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Antibiotic susceptibility profiles of *Mycoplasma hyorhinis* strains isolated from swine in Hungary

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ABSTRACT

Mycoplasma hyorhinis is a common pathogen of swine causing mainly polyserositis and arthritis, but it has also been implicated as a cause of pneumonia. The economic losses due to *M. hyorhinis* infection could be reduced by antibiotic treatment. The aim of this study was to determine minimal inhibitory concentrations (MIC) of antibiotics potentially used to combat *M. hyorhinis* in swine production.

Thirty-eight Hungarian *M. hyorhinis* strains isolated between 2014 and 2017 were examined by microbroth dilution tests for fifteen antimicrobial agents. Low MIC values of tetracyclines (MIC_{50} 0.078 μg/ml for doxycycline, ≤0.25 μg/ml for oxytetracycline) and pleuromutilins (MIC_{50} 0.156 μg/ml for tiamulin, ≤0.039 μg/ml for valnemulin) were detected against all strains. Fluoroquinolones (MIC_{50} 0.625 μg/ml), gentamicin (MIC_{50} 1 μg/ml) and florfenicol (MIC_{50} 2 μg/ml) inhibited the growth of Hungarian isolates at moderate MIC values. Most of the strains were inhibited by spectinomycin with low or moderate MIC values (MIC_{50} 4 μg/ml) except one strain (> 64 μg/ml). Numerous isolates showed decreased susceptibility to macrolides and lincomycin (MIC_{90} > 64 for tylosin, tilmicosin, tulathromycin, gamithromycin, lincomycin, 8 μg/ml for tylvalosin).

This study serves as evidence for the increasing resistance to macrolides and lincomycin in mycoplasmas, and also reports the occurrence of strains with extremely high MIC values to spectinomycin thus emphasizes the importance of the prudent use of antibiotics. Based on our results, tetracyclines and pleuromutilins are the most active compounds *in vitro* against the Hungarian *M. hyorhinis* strains.

1. Introduction

Mycoplasma hyorhinis, first isolated in 1953 (Carter and McKay, 1953), is a frequently occurring pathogenic microorganism in swine. It is a common inhabitant of the ciliated upper respiratory tract (Switzer, 1955; Friis, 1971; Friis and Feenstra, 1994). Under certain conditions *M. hyorhinis* can cause systemic infections, mainly fibrinous polyserositis and arthritis, but it has also been implicated as a cause of pneumonia (Kobisch and Friis, 1996; Kobayashi et al., 1996). *M. hyorhinis* has recently emerged as one of the main concerns of swine veterinarians dealing with post-weaning morbidity and mortality (Clavijo et al., 2017).

Up to the present day, no effective vaccine is commercially available against *M. hyorhinis* infection, thus adequate housing conditions are crucial in the prevention. Economic losses caused by *M. hyorhinis* infection can be reduced by appropriate antibiotic treatment. Mycoplasmas are cell-wall-less microorganisms, thus they are intrinsically resistant to β-lactam antimicrobials. Sulphonamides and trimethoprim are also ineffective against *Mycoplasma* species as they do not synthesize folic acid (McCormack, 1993). Mycoplasmas are generally susceptible to antibiotics that affect protein or nucleic acid synthesis (Hannan et al., 1989). However, decreased effectiveness of a growing number of antimicrobial agents used in the therapy of porcine mycoplasma infections such as fluoroquinolones, macrolides or

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Table 1
Background information and MIC data of the 38 Hungarian *Mycoplasma hyorhinis* strains isolated from swine in Hungary and the *M. hyorhinis* reference strain ATCC 17981. MIC values are expressed in μg/ml.

Sample ID	Origin of herd	Date	Sample source	Fluoroquinolone		Tetracycline		Aminoglycoside and Aminocyclitol	
				Enrofloxacin	Marbofloxacin	Doxycycline	Oxytetracycline	Gentam.	Spectinom.
ATCC 17981				0.625	0.625	≤0.039	≤0.25	1	4
MycSu21	Heves	2014	lung	0.625	1.25	0.078	≤0.25	0.5	4
MycSu23	Bácsalmás	2014	lung	0.625	0.625	≤0.039	≤0.25	0.5	2
MycSu24	Hajdúszoboszló	2014	lung	0.625	1.25	0.078	≤0.25	1	4
MycSu25	Hajdúdorog	2014	lung	0.625	0.625	0.078	≤0.25	1	4
MycSu27	Hajdúszoboszló	2015	lung	1.25	1.25	0.312	2	1	4
MycSu29	Bácsalmás	2015	lung	1.25	1.25	0.156	1	1	4
MycSu32t	Bácsalmás	2016	tonsil	0.625	1.25	0.312	2	2	4
MycSu54	Lovasberény	2016	lung	0.625	0.625	≤0.039	≤0.25	2	8
MycSu55	Felsőbabád	2016	lung	0.625	1.25	≤0.039	≤0.25	1	4
MycSu56	Nemesnádudvar	2016	lung	0.625	1.25	0.156	0.5	1	4
MycSu57	Osli	2016	lung	0.312	0.625	0.078	0.5	0.5	2
MycSu58	Somogycsicsó	2017	lung	0.625	1.25	0.625	2	1	4
MycSu59	Felsőszentiván	2017	lung	0.625	1.25	0.312	1	1	4
MycSu61	Pápa	2017	lung	0.625	1.25	0.078	0.5	1	4
MycSu62	Beremend	2017	lung	0.625	0.625	0.078	0.5	0.5	4
MycSu63	Sárbogárd	2017	pleural exudate	0.625	1.25	≤0.039	≤0.25	1	4
MycSu64	Csongrád	2017	pleural exudate	0.312	0.625	0.078	≤0.25	0.5	2
MycSu65	Borota	2017	nasal swab	0.312	0.625	≤0.039	≤0.25	0.5	4
MycSu75	Mohács	2017	synovial fluid	0.312	0.625	0.078	≤0.25	1	4
MycSu76	Hajdúszoboszló	2017	pleural exudate	0.625	0.625	≤0.039	≤0.25	0.5	2
MycSu86	Jánoshalma	2017	lung	0.625	0.625	≤0.039	≤0.25	0.5	2
MycSu88	Derecske	2017	synovial fluid	0.312	0.625	≤0.039	≤0.25	0.5	1
MycSu90	Orosháza	2017	lung	0.625	0.625	0.156	1	0.5	4
MycSu92	Baracska	2017	lung	0.625	0.625	0.078	0.5	1	4
MycSu94	Fadd	2017	nasal swab	0.625	1.25	0.312	2	1	>64
MycSu97	Alsómocsolád	2017	nasal swab	0.312	0.625	0.078	≤0.25	0.5	4
MycSu99	Szalánta	2017	nasal swab	0.625	1.25	0.156	1	1	4
MycSu101	Bácsalmás	2017	pericardial matter	0.312	0.625	0.078	≤0.25	0.5	4
MycSu103	Jánoshalma	2017	lung	0.625	0.625	0.078	≤0.25	1	4
MycSu105	Tiszainoka	2017	meninx	0.312	0.625	0.078	≤0.25	1	4
MycSu106	Létavértes	2017	bursa	0.625	0.625	0.156	0.5	1	4
MycSu107	Nagymágocs	2017	pericardial matter	0.312	0.625	≤0.039	≤0.25	0.5	2
MycSu109	Bábolna	2017	cerebra	0.312	0.625	0.078	≤0.25	1	2
MycSu110	Városföld	2017	pericardium	0.312	0.625	0.078	≤0.25	0.5	2
MycSu111	Óbánya	2017	lung	0.312	0.625	≤0.039	≤0.25	0.5	2
MycSu112	Vásárosnamény	2017	conjunctival swab	0.312	0.625	≤0.039	≤0.25	0.5	2
MycSu113	Hajdúnánás	2017	pleura	0.625	0.625	0.078	≤0.25	1	4
MycSu114	Szőny	2017	synovial fluid	0.625	1.25	≤0.039	0.5	0.5	4
Range				**0.312 to 1.25**	**0.625 to 1.25**	**≤0.039 to 0.625**	**≤0.25 to 2**	**0.5 to 2**	**1 to >64**
Mode				**0.625**	**0.625**	**0.078**	**≤0.25**	**1**	**4**

(*continued on next page*)

Table 1 (*continued*)

Sample ID	Macrolide					Pleuromutilin		Lincosamide	Phenicol
	Tylosin	Tilmicosin	Tylvalosin	Tulathrom.	Gamithrom.	Tiamulin	Valnemulin	Lincomycin	Florfenicol
ATCC 17981	≤0.25	1	≤0.25	2	0.5	0.078	≤0.039	0.5	1
MycSu21	≤0.25	2	≤0.25	2	0.5	0.078	≤0.039	0.5	2
MycSu23	0.5	1	≤0.25	4	0.5	0.156	≤0.039	0.5	0.5
MycSu24	64	> 64	8	> 64	> 64	0.312	≤0.039	> 64	2
MycSu25	0.5	2	≤0.25	4	2	0.156	≤0.039	1	2
MycSu27	> 64	> 64	8	> 64	> 64	0.625	≤0.039	> 64	4
MycSu29	1	4	≤0.25	4	2	0.156	≤0.039	1	2
MycSu32t	> 64	> 64	8	> 64	> 64	0.312	≤0.039	> 64	2
MycSu54	0.5	2	≤0.25	2	1	0.078	≤0.039	≤0.25	1
MycSu55	≤0.25	2	≤0.25	2	1	0.156	≤0.039	≤0.25	1
MycSu56	0.5	4	≤0.25	4	2	0.156	≤0.039	0.5	2
MycSu57	64	> 64	4	> 64	> 64	0.312	≤0.039	> 64	1
MycSu58	0.5	4	≤0.25	4	2	0.156	≤0.039	0.5	2
MycSu59	0.5	2	≤0.25	4	2	0.156	≤0.039	0.5	2
MycSu61	0.5	2	≤0.25	4	1	0.156	≤0.039	0.5	1
MycSu62	> 64	> 64	8	> 64	> 64	0.625	≤0.039	> 64	2
MycSu63	0.5	4	≤0.25	4	1	0.156	≤0.039	0.5	2
MycSu64	≤0.25	1	≤0.25	2	0.5	0.078	≤0.039	≤0.25	1
MycSu65	0.5	2	≤0.25	4	1	0.156	≤0.039	0.5	1
MycSu75	0.5	2	≤0.25	4	1	0.156	≤0.039	0.5	1
MycSu76	≤0.25	1	≤0.25	4	1	0.156	≤0.039	0.5	1
MycSu86	> 64	> 64	4	> 64	> 64	0.312	≤0.039	> 64	1
MycSu88	≤0.25	1	≤0.25	4	1	0.156	≤0.039	≤0.25	1
MycSu90	> 64	> 64	4	> 64	> 64	0.156	≤0.039	> 64	0.5
MycSu92	1	2	≤0.25	2	1	0.156	≤0.039	0.5	1
MycSu94	1	16	≤0.25	4	2	0.312	≤0.039	≤0.25	0.5
MycSu97	≤0.25	2	≤0.25	4	1	0.078	≤0.039	0.5	2
MycSu99	0.5	2	≤0.25	2	0.5	0.078	≤0.039	0.5	0.5
MycSu101	> 64	> 64	4	> 64	> 64	0.156	≤0.039	> 64	2
MycSu103	64	> 64	4	> 64	> 64	0.312	≤0.039	> 64	2
MycSu105	0.5	2	≤0.25	2	1	0.078	≤0.039	≤0.25	1
MycSu106	> 64	> 64	4	> 64	> 64	0.312	≤0.039	> 64	2
MycSu107	≤0.25	1	≤0.25	4	2	0.156	≤0.039	1	2
MycSu109	≤0.25	1	≤0.25	4	2	0.156	≤0.039	1	2
MycSu110	≤0.25	1	≤0.25	4	1	0.156	≤0.039	0.5	2
MycSu111	≤0.25	1	≤0.25	4	1	0.156	≤0.039	0.5	2
MycSu112	≤0.25	1	≤0.25	4	1	0.078	≤0.039	0.5	2
MycSu113	> 64	> 64	4	> 64	> 64	0.156	≤0.039	> 64	2
MycSu114	> 64	> 64	4	> 64	> 64	0.156	≤0.039	> 64	1
Range	**≤0.25 to > 64**	**1 to > 64**	**≤0.25 to 8**	**2 to > 64**	**0.5 to > 64**	**0.078 to 0.625**	**≤0.039**	**≤0.25 to > 64**	**0.5 to 4**
Mode	**0.5**	**2; > 64**	**≤0.25**	**4**	**1**	**0.156**	**≤0.039**	**0.5**	**2**

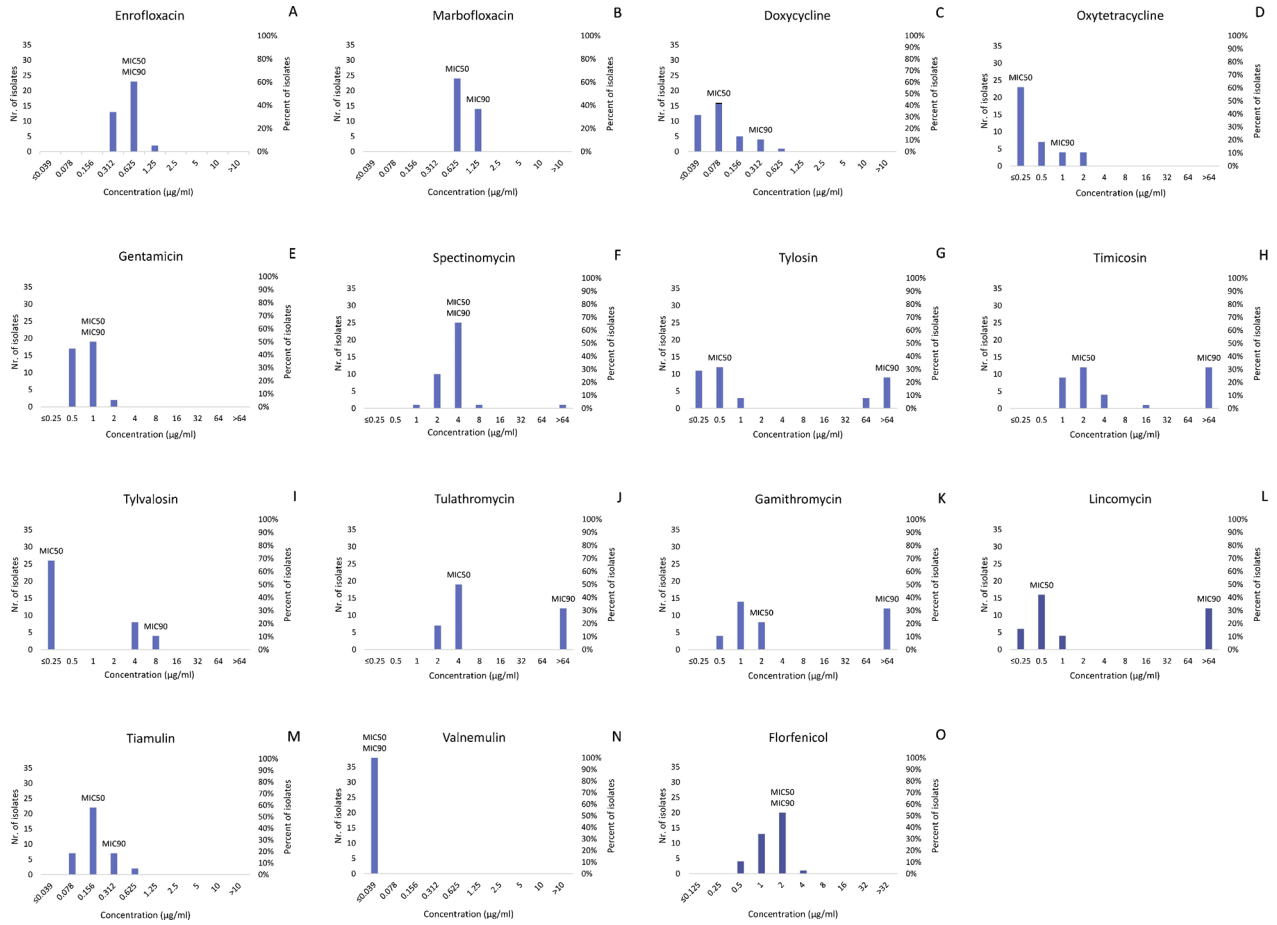

Fig. 1. MIC distribution of the Hungarian isolates for each antibiotic tested in this study.

lincomycin has been reported (Kobayashi et al., 2005; Aarestrup and Kempf, 2006; Lee et al., 2014).

Data originating from *in vitro* determination of antibiotic susceptibility are essential for the choice of the appropriate antimicrobial agent in the therapy. However, information about the antibiotic susceptibility profile of Hungarian or even European *M. hyorhinis* strains are scarce in the literature. The aim of this study was to determine the susceptibility of 38 Hungarian *M. hyorhinis* isolates to fifteen antibiotics approved for therapeutic applications in veterinary use in Hungary (ECDC/EFSA/EMA, 2015).

2. Materials and methods

Thirty-eight *M. hyorhinis* strains originating from different parts of Hungary were tested in this study (Table 1). The samples were collected between 2014 and 2017. Ethical approval was not required for the study as all samples were collected during routine diagnostic examinations or necropsies with the consent of the owners. *M. hyorhinis* strains were isolated from various types of clinical samples (tissue and swab samples) (Table 1). Samples were homogenized in 2 ml of liquid Mycoplasma medium (Mycoplasma Experience Ltd., UK) and cultured at 37 °C in a 5% CO_2 atmosphere. Following colour change (red to yellow shift) the cultures were inoculated onto solid Mycoplasma media (Mycoplasma Experience Ltd., UK) and were incubated at 37 °C and 5% CO_2 until visible colonies appeared. Cultures were filter cloned only once to minimize *in vitro* mutations of the isolates. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturers' instructions for Gram-negative bacteria. All isolates were identified by polymerase chain reaction (PCR) (Caron et al., 2000; Assunção et al., 2005). In order to exclude other *Mycoplasma* spp. contamination the samples were submitted to a universal Mycoplasma PCR system (Lauerman et al., 1995) followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, USA), sequence analysis and BLAST search. Aliquots of the purified cultures were stored frozen at −70 °C until required. The number of colour changing units (CCU) was calculated by microplate dilution method (Hannan, 2000).

The following antimicrobial agents were investigated: two fluoroquinolones: enrofloxacin and marbofloxacin; one aminoglycoside: gentamicin; one aminocyclitol: spectinomycin, two tetracyclines: doxycycline and oxytetracycline; five macrolides: tylosin, tilmicosin, tylvalosin, tulathromycin and gamithromycin; one lincosamide: lincomycin; two pleuromutilins: tiamulin and valnemulin; and one phenicol: florfenicol. Tylvalosin originated from ECO Animal Health Ltd., UK (Aivlosin), tulathromycin originated from Zoetis Inc., USA, and the rest of antimicrobial agents originated from VETRANAL, Sigma-Aldrich, Germany. They were diluted and stored according to the recommendations of Hannan (2000). Twofold dilutions were freshly prepared for each microtest in the range 0.039–10 µg/ml for fluoroquinolones, doxycycline and pleuromutilins, 0.125–32 µg/ml for florfenicol, and 0.25–64 µg/ml for gentamicin, spectinomycin, oxytetracycline, macrolides and lincomycin.

The microbroth dilution test was performed in 96-well microtiter plates containing twofold dilution series of the antibiotic, growth control, sterility control and pH control wells. Liquid Mycoplasma medium (Mycoplasma Experience Ltd., UK) was used as a culture medium. The duplicates of three clinical isolates and as quality control, the duplicate of the type strain (ATCC 17981) were tested on each plate.

The MIC (minimal inhibitory concentration) value of each isolate was defined as the lowest concentration of the antibiotic that

completely inhibits the growth in the broth (no pH and colour change), recorded as soon as the growth controls changed colour (Ter Laak et al., 1991; Hannan, 2000). MIC_{50} and MIC_{90} values were defined as the lowest concentrations that inhibit 50% and 90% of bacterial isolates.

3. Results

In the present study, MIC values of *M. hyorhinis* type strain ATCC 17981 (Table 1) were similar or within the range of values previously obtained for enrofloxacin (0.5–2 μg/ml), doxycycline (≤0.03 μg/ml), oxytetracycline (0.05-0.25 μg/ml), gentamicin (2–16 μg/ml), spectinomycin (2–4 μg/ml), tylosin (0.06-0.5 μg/ml), tilmicosin (2 μg/ml), lincomycin (0.4–2 μg/ml) and tiamulin (≤0.03-0.1 μg/ml) in studies using the microbroth dilution method (Ter Laak et al., 1991; Kobayashi et al., 1996; Hannan et al., 1997; Hannan, 2000; Wu et al., 2000; Lee et al., 2014). For the rest of the antimicrobial agents, data determined by microbroth dilution test were not available. Our results for type strain ATCC 17981 were consistent throughout the study indicating good reproducibility of the test.

The MIC values of the fifteen antimicrobial agents obtained from the examinations of the Hungarian *M. hyorhinis* isolates are shown in Fig. 1, and listed in Table 1.

In this study *M. hyorhinis* strains were isolated from various clinical samples, but no correlation could be observed between the type of the samples and the antibiotic susceptibility profile of the *M. hyorhinis* isolates. Some strains were isolated from the same farm with at least six months between the sampling, but no consequent tendency was found in the results of their susceptibility tests.

The MIC values for enrofloxacin and marbofloxacin clustered steadily around the MIC_{50} value (0.625 μg/ml). All of the strains had low MIC values to doxycycline (≤0.625 μg/ml), oxytetracycline and gentamicin (≤2 μg/ml). In case of spectinomycin, the isolates had MIC values ≤8 μg/ml, except one strain which yielded extremely high MIC value (> 64 μg/ml). MIC values of macrolides and lincomycin divided the strains into two distinct populations. Most of the strains had MIC values clustering around MIC_{50}, while the rest of the strains were inhibited by these antimicrobial agents with higher MIC values close to the MIC_{90}. All of the strains were inhibited with MIC values ≤0.625 μg/ml by tiamulin and ≤0.039 μg/ml by valnemulin. In case of florfenicol, the Hungarian strains had MIC values in the range of 0.5–4 μg/ml.

4. Discussion

Relevant data about antibiotic susceptibility of *M. hyorhinis* strains are scarce in the literature, especially concerning European strains. *In vitro* determination of antibiotic susceptibility of mycoplasmas is difficult and time-consuming, thus it is not performed routinely in practice (Hannan, 2000). However, these data would be essential for the adequate therapy with taking into account that the results of *in vitro* antibiotic susceptibility tests can only predict the expected *in vivo* efficacy of the antibiotics. It causes more difficulties that standard breakpoints of susceptible, intermediate and resistant categories to antimicrobial agents concerning *M. hyorhinis* have not been defined yet. In the lack of official breakpoints, the data of the present study are evaluated by comparing them to MIC values previously reported in other publications (Ter Laak et al., 1991; Kobayashi et al., 1996; Hannan et al., 1997; Hannan, 2000; Wu et al., 2000; Kobayashi et al., 2005; Makhanon et al., 2006; Lee et al., 2014; Wanjiru Maingi et al., 2014; Jang et al., 2016; Gautier-Bouchardon, 2018).

In accordance with previous reports, pleuromutilins showed markedly low MIC values against *M. hyorhinis* (Gautier-Bouchardon, 2018; Wanjiru Maingi et al., 2014; Jang et al., 2016). Likewise, all of the Hungarian isolates were inhibited by low MIC values of doxycycline. MIC values of oxytetracycline were within the range recently reported in the review of Gautier-Bouchardon (2018). Susceptibility to tetracyclines was reported previously in Japan and the Netherlands also (Ter Laak et al., 1991; Kobayashi et al., 1996, 2005); however, *M. hyorhinis* strains with higher MIC values were found in China, Korea and Thailand (Makhanon et al., 2006; Wanjiru Maingi et al., 2014; Lee et al., 2014). The reason of this discrepancy might be the difference in the general antibiotic usage in the countries.

The Hungarian strains were inhibited by similar MIC_{50} values, but lower MIC_{90} values of gentamicin when compared to isolates originating from Korea and the USA (Gautier-Bouchardon, 2018; Lee et al., 2014). Though gentamicin has not been licensed for the treatment of mycoplasmosis yet, it would be worth considering due to the results of the present study. Likewise, florfenicol also could be a good choice, as Hungarian *M. hyorhinis* strains showed low or intermediate MIC values, identical to that which has been reported by Ter Laak et al. (1991), investigating Dutch *M. hyorhinis* isolates.

In this study, *M. hyorhinis* strains had moderate MIC values for fluoroquinolones within the range published by Gautier-Bouchardon (2018). Fluoroquinolones are highly active, broad spectrum antibiotics with many uses in veterinary medicine and their importance is critical in the therapy of humans as well. Since *M. hyorhinis* isolates with reduced susceptibility are evidenced (Gautier-Bouchardon, 2018) and considering their importance for human medicine, fluoroquinolones should not be the first choice when treating porcine mycoplasma infections.

According to Gautier-Bouchardon (2018), macrolides and lincosamides still has good *in vitro* activity against most *M. hyorhinis* strains, but there is also an evidence for the selection of resistant strains in the recent years. In Japan, *M. hyorhinis* strains with high MIC values to macrolides have been detected already in the 1990s, then incidence of these strains has been further increased for the 2000s (Kobayashi et al., 1996, 2005). Although MIC_{50} values of the tested Hungarian *M. hyorhinis* strains are low, the observed higher MIC_{90} values may indicate shifts in the susceptibility of the bacterial population (Lysnyansky et al., 2015).

Most of the Hungarian isolates showed low or moderate MIC values to spectinomycin in agreement with previous reports from other countries (Ter Laak et al., 1991; Wu et al., 2000). However, the outlier Hungarian *M. hyorhinis* strain (MIC ≥ 64 μg/ml) along with previously published Korean strains with similarly high MIC values (Lee et al., 2014) indicate that *M. hyorhinis* might have decreasing susceptibility to spectinomycin.

5. Conclusion

The present study determined the antibiotic susceptibility profiles of 38 Hungarian *M. hyorhinis* strains. Based on previous literature data and our results, pleuromutilins and tetracyclines, especially doxycycline are the most effective agents against *M. hyorhinis* infections in Hungary, nevertheless, gentamicin and florfenicol also could be a good choice in the therapy. Our results confirm the increasing resistance to macrolides and lincomycin and indicate emerging resistance to spectinomycin as well.

The present study provides information about antibiotic susceptibility of *M. hyorhinis* strains in the central European region. The results also highlight the importance of the systematic monitoring and regular testing when antibiotic treatment is required in order to avoid the development of resistance and preserve critical antimicrobials (*e.g.* fluoroquinolones) for the therapy of humans.

Conflict of interest

The authors declare that they have no competing interests.

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