This is the second CEESA study on *M. bovis*; the first was carried out on British, French, Hungarian and Spanish isolates obtained between 2010 and 2012 (Klein et al., 2017). Therefore a

Table 1
Minimal Inhibitory Concentration (MIC) values for ten antimicrobial agents against 232 *Mycoplasma bovis* isolates. Total values and value for each of the five different countries.

Result (mg/L)											
Country of Origin	MIC Parameter	Danofloxacin	Enrofloxacin	Marbofloxacin	Gamithromycin	Tulathromycin	Spiramycin	Tilmicosin	Tylosin	Florfenicol	Oxytetracycline
France 35 isolates	MIC Range	0.125–2	0.25–16	0.5–16	> 64– > 64	0.5– > 64	0.5– > 64	0.5– > 64	0.5– > 64	0.5–16	1– > 64
	MIC_{50}	0.25	0.5	1	> 64	> 64	16	> 64	> 64	2	32
	MIC_{90}	2	0.5	1	> 64	> 64	64	> 64	> 64	8	64
Great Britain 62 isolates	MIC Range	0.125–4	0.125–16	0.5– > 64	0.5– > 64	0.032– > 64	0.032– > 64	4– > 64	2– > 64	0.5–16	0.25–64
	MIC_{50}	0.25	0.25	1	> 64	1	0.25	> 64	8	4	8
	MIC_{90}	0.5	0.5	1	> 64	> 64	8	> 64	> 64	8	32
Hungary 61 isolates	MIC Range	0.064–8	0.25–32	0.25–16	1– > 64	0.032– > 64	0.064–32	0.25 - > 64	0.5 - > 64	0.5–16	0.25–32
	MIC_{50}	0.25	0.5	0.5	> 64	> 64	4	> 64	> 64	4	4
	MIC_{90}	1	1	1	> 64	> 64	16	> 64	> 64	8	8
Italy 56 isolates	MIC Range	0.125–4	0.5–32	0.5–64	> 64– > 64	1– > 64	4– > 64	16– > 64	4– > 64	0.5–8	4–64
	MIC_{50}	0.25	0.5	1	> 64	> 64	32	> 64	32	4	16
	MIC_{90}	2	16	8	> 64	> 64	> 64	> 64	> 64	8	32
Spain 18 isolates	MIC Range	0.25–4	0.25–32	0.25–64	16– > 64	1– > 64	4– > 64	32– > 64	8– > 64	4 – 32	4–64
	MIC_{50}	0.5	0.5	1	> 64	> 64	16	> 64	> 64	8	16
	MIC_{90}	4	32	8	> 64	> 64	32	> 64	> 64	16	32
All 232 isolates	MIC Range	0.064–8	0.125–32	0.25– > 64	0.5– > 64	0.032– > 64	0.032– > 64	0.25– > 64	0.5– > 64	0.5–32	0.25– > 64
	MIC_{50}	0.25	0.5	1	> 64	> 64	8	> 64	64	4	8
	MIC_{90}	1	8	4	> 64	> 64	16	> 64	> 64	8	32
QC[a]	MIC Range	0.125–0.25	0.25–0.5	0.5–1	> 64	0.064–0.25	4–8	0.064	0.064	2–4	0.5–4

[a] MICs of Quality Control strain ATCC 25,523/NCTC 10,131.

Table 2
Minimal Inhibitory Concentration (MIC) distribution for ten antimicrobial agents against all 232 *Mycoplasma bovis* isolates from *Mycoplasma* respiratory infections in cattle.

Antimicrobial	MIC (mg/L)																	MIC_{50}	MIC_{90}	Range
agent	≤0.001	0.002	0.004	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	> 64	(mg/L)	(mg/L)	(mg/L)
Danofloxacin							1	73	109	16	11	6	15	1					0.25	1	0.064 - 8
Enrofloxacin								1	51	135	15		6	6	10	8			0.5	8	0.125 - 32
Marbofloxacin									4	68	129	6	6	11	2	2	3	1	1	4	0.25 - > 64
Gamithromycin										1	5	3	7	10	6	1		199	> 64	> 64	0.5 - > 64
Tulathromycin						2	8	9	7	11	11	4	5	4	9	7		155	> 64	> 64	0.032 - 64
Spiramycin						1	1	17	26	9	6	14	36	57	42	9	8	6	8	16	0.032 - > 64
Tilmicosin									1	2		1	4	17	18	34	36	119	> 64	> 64	0.25 - > 64
Tylosin										4	2	5	27	23	11	38	17	105	64	> 64	0.5 - > 64
Florfenicol										8	23	47	92	52	9	1			4	8	0.5 - 32
Oxytetracycline									3	5	9	21	49	33	56	48	6	2	8	32	0.25 - > 64

Table 3
Comparison of Minimal Inhibitory Concentration (MIC) data of *Mycoplasma bovis* between this study (MycoPath II) and the previous study (MycoPath I; Klein et al., 2017).

Antimicrobial Agent	MycoPath I (Klein et al., 2017) MIC_{50} in mg/L (156 isolates)	MycoPath II (This study) MIC_{50} in mg/L (232 isolates)	MycoPath I (Klein et al., 2017) MIC_{90} in mg/L (156 isolates)	MycoPath II (This study) MIC_{90} in mg/L (232 isolates)
Danofloxacin	0.25	0.25	1	1
Enrofloxacin	0.25	0.5	4	8
Marbofloxacin	1	1	4	4
Gamithromycin	> 64	> 64	> 64	> 64
Tulathromycin	> 64	> 64	> 64	> 64
Spiramycin	4	8	16	16
Tylosin	32	64	> 64	> 64
Florfenicol	2	4	4	8
Oxytetracycline	4	8	> 64	32

comparison between that data and the present study can be made (see Table 3). Table 3 shows a one dilution increase in the MIC_{50} values of enrofloxacin, spiramycin, tylosin, florfenicol and oxytetracycline; and a one dilution increase in the MIC_{90} value of enrofloxacin and florfenicol; but at least a double dilution decrease from > 64 mg/L to 32 mg/L for oxytetracycline when comparing this present study with the previous one (Klein et al., 2017). In the present study (MycoPath II), isolates were also collected in Italy, and tilmicosin has additionally been included. The only difference in procedure was the use of Eaton's media with phenol red as an indicator in MycoPath II, which made reading the MIC easier compared with MycoPath I when modified Hayflicks medium (Hayflick, 1965) with 5% Alamar Blue and 0.01% Nicotinamide Adenine Dinucleotide was used (Rosenbusch et al., 2005). As recommended by Hannan (2000) the growth medium used for antimicrobial susceptibility should give optimum growth of the mycoplasma species being tested and *M. bovis* grows well in both Hayflick and Eaton's medium. It was observed that the MIC for the NCTC control differed particularly for gamithromycin between the two studies (2–4 mg/L in MycoPath I vs. > 64 mg/L in MycoPath II for gamithromycin). This discrepancy might specifically apply to the control strain because the colour changes were weak for the controls of MycoPath I in contrast to those for the field isolates. Additional studies with a fresh NCTC isolate confirmed the gamithromycin QC MICs of 64–128 mg/L. Furthermore, repeat MIC determinations of a selection of the field isolates confirmed the comparability of the two studies. Generally comparison of the MIC values of the two surveys should be prudently conducted.

Generally, MIC ranges for a given antimicrobial were comparable between countries. One exception was the much lower MIC_{50} values for the British isolates for tulathromycin at 1 mg/L compared to > 64 mg/L; spiramycin at 0.25 mg/L compared to a range of 4 to 16 mg/L; and tylosin at 8 mg/L compared to 32 to > 64 mg/L for the other countries. However the MIC_{90} values for tulathromycin and tylosin were > 64 mg/L for all countries. Spiramycin MIC_{90} values ranged from 8 to > 64 mg/L for the five countries. Both Italy and Spain had higher MIC_{90} values for fluoroquinolones compared to the other countries. Higher MIC values for fluoroquinolones in Mediterranean countries have been observed in numerous studies in both target pathogens and zoonotic/commensal organisms. This is frequently associated with the high consumption of antimicrobials including fluoroquinolones over at least three decades in these countries (ESVAC, 2015). The other main between-country differences observed in this study were the MIC_{50} and MIC_{90} ranges from 4 to 32 mg/L and 8 to 64 mg/L respectively for oxytetracycline with Hungary exhibiting the lowest and France the highest values within these ranges.

When comparing the previous MIC results (Klein et al., 2017) with the present study, the majority of MIC values for each country and each antimicrobial are within two dilutions with the following exceptions: for spiramycin, France MIC_{90} increased from 8 to 64 mg/L, Great Britain's MIC_{50} decreased from 4 to 0.25 mg/L; for tylosin, Hungary MIC_{50} increased from 1 to > 64 mg/L; for oxytetracycline, France MIC_{50} increased from 4 to 32 mg/L and Hungary's MIC_{90} decreased from > 64 to 8 mg/L. Although no substantial changes were observed in MIC values, some potential underlying trends which varied between countries would benefit from continued monitoring so that the most effective antimicrobials could be used *in vivo*.

In the past few years other workers have also published antimicrobial susceptibility results for *M. bovis*. Although slightly different methods have been used some comparisons can be made. On British respiratory and mastitis isolates obtained from cattle between 2004 and 2009 Ayling et al. (2014) reported increasing trends in MIC values against oxytetracycline, danofloxacin and enrofloxacin. However in this MycoPath II study a similar MIC range, MIC_{50} and MIC_{90} was recorded for British isolates to oxytetracyline, and with the exception of one isolate for enrofloxacin at 16 mg/L, the higher MIC values > 4 mg/L were absent for danofloxacin and enrofloxacin. Barberio et al. (2016) tested isolates from mastitis cases originating in Belgium, Germany and Italy, and reported high percentages of non-wild type to macrolides on isolates from those three countries, with MIC_{mode} of spiramycin at 16 mg/L, and tilmicosin and tylosin both > 32 mg/L, while enrofloxacin, florfenicol and oxytetracycline were 0.25, 1 and 4 mg/L respectively. In the Netherlands when isolates from different pathologies were tested, tulathromycin was the only antimicrobial with a significant difference ($p < 0.05$) in MIC_{50} values obtained for isolates from lungs versus milk or synovial fluid with MIC_{50} values of 8, 0.5 and 1 mg/L respectively. The corresponding MIC_{90} values were 1024, 4 and 2 mg/L respectively (Heuvelink et al., 2016). The other MIC_{50} values they obtained for the three sample types were: enrofloxacin all 0.25 mg/L, oxytetracycline 4, 4 and 8 mg/L, tilmicosin 512, 256, and 256 mg/L, and tylosin 64, 32 and 32 mg/L respectively.

Anholt et al. (2017) tested 226 Canadian respiratory *M. bovis* isolates; all had MICs below 2 mg/L for danofloxacin and enrofloxacin, whereas more than 90% of the isolates had MICs above 32 mg/L for the macrolides tilmicosin, tulathromycin and tylosin. Oxytetracycline had a maximum MIC of 8 mg/L which is lower than obtained here, whilst florfenicol had $MIC_{50/90}$ values of 8 mg/L compared to 4 and 8 mg/L in this study. Cai et al. (2019) reported stable MIC_{50} values for the fluoroquinolones over a period of three decades, but increased MIC values to the macrolides and tetracycline, although spectinomycin and tulathromycin MICs subsequently declined. In contrast the fluoroquinolones, tetracyclines and florfenicol failed to inhibit growth of *M. bovis* isolates from *Bison bison* in Canada (Suleman et al., 2016). In Japan, Sato et al. (2017) carried out MIC testings against the macrolides, tylosin and tilmicosin and all 58 isolates tested had MICs above 64 mg/L. Gautier-Bouchardon (2018) reported MIC ranges on French isolates, with many of the recent isolates having high MIC values to the same antimicrobials used in this study. Many of the isolates with high MICs had genetic mutations associated with resistance to the macrolides and fluoroquinolones which was also shown by Khalil et al. (2016). This also links in with the report by Becker et al. (2015), who reported a loss of diversity in French isolates possibly due to selection of a multi-resistant clone.

Although no breakpoints are published for *M. bovis* MICs, it has been established that high MIC values for oxytetracycline (Amram et al., 2015), tylosin and tilmicosin (Lerner et al., 2014; Sato et al., 2017) and the fluoroquinolones (Lysnyansky et al., 2009) are related to genetic mutations (Gautier-Bouchardon, 2018; Lysnyansky and Ayling, 2016; Sulyok et al., 2017) that are consistent with antimicrobial resistance in other bacteria. Sulyok et al. (2018) used two PCR methods, a mismatch amplification mutation assays (MAMA-PCR), which detects single nucleotide polymorphisms (SNP's) and high resolution melting (HRM) tests to detect antimicrobial resistance markers. They designed the tests to detect resistance in the fluoroquinolones, tetracyclines, macrolides and spectinomycin.

Godinho (2008) published the importance of maintaining the pH of the culture media within the range pH 7.2–7.4 in obtaining consistent tulathromycin MIC data, hence the use of buffered tulathromycin in this study as recommended by the manufacturer. Many of the MIC values are high for tulathromycin, but an experiment over a four-week period demonstrated that it was still effective when calves were infected with isolates with high tulathromycin MIC values (Godinho et al., 2005; Bartram et al., 2016).

From the published literature it does appear that the greatest variation in MIC levels is due to difference between host species, i.e. bison when compared with cattle; and the different clinical sites that *M. bovis* has been isolated from. The number of antimicrobials that have elevated MIC levels, and therefore possibly reduced *in vivo* effectiveness against *M. bovis,* supports initiatives that promote prudent use of antimicrobials in agriculture (Cai et al., 2019).

There are guidelines about antimicrobial testing of veterinary mycoplasma species (Hannan, 2000), but there are still no standard methods, or quality control ranges. It is encouraging that despite different methods being used, similar antimicrobial susceptibility results are being described from laboratories in different countries. As highlighted by Calcutt et al. (2018) there are still many gaps in our knowledge about *M. bovis,* as a pathogen, the disease course, the way it is transmitted, its pathogenicity, and control methods. Some of those gaps are apparent from these antimicrobial studies and include standardising the interpretation of the *in vitro* results in relation to their potential effectiveness *in vivo*. There is still an urgent need for standard veterinary *Mycoplasma*-specific laboratory methods, control strains with known MIC values, and clinical breakpoints for MIC data interpretation of *Mycoplasma* species. As in this study, use of a central laboratory to carry out the MIC testing reduces the risk of inter-laboratory test variations. The development of determining genetic mutants as antimicrobial resistant markers is a major step forward and would also benefit from standardisation.

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Declaration of Competing Interest

Some authors are connected with pharmaceutical companies, however the testing, interpretation of results and preparation of the manuscript have been carried out independently.

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