Interpretive Summary

***In vitro* antimicrobial susceptibility of *Mycoplasma bovis* strains isolated from dairy cattle in Belgium, Germany and Italy** by Antonio Barberio et al. The aim of this study was to detect the Minimum Inhibitory Concentrations (MIC) in 73 strains of *Mycoplasma bovis* isolated from milk in 3 European countries, and to compare the levels of antimicrobial resistance among them.

Running Head: SHORT COMMUNICATION*: MYCOPLASMA BOVIS* ANTIMICROBIAL SUSCEPTIBILITY

Short communication: *In vitro* antimicrobial susceptibility of *Mycoplasma bovis* strains isolated from dairy cattle in Belgium, Germany and Italy.

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ABSTRACT

The objective of this study was to assess the *in vitro* antimicrobial susceptibility of 73 isolates of *Mycoplasma bovis* isolated from milk of dairy cattle herds of Belgium, Germany, and Italy. Minimal inhibitory concentration (MIC) values were determined by the microbroth dilution method for the following antimicrobials: erythromycin, spiramycin, tilmicosin, tylosin, lincomycin, enrofloxacin, doxycycline, oxytetracycline, florfenicol, tiamulin. Among the agents of the different antimicrobial classes, the macrolides showed the highest MIC_{90} values, all above the highest concentration tested: > 8 μg/mL for erythromycin, > 16 μg/mL for spiramycin, > 32 μg/mL for tilmicosin and tylosin. Also the MIC_{90} of lincomycin was above the highest concentration tested (> 32 μg/mL) but the distribution of the MIC values was almost perfectly bimodal: 41 isolates had a MIC ≤ 0.5 μg/mL, and 30 isolates > 32 μg/mL. Oxytetracycline had a 2-fold higher MIC_{50} (2 vs 0.5 μg/mL) and 1-fold higher MIC_{90} (4 vs 2 μg/mL) than doxycycline. Enrofloxacin and florfenicol had both a MIC_{90} of 2 μg/mL, while tiamulin had a MIC_{90} of 0.5 μg/mL. Significant differences on the MIC values were found among the 3 countries for several

antimicrobials: Belgium and Italy showed, compared to Germany, significantly higher MICs for lincomycin, spiramycin, and tylosin, and lower for oxytetracycline and florfenicol. The Belgian isolates showed the lowest MICs for enrofloxacin, compared to Germany and Italy. The MICs results obtained in our study suggest the presence of a high level of resistance of *Mycoplasma bovis* isolates originating from milk to macrolides in all countries involved in this study. Oppositely, a low level of resistance was found against the antimicrobials that are not used in cattle such as tiamulin and doxycycline, highlighting a possible link between antimicrobial treatments and development of resistance in the studied *Mycoplasma bovis* population.

Key words: *Mycoplasma bovis*, minimum inhibitory concentration, antimicrobials, dairy cattle

Short Communication

Mycoplasma bovis is a wall-less microorganism belonging to the class of Mollicutes. In cattle it can induce respiratory disease, otitis media, arthritis, mastitis, and keratoconjunctivitis (Maunsell et al., 2011). This bacterium is considered to be one of the major emerging pathogens in cattle herds of industrialized countries threatening livestock production (Nicholas, 2011).

Mycoplasma bovis mastitis can occur with clinical signs, like milk abnormalities, such as flaky sediments in a watery or serous fluid, swelling and udder induration (Bushnell 1984; Maunsell et al., 2011), but also subclinical cases of mastitis have been described (Gonzales and Wilson, 2003; Maunsell et al., 2011). *Mycoplasma bovis* mastitis has been considered contagious in nature and is transmitted from infected to uninfected udders mostly at milking time. Still, the transmission

may less commonly occur from pathways others than those associated with milking (Fox et al., 2003). Some studies have highlighted the ability of *Mycoplasma bovis* to colonize multiple body sites (Punyapornwithaya et al., 2010), and some authors postulated that transmission of *Mycoplasma bovis* associated with bovine intramammary infection (IMI) may occur within the cow internally, from one infected organ site to the udder or vice versa. Also, between-cow transmission by shedding of the pathogen through external mucosal surfaces of an infected or colonized animal to a naïve animal has been mentioned (Fox, 2012).

To date there is no effective vaccine to prevent *Mycoplasma bovis* infection in cattle (Mulongo et al., 2013) and antibiotic treatment is used to control the disease, yet only in case of respiratory syndrome or arthritis. Increasing antimicrobial resistance of *Mycoplasma bovis* isolates has been reported in several European countries and in the USA: high levels of resistance were found especially against tylosin, tilmicosin, ampicillin, ceftiofur, both in the USA and in Europe (Rosenbush et al., 2005; Soehnlen et al., 2011; Ayling et al., 2014; Gautier-Bouchardon et al., 2014; Sulyok et al., 2014). The large majority of the studies on the antimicrobial susceptibility have been performed on isolates obtained from the respiratory tract, while few data are available on mastitis isolates (Soehnlen et al., 2011; Kawai et al., 2014). This is most likely because, since after the first description of *Mycoplasma bovis* as a cause of mastitis, antimicrobial treatment of mastitis was shown to be unsuccessful (Gonzales and Wilson, 2003). The evaluation of the antimicrobial susceptibility profiles of *Mycoplasma bovis* isolated from bovine milk samples could be helpful to assess the levels of antimicrobial resistance originating from distinct dairy cattle populations, given that *Mycoplasma bovis* mastitis, when recognized, is typically not treated with antibiotic therapy or, if the pathogen is undetected, is treated with unsuitable antimicrobials, like betalactams.

The objective of this study was to assess the *in vitro* antimicrobial susceptibility of 73 strains of *Mycoplasma bovis* isolated from milk samples of dairy herds of Belgium, Germany, Italy, and to evaluate the overall patterns of antimicrobial resistance and the differences among the 3 countries.

The isolates (73) used in this study were part of the laboratory culture collection of 5 European laboratories located in Belgium (Ghent University and Flanders Milk Control Center, Lier), in Germany (University of Applied Science and Arts – Microbiology, Hannover), and in Italy, (Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), and Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia). The strains were isolated from 68 cows and 4 bulk tank milk samples collected in 22 Belgian, 17 German, and 34 Italian dairy farms. All the antimicrobial susceptibility tests were performed at the laboratory of IZSVe and all isolates were grown to reach the "log phase", stored in PPLO broth medium with the addition of sterile glycerol (5% v/v), frozen at -20° C at the different labs, and shipped to the IZSVe laboratory for MIC determination. The MIC were determined following the guidelines for Mycoplasma MIC testing (Hannan, 2000), and the Clinical and Laboratory Standard Institute (CLSI) guidelines for performing MIC in Human Mycoplasma isolates (CLSI, 2011).

All the isolates, after their arrival, were inoculated in 2 mL of PPLO broth medium added with phenol red (Acumedia-Neogen, Lansing, MI, USA), and incubated at 37±1 °C under 5% CO_2 conditions. Broths were checked daily for changes in color and/or turbidity; then they were inoculated onto PPLO agar medium (Acumedia-Neogen, Lansing, MI, USA) and checked daily for the presence of *Mycoplasma bovis* suspected colonies.

In order to confirm the identification of *Mycoplasma bovis*, DNA was extracted from broth of suspect samples and a 16S-rDNA PCR and Denaturing Gradient Gel Electrophoresis (DGGE) method were performed as described by McAuliffe et al. (2005).

Each *Mycoplasma bovis* isolate was submitted to 3 consecutive clonal passages both in liquid and solid PPLO media, and finally propagated in 10 mL of PPLO broth without inhibitors.

The bacterial stock solution was spiked in 10 subaliquotes and stored at least 24h at -80 °C. The day after, an aliquot was thawed and used for the evaluation of the titer by the Unit Changing Colour (UCC/mL) (Hannan, 2000; Blodgett, 2010).

When the titer was available, a new subaliquote of approximately 10^4 UCC/mL was inoculated in each well of a 96 wells commercial ready for use plate (Merlin Diagnostika, Micronaut-S, Berlin, Germany) that contained 10 freeze-dried antimicrobials (see Table 1).

Plates were incubated aerobically at 37 ± 1° C for at least 18 hours and checked daily, in order to evidence the growth of the positive control well. Each strain was tested in duplicate and reference strain ATCC 25523 was tested as quality control. The MIC was determined when the positive control well showed an evident growth.

The MIC value of each isolate, expressed as μg/mL, was defined as the lowest concentration of the antibiotic that completely inhibited the growth after the incubation period. When the growth was not inhibited at the highest antimicrobial concentration, the MIC was expressed as greater (>) than the highest concentration; when the growth was inhibited at the lowest concentration, the MIC was expressed as lower or equal (≤) to this concentration.

For each tested antimicrobial the following parameters were calculated: MIC dilution range used, minimum and maximum MIC obtained, mode, MIC_{50}, and MIC_{90}, defined respectively as the lowest concentrations that inhibit 50% and 90% of the isolates.

Statistical analysis was performed to evaluate the differences among MICs of the 3 countries using SPSS 22.0 software for windows (IBM, SPSS Inc., Chicago IL, USA). The overall variance among the isolates MICs was evaluated using Kruskall-Wallis test for each antimicrobial, and then "post-hoc" comparisons among the MICs of the 3 countries were performed using the Wilcoxon rank-sum test. *P*-values < 0.05 were considered statistically significant for Kruskall-Wallis test, while Wilcoxon test was considered significant only with a *P*-value < 0.0167, corresponding to the value of 0.05 divided by the number of comparisons (3) to be tested (Belgium versus Germany, Belgium versus Italy, Germany versus Italy).

The antimicrobial susceptibility profiles of all the isolates are summarized in Table 1. Among the antimicrobials tested, all macrolides (erythromycin, spiramycin, tilmicosin and tylosin) showed the highest MIC_{90} values, all above the highest concentration tested. This is in agreement with previous report on *Mycoplasma bovis* isolates originating from milk (Soehnlen et al., 2011; Kawai et al., 2014) and the respiratory tract (Rosenbush et al., 2005; Uemura R., 2010; Soehnlen et al., 2011; Ayling et al., 2014; Gautier-Bouchardon et al., 2014; Sulyok et al., 2014). The macrolide with the lowest MIC_{50} was spiramycin, with 27 isolates (37%) having a MIC ≤ 0,5 μg/mL, the lowest concentration tested. Among macrolides, it's interesting to mention the differences between tylosin and tilmicosin: for tilmicosin all the isolates but one had a MIC above the highest concentration tested, while the distribution of MIC values for tylosin was wider, with 43 isolates (59%) ranging from 0.125 μg/mL to 32 μg/mL, and 30 isolates (61%) > 32 μg/mL. A different level of resistance among these antimicrobials has already been reported (Gerchman et al., 2009;

158 Sulyok et al., 2014; Ayling et al., 2014). In a previous study (Lerner et al., 2014) it has been
159 highlighted that, although tylosin and tilmicosin share the same ribosomal binding site, a
160 combination of point mutations in the 23S rRNAs genes is necessary to achieve a high level of
161 resistance to tylosin, while mutations only in a single domain may alone confer high resistance to
162 tilmicosin. Nevertheless this difference is peculiar because tilmicosin in the European Union (EU)
163 is not used at all in dairy cows due to the long milk withdrawal time, while tylosin is frequently
164 used also in dairy cows. A possible explanation for this difference is the circulation of *Mycoplasma*
165 *bovis* strains in the bovine population between young stock and cows, and also between beef and
166 dairy cattle, considering that tilmicosin is the most widely used product for bovine respiratory
167 disease (BRD) treatment in cattle, with the exception of dairy cows, but further studies are needed
168 to evaluate this hypothesis.

169 Lincomycin, an antimicrobial belonging to the class of lincosamides, showed an almost perfect
170 bimodal distribution of the MIC values (Fig. 1): 41 isolates (56%) had a MIC $\leq$ 0.5 μg/mL, and 30
171 (41%) > 32 μg/mL. Resistance to lincosamides is commonly associated with resistance to
172 macrolides and streptogramins group B (MLSB), and this is due to isolates harbouring *erm* genes
173 and producing an enzyme that methylates the 23S rRNA (Gigueré et al., 2013). The different
174 resistance pattern observed in lincomycin, compared to macrolides, could be explained by the
175 presence of a dissociated inducible cross-resistance in which bacteria resistant to macrolides are
176 fully susceptible to lincosamides but can rapidly develop resistance also to lincosamides when
177 exposed to macrolides (Gigueré et al., 2013). The MIC values distribution for enrofloxacin (Fig.
178 1) was unimodal and both MIC_{50} (0.25 μg/mL) and MIC_{90} (2 μg/mL) were much lower compared
179 to the macrolides. These results were in agreement with the data of previous studies performed in
180 Belgium, France and Japan (Thomas et al., 2003; Gautier-Bouchardon et al., 2014; Kawai et al.,

2014), irrespective wherever the isolates originated from the respiratory tract or from milk. The enrofloxacin MIC_{90} found in this study was 2-fold higher compared to those mentioned in some previous studies performed in Hungary, on respiratory isolates (Sulyok et al., 2014), and in the USA on respiratory and milk isolates (Rosenbush et al., 2005; Soehnlen et al., 2011), but much lower compared to the MIC_{90} reported from a UK study (Ayling et al., 2014), where a MIC_{90} of 32 μg/mL was found. Two tetracyclines were tested, oxytetracycline and doxycycline, the latter not approved in the EU for the treatment in dairy cattle. Oxytetracycline had a 2-fold higher MIC_{50} (2 versus 0.5 μg/mL) and 1-fold higher MIC_{90} (4 versus 2 μg/mL) than doxycycline (Table 1). Both the antimicrobials showed a bimodal distribution, but with a cut-off of the 2 peaks of the distribution between 0.25 and 0.5 μg/mL for doxycycline, and between 2 and 4 μg/mL for oxytetracycline. The MIC values for oxytetracycline were lower than those reported in other studies (Thomas et al., 2003; Soehnlen et al., 2011; Gautier-Bouchardon et al., 2014; Kawai et al., 2014; Sulyok et al., 2014), in which MIC_{50} and MIC_{90} were also 4-fold greater than in our study. For florfenicol, an antimicrobial used for BRD treatment in cattle, the MIC_{50} (1 μg/mL) and the MIC_{90} (2 μg/mL) were much lower than those reported in other studies, also when the isolates were obtained from milk (Soehnlen et al., 2011; Gautier-Bouchardon et al., 2014). Also tiamulin, an antimicrobial belonging to the class of pleuromutilins and currently approved only for treatment of swine and poultry in the EU, was tested and deemed very active against *Mycoplasma* species. Both MIC_{50} (0.25 μg/mL) and MIC_{90} (0.5 μg/mL) values of tiamulin were very low, in agreement with the values reported in literature (Gigueré et. al, 2013).

In Table 2 the values of the MIC_{50} and MIC_{90} of the isolates grouped by countries are shown. Significant differences ($P < 0.05$) of the MIC values were found among the 3 countries for the following compounds: enrofloxacin, florfenicol, lincomycin, oxytetracycline, spiramycin,

tiamulin and tylosin. In detail Belgium and Italy showed, compared to Germany, significantly ($P < 0.0167$) higher MICs for lincomycin, spiramycin, and tylosin, and lower for oxytetracycline and florfenicol. The Belgian isolates showed the lowest MICs for enrofloxacin, compared to Germany and Italy.

Taking into account the limited number of isolates tested, this evaluation can only provide some considerations about differences in resistance among the 3 countries. First of all there wasn't a single country that showed a greater level of resistance for all the antimicrobials, yet the differences among the 3 countries were limited to some specific antimicrobials. For example Germany had higher level of MICs for oxytetracycline compared to the other countries, while Italy showed higher level of MICs for spiramycin compared to Belgium and Germany. The different MIC patterns in the 3 countries could reflect different market availability for the different antimicrobials or different treatment procedures for diseases.

Because of the absence of CLSI approved and standardized breakpoints for *Mycoplasma bovis*, the isolates were not classified as susceptible, intermediate or resistant to the different antimicrobials. Nevertheless the MICs results obtained in our study suggest the presence of a high level of resistance to macrolides in all the countries involved in this study. This is remarkable especially for tilmicosin and tylosin, in relation to the fact that they are considered the first choice for *Mycoplasma bovis* therapy in respiratory disease outbreaks. Oppositely, low levels of resistance were found against all the antimicrobials that are typically not used in cattle such as tiamulin and doxycycline, highlighting a possible link between antimicrobial treatments and development of resistance in *Mycoplasma bovis* population. Further studies are needed to evaluate whether the increasing resistance of *Mycoplasma bovis* to macrolides in dairy cows population is

226 due mainly to an indiscriminate use of these drugs, or whether the circulation of *Mycoplasma bovis*
227 strains between beef and dairy cattle population play a role in this phenomenon.

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289 **Table 1**: Antimicrobial susceptibility patterns of the 73 *Mycoplasma bovis* strains isolated from
290 milk tested: for each antibiotic dilution range, MIC range, MIC mode, MIC_{50} and MIC_{90} are
291 listed. All the values are expressed in μg/mL. MIC values that were above the dilution range
292 are marked with the sign >, MIC values below the dilution range are marked with the sign ≤

Antimicrobial tested	Dilution range μg/mL	MIC min value μg/mL	MIC max value μg/mL	Mode μg/mL	MIC 50 μg/mL	MIC 90 μg/mL
Doxycycline	0.125 - 32	0.25	32	0.5	0.5	2
Enrofloxacin	0.125 - 32	≤0.125	16	0.25	0.25	2
Erythromycin	0.5 - 8	> 8	>8	> 8	>8	>8
Florfenicol	0.5 - 16	0.5	4	1	1	2
Lincomycin	0.5 - 32	≤0.5	> 32	≤0.5	≤0.5	>32
Oxytetracyclin	0.5 - 32	1	> 32	4	2	4
Spiramycin	0.5 - 16	≤0.5	> 16	16	8	>16
Tiamulin	0.625 - 32	625	0.5	0.125	0.25	0.5
Tilmicosin	0.625 - 32	2	> 32	>32	>32	>32
Tylosin	0.625 - 32	0.125	> 32	>32	32	>32

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295 **Table 2**: Comparison among the MIC_{50} and MIC_{90} of the antimicrobials tested. All the values
296 are expressed in μg/mL. MIC_{50} and MIC_{90} values that were above the dilution range are marked
297 with the sign >, MIC_{50} and MIC_{90} values below the dilution range are marked with the sign ≤

	MIC50 (μg/mL)			MIC 90 (μg/mL)		
Antimicrobial	Belgium	Germany	Italy	Belgium	Germany	Italy
Doxicycline	0.5	0.5	0.25	1	1	8
Enrofloxacin	≤0.125	0.25	0.25	0.25	0.5	4
Erythromycin	>8	>8	>8	>8	>8	>8
Florfenicol	1	2	1	2	2	2
Lincomycin	>32	≤0.5	1	>32	>32	>32
Oxytetracyclin	2	4	2	4	4	32
Spiramycin	8	≤0.5	16	>16	8	>16
Tiamulin	0.25	0.125	0.25	0.5	0.25	0.5
Tilmicosin	>32	>32	>32	>32	>32	>32
Tylosin	32	16	32	>32	>32	>32

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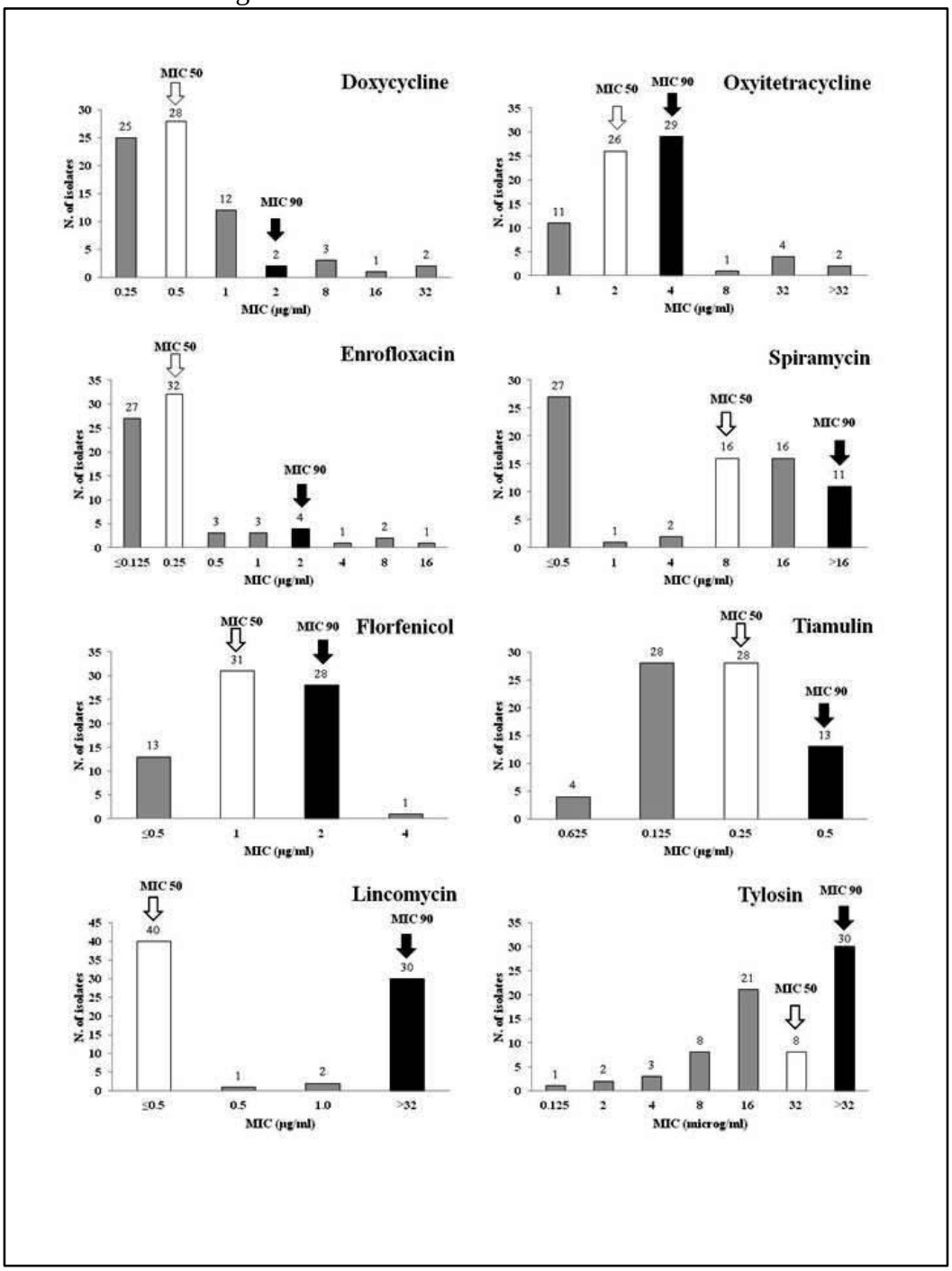
Figure 1: MIC distribution (μg/mL) of the 73 *Mycoplasma bovis* for the following antimicrobials: doxycycline, enrofloxacin, florfenicol, lincomycin, oxytetracycline, spiramycin, tiamulin, tylosin. White arrows indicate the MIC_{50} values, and black arrows the MIC_{90} values (erythromycin and tilmicosin have not been included because their distribution was one-dimensional)

304 Antonio Barberio Figure 1



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