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Antibiotic susceptibility profiles of *Mycoplasma* sp. 1220 strains isolated from geese in Hungary

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Abstract

Background: *Mycoplasma* sp. 1220 can induce inflammation primarily in the genital and respiratory tracts of waterfowl, leading to serious economic losses. Adequate housing and appropriate antibiotic treatment are promoted in the control of the disease. The aim of the present study was to determine the in vitro susceptibility to thirteen different antibiotics and an antibiotic combination of thirty-eight *M.* sp. 1220 strains isolated from geese and a duck in several parts of Hungary, Central Europe between 2011 and 2015.

Results: High MIC_{50} values were observed in the cases of tilmicosin (>64 μg/ml), oxytetracycline (64 μg/ml), norfloxacin (>10 μg/ml) and difloxacin (10 μg/ml). The examined strains yielded the same MIC_{50} values with spectinomycin, tylosin and florfenicol (8 μg/ml), while enrofloxacin (MIC_{50} 5 μg/ml), doxycycline (MIC_{50} 5 μg/ml), lincomycin (MIC_{50} 4 μg/ml) and lincomycin-spectinomycin (1:2) combination (MIC_{50} 4 μg/ml) inhibited the growth of the bacteria with lower concentrations. Tylvalosin (MIC_{50} 0.5 μg/ml) and two pleuromutilins (tiamulin MIC_{50} 0.625 μg/ml; valnemulin MIC_{50} ≤ 0.039 μg/ml) were found to be the most effective drugs against *M.* sp. 1220. However, strains with elevated MIC values were detected for all applied antibiotics.

Conclusions: Valnemulin, tiamulin and tylvalosin were found to be the most effective antibiotics in the study. Increasing resistance was observed in the cases of several antibiotics. The results highlight the importance of testing *Mycoplasma* species for antibiotic susceptibility before therapy.

Keywords: Antibiotic resistance, Duck, Goose, MIC, Microbroth dilution, *Mycoplasma* sp. 1220

Abbreviations: MIC, Minimal inhibitory concentrations

Background

Mycoplasma sp. 1220 was first described as a new *Mycoplasma* species by Stipkovits et al. in 1986 [1]. This *Mycoplasma* species causes cloaca and phallus inflammation and testicular atrophy in the ganders [1, 2]. In the infected geese salpingitis and vaginitis are the main symptoms [1, 3]. The egg production activates the pathogen and the flared up mycoplasmosis could induce lethal pathological changes in the embryos [1, 4]. Airsacculitis and peritonitis are also common, and general

symptoms such as changes in thirst, decreased food consumption, body weight losses, weakness, nasal discharge, impaired breathing, conjunctivitis, diarrhoea and nervous signs were also described in the affected waterfowl flocks [2, 5–8]. *Mycoplasma* infection of the birds can aggravate diseases caused by other agents and could lead to serious economic losses [3, 6]. Since there is no commercially available vaccine against *M.* sp. 1220, adequate housing and appropriate antibiotic treatment are promoted in the control of the diseases caused by this agent. Prophylactic medication could prevent economic losses if appropriate antibiotics are administered in the early weeks of life and in expected stress periods. Medication of the layers is essential to reduce the vertical transmission of *M.* sp. 1220 [2].

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Mycoplasmas are resistant to β-lactam antimicrobials because of the lack of cell-wall and the bacteria are also resistant to membrane synthesis inhibitors [2, 9]. Antibiotics such as quinolones, tetracyclines, macrolides and pleuromutilins which induce DNA fragmentation or inhibition at the level of protein synthesis are the drugs of choice for the therapy of mycoplasmosis. Among the macrolides, erythromycin showed high effectiveness against *Mycoplasma* strains which could ferment glucose (e.g. *M.* sp. 1220), while arginine-hydrolysing strains proved to be less susceptible to this compound [2, 10]. *Mycoplasma* infected waterfowl and poultry flocks are usually treated with macrolides, pleuromutilins or with the combination of lincomycin and spectinomycin [3, 11–18].

The aim of this study was to determine the susceptibility of 38 Hungarian *M.* sp. 1220 isolates to thirteen antibiotics and a drug combination using the microbroth dilution method.

Methods

A total of 38 *M.* sp. 1220 strains isolated from geese and a duck originating from different parts of Hungary were tested in the study (Table 1, Fig. 1). The samples were collected during routine diagnostic examinations or necropsies between 2011 and 2015, thus ethical approval was not required for the study. Phallus lymph, cloaca swabs, tracheal swabs, follicules and lung samples were washed in 2 ml of *Mycoplasma* broth medium (pH 7.8) (ThermoFisher Scientific Inc./Oxoid Inc./, Waltham, MA) supplemented with 0.5 % (w/v) sodium pyruvate, 0.5 % (w/v) glucose and 0.005 % (w/v) phenol red and incubated at 37 °C in a 5 % CO_2 atmosphere. The cultures were inoculated onto solid *Mycoplasma* media (Thermo Fisher Scientific Inc./Oxoid Inc./) after colour change of the broth, and were incubated at 37 °C and 5 % CO_2 until visible colonies appeared (1–2 days). Purification of mixed cultures was performed by one-time filter cloning, minimizing the in vitro mutations of the isolates. The QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany) was used for DNA extraction according to the manufacturers' instructions for Gram-negative bacteria. The purity of the cultures was confirmed by a universal *Mycoplasma* PCR system targeting the 16S/23S rRNA intergenic spacer region in *Mycoplasmatales* followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA), sequence analysis and BLAST search [19]. The number of colour changing units (CCU) was calculated by microbroth dilution method, from the lowest dilution showing colour change after one week of incubation [9].

The following antimicrobial agents were examined during the microbroth dilution tests: the fluoroquinolones: enrofloxacin (batch SZBA336XV), difloxacin (SZBD178XV) and norfloxacin (batch SZBD099XV);

the aminoglycoside: spectinomycin (batch SZBB166XV); the lincosamide: lincomycin (batch SZBC340XV); the tetracyclines: doxycycline (batch SZBD007XV) and oxytetracycline (batch SZBC320XV); the macrolides: tilmicosin (batch SZBC345XV) and tylosin (batch SZBB160XV); the pleuromutilins: tiamulin (batch SZBC026XV) and valnemulin (batch SZBE127XV); and the phenicol: florfenicol (batch SZBC223XV); all products originated from VETRANAL, Sigma-Aldrich, Germany. The macrolide tylvalosin (Aivlosin, ECO Animal Health Ltd., UK; LOT M102A) was also included in the examinations. Lincomycin and spectinomycin were applied also in combination at a ratio of 1:2. The antibiotics were diluted and stored according to the recommendations of Hannan [9]. Stock solutions of 1 mg/ml fluoroquinolones were prepared in 0.1 M NaOH; stock solution of 1 mg/ml florfenicol was prepared in 96 % ethanol and in sterile distilled water; and the rest of the stock solutions of 1 mg/ml were prepared in sterile distilled water. Dilutions of the antibiotics were freshly prepared for each microtest from the aliquots stored at −70 °C. Twofold dilutions were prepared in the range 0.039–10 µg/ml for fluoroquinolones, doxycycline and pleuromutilins, 0.25–64 µg/ml for spectinomycin, lincomycin, lincomycin-spectinomycin (1:2) combination, oxytetracycline and macrolides and 0.125–32 µg/ml for florfenicol.

The microbroth dilution examinations on 10^4–10^5 CCU/ml of the strains were performed according to Hannan [9]. *Mycoplasma* broth medium was used in the tests as well, and each 96-well microtiter plates contained growth controls (broth medium without antibiotic), sterility controls (broth medium without antibiotic and *Mycoplasma* inoculum) and pH controls (broth medium adjusted to pH 6.8). One clinical isolate (strain MYCAV 65) was selected to be used as quality control of minimal inhibitory concentration (MIC) determination throughout the experiments. The duplicates of three clinical isolates and the duplicate of the selected strain (MYCAV 65) were tested on each 96-well microtiter plates.

The MIC values were determined from the lowest concentration of the antibiotics where no pH and colour change of the broth was detected after one week of incubation, meaning that the growth of the bacteria was completely inhibited in the broth. MIC_{50} and MIC_{90} values were defined as the lowest concentrations that inhibited the growth of 50 % or 90 % of the strains [9].

Results

The quality control strain (MYCAV 65) showed consistent results throughout the study. Strains with elevated MIC values were found in the cases of all tested antibiotics (Tables 1 and 2).

Among the fluoroquinolones, the MIC values of enrofloxacin and difloxacin showed a wide range (1.25 to

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Table 1 Background data and MIC values of the isolated *Mycoplasma* sp. 1220 strains

Sample ID	Sample source	Place	Animal	Date	MIC values (µg/ml)					
					Fluoroquinolones			Aminoglycoside	Lincosamide	
					Enrofloxacin	Norfloxacin	Difloxacin	Spectinomycin	Lincomycin	Lincomycin-spectinomycin (1:2) combination
MYCAV 65	Phallus lymph	Rém	goose	2014	5	>10	10	16	4	4
MYCAV 34	Phallus lymph	Szentes	goose	2011	5	>10	10	8	4	2
MYCAV 35	Phallus lymph	Rém	goose	2012	5	>10	10	>64	4	4
MYCAV 36	Cloaca	Hajdúböszörmény	goose	2012	5	>10	>10	64	4	4
MYCAV 38	Cloaca	Kelebia	goose	2012	2.5	>10	10	8	2	4
MYCAV 44	Cloaca	Nagykamarás	goose	2012	5	>10	10	8	4	4
MYCAV 47	Lung	Tázlár	duck	2012	>10	>10	>10	16	>64	16
MYCAV 49	Phallus lymph	Tiszavasvári	goose	2013	5	>10	10	16	4	4
MYCAV 50	Phallus lymph	Cered	goose	2013	>10	>10	>10	16	4	4
MYCAV 51	Phallus lymph	Derekegyház	goose	2013	5	>10	10	32	4	4
MYCAV 53	Phallus lymph	Szentes	goose	2013	5	>10	10	16	4	4
MYCAV 54	Follicule	Hódmezővásárhely	goose	2013	5	>10	10	8	4	4
MYCAV 55	Follicule	Kiskunmajsa	goose	2013	10	>10	10	8	4	4
MYCAV 56	Phallus lymph	Sükösd	goose	2013	1.25	>10	1.25	8	4	4
MYCAV 59	Follicule	Rém	goose	2013	5	>10	10	8	4	4
MYCAV 61	Phallus lymph	Tatárszentgyörgy	goose	2013	5	>10	10	16	2	4
MYCAV 63	Trachea	Sükösd	goose	2013	1.25	10	1.25	8	2	2
MYCAV 66	Phallus lymph	Tiszaföldvár	goose	2014	5	>10	10	16	4	4
MYCAV 67	Phallus lymph	Szentes	goose	2014	5	>10	10	8	>64	16
MYCAV 68	Phallus lymph	Érpatak	goose	2014	5	>10	10	8	>64	32
MYCAV 69	Phallus lymph	Ludas	goose	2014	5	>10	10	4	4	4
MYCAV 70	Phallus lymph	Cered	goose	2014	>10	>10	>10	16	4	4
MYCAV 71	Phallus lymph	Sükösd	goose	2014	1.25	>10	1.25	8	2	4
MYCAV 72	Phallus lymph	Nagykamarás	goose	2014	5	>10	10	8	4	4
MYCAV 75	Phallus lymph	Dömsöd	goose	2014	5	>10	10	16	4	4
MYCAV 76	Phallus lymph	Tiszabábolna	goose	2014	5	>10	10	32	8	4
MYCAV 91	Phallus lymph	Hajdúsámson	goose	2011	10	>10	>10	8	8	4
MYCAV 93	Phallus lymph	Bojt	goose	2014	2.5	>10	1.25	8	2	4
MYCAV 94	Cloaca	Tiszabábolna	goose	2012	2.5	>10	5	16	4	4
MYCAV 160	Phallus lymph	Érpatak	goose	2015	>10	>10	>10	16	4	4
MYCAV 161	Phallus lymph	Szilaspogony	goose	2015	>10	>10	>10	8	4	4
MYCAV 162	Phallus lymph	Encsencs	goose	2015	2.5	>10	10	8	4	4
MYCAV 176	Phallus lymph	Cered	goose	2015	10	>10	5	8	4	4
MYCAV 177	Phallus lymph	Cered	goose	2015	>10	>10	10	8	4	4
MYCAV 178	Follicule	Cered	goose	2015	5	>10	10	8	2	4
MYCAV 179	Trachea	Apátfalva	goose	2015	10	>10	10	16	4	4
MYCAV 180	Phallus lymph	Kisbér	goose	2015	5	>10	10	>64	4	4
MYCAV 202	Cloaca	Kelebia	goose	2015	5	>10	5	16	4	4

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| | MIC values (μg/ml) | | | | | | | |
| | Tetracyclines | | Macrolides | | | Pleuromutilines | | Phenicol |
Sample ID	Oxytetracycline	Doxycycline	Tylosin	Tilmicosin	Tylvalosin	Tiamulin	Valnemulin	Florfenicol
MYCAV 65	32	5	0.5	0.5	≤0.25	1.25	0.078	8
MYCAV 34	64	2.5	1	4	≤0.25	0.625	≤0.039	4
MYCAV 35	64	10	1	4	≤0.25	1.25	≤0.039	8
MYCAV 36	64	>10	1	4	≤0.25	1.25	≤0.039	8
MYCAV 38	4	0.156	≤0.25	≤0.25	≤0.25	0.625	≤0.039	4
MYCAV 44	8	0.312	8	>64	0.5	1.25	≤0.039	8
MYCAV 47	>64	5	16	>64	1	2.5	0.312	8
MYCAV 49	64	5	8	>64	0.5	0.625	≤0.039	8
MYCAV 50	>64	5	2	2	≤0.25	0.625	≤0.039	8
MYCAV 51	>64	10	8	>64	0.5	0.625	≤0.039	8
MYCAV 53	>64	10	8	>64	0.5	0.625	≤0.039	8
MYCAV 54	>64	5	8	>64	0.5	0.625	≤0.039	8
MYCAV 55	8	0.312	≤0.25	≤0.25	≤0.25	0.625	≤0.039	4
MYCAV 56	4	0.312	8	>64	0.5	0.625	≤0.039	4
MYCAV 59	32	2.5	0.5	≤0.25	≤0.25	1.25	0.078	2
MYCAV 61	2	0.078	≤0.25	≤0.25	≤0.25	0.312	≤0.039	4
MYCAV 63	4	0.312	4	64	≤0.25	0.156	≤0.039	4
MYCAV 66	>64	>10	≤0.25	≤0.25	≤0.25	0.625	≤0.039	4
MYCAV 67	>64	5	>64	>64	16	2.5	0.078	4
MYCAV 68	>64	10	>64	>64	16	5	≤0.039	8
MYCAV 69	>64	5	8	>64	1	0.625	≤0.039	4
MYCAV 70	>64	>10	16	>64	1	0.625	≤0.039	8
MYCAV 71	8	0.625	8	>64	0.5	0.625	≤0.039	4
MYCAV 72	4	0.312	8	>64	0.5	0.625	≤0.039	4
MYCAV 75	>64	10	≤0.25	≤0.25	≤0.25	0.625	≤0.039	8
MYCAV 76	64	5	8	>64	0.5	1.25	≤0.039	8
MYCAV 91	64	2.5	≤0.25	≤0.25	≤0.25	0.625	≤0.039	8
MYCAV 93	8	0.312	≤0.25	≤0.25	≤0.25	0.312	≤0.039	8
MYCAV 94	>64	>10	≤0.25	≤0.25	≤0.25	0.625	≤0.039	8
MYCAV 160	>64	10	>64	>64	2	0.625	≤0.039	8
MYCAV 161	>64	>10	16	>64	0.5	0.625	≤0.039	8
MYCAV 162	>64	5	16	>64	0.5	0.625	≤0.039	4
MYCAV 176	>64	5	64	>64	4	0.625	≤0.039	16
MYCAV 177	>64	10	>64	>64	4	0.625	≤0.039	32
MYCAV 178	>64	5	4	>64	0.5	0.312	≤0.039	4
MYCAV 179	4	0.312	4	4	0.5	1.25	≤0.039	8
MYCAV 180	4	0.312	32	>64	1	1.25	≤0.039	8
MYCAV 202	32	2.5	0.5	0.5	≤0.25	1.25	≤0.039	8

>10 μg/ml), while all strains had very high MIC values for norfloxacin (≥10 μg/ml) (Fig. 2a, b and c). The MIC_{50} was 8 μg/ml for spectinomycin and most of the strains yielded the MIC_{50} or higher MIC values (Fig. 2d). The MICs for lincomycin clustered around the MIC_{50} value (4 μg/ml) as well, but high MIC values (>64 μg/ml) were yielded in the case of three isolates (Fig. 2e). The MIC_{50} and the MIC_{90} values (4 μg/ml) for lincomycin-spectinomycin (1:2) combination was the same as the MIC_{50} value for lincomycin. In the case of lincomycin-
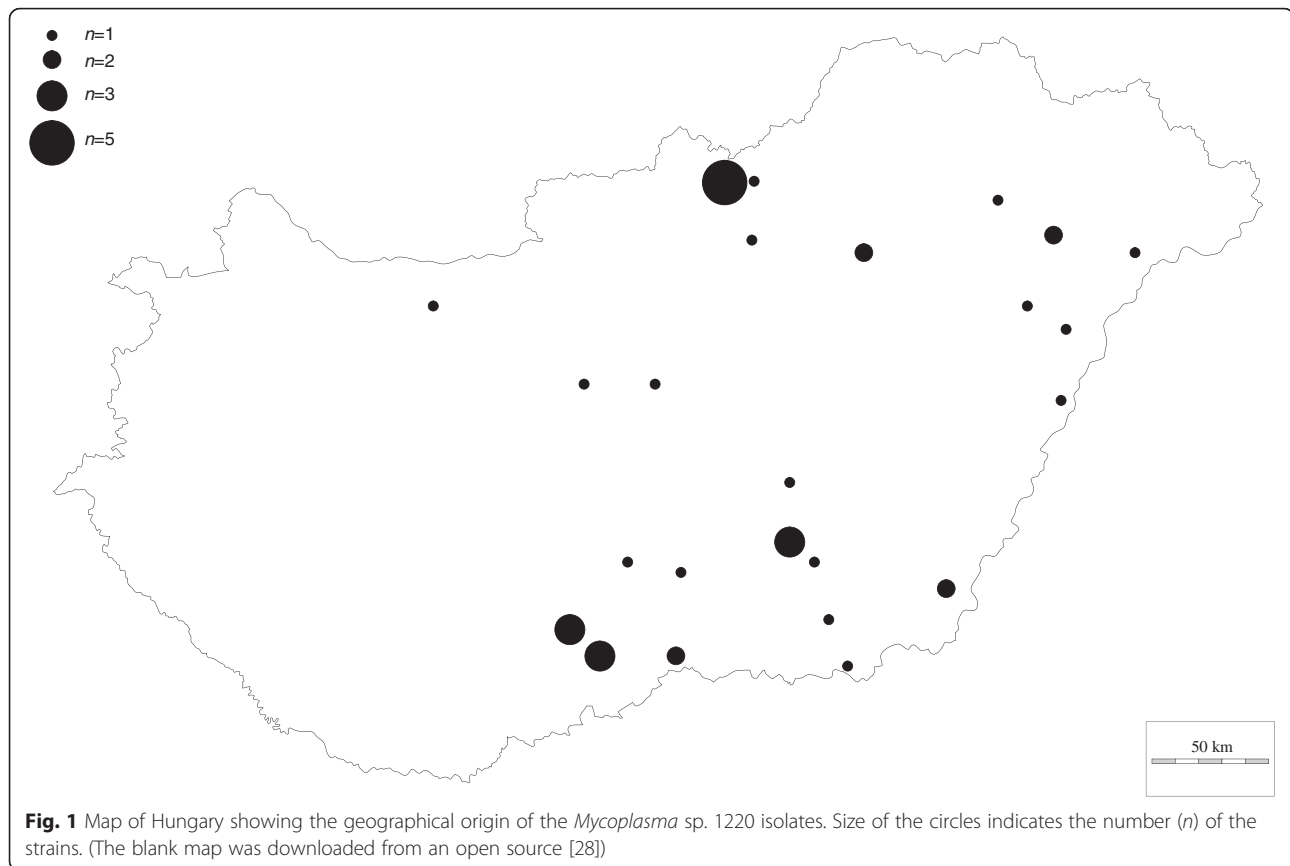
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Fig. 1 Map of Hungary showing the geographical origin of the *Mycoplasma* sp. 1220 isolates. Size of the circles indicates the number (*n*) of the strains. (The blank map was downloaded from an open source [28])

Table 2 Summary of MIC range, MIC_{50} and MIC_{90} values of the isolated *Mycoplasma* sp. 1220 strains

Antibiotic class	Antibiotic agent	Range	MIC_{50}	MIC_{90}
Fluoroquinolones	Enrofloxacin	1.25 to >10	5	>10
	Norfloxacin	10 to >10	>10	>10
	Difloxacin	1.25 to >10	10	>10
Aminoglycoside	Spectinomycin	4 to >64	8	32
Lincosamide	Lincomycin	2 to >64	4	8
	Lincomycin-spectinomycin (1:2) combination	2 to 32	4	4
Tetracyclines	Oxytetracycline	2 to >64	64	>64
	Doxycycline	0.078 to >10	5	>10
Macrolides	Tylosin	≤0.25 to >64	8	>64
	Tilmicosin	≤0.25 to >64	>64	>64
	Tylvalosin	≤0.25 to 16	0.5	4
Pleuromutilins	Tiamulin	0.156 to 5	0.625	1.25
	Valnemulin	≤0.039 to 0.312	≤0.039	0.078
Phenicol	Florfenicol	2 to 32	8	8

spectinomycin (1:2) combination the highest concentration needed for inhibition was 32 μg/ml (Fig. 2f). Broad ranges of the MIC values were observed for tetracyclines (2 to >64 μg/ml for oxytetracycline and 0.078 to >10 μg/ml for doxycycline) with high MIC_{50} and MIC_{90} values (Fig. 2g and h). The broadest ranges of MIC values were detected for tylosin and tilmicosin (≤0.25 to >64 μg/ml) with high MIC_{50} and MIC_{90} values in the case of tilmicosin (Fig. 2i and j). While the MIC values for tylosin showed diverse distribution, the strains' susceptibility profiles formed three groups in the case of tilmicosin (≤0.25, 4 and >64 μg/ml) (Fig. 2j). Among the examined three macrolides (tylosin, tilmicosin and tylvalosin), tylvalosin showed the lowest MIC_{50} value (0.5 μg/ml) against the strains (Fig. 2k). From the pleuromutilins the MIC values of tiamulin were higher than those of valnemulin, and the latter compound was found to be the most active antibiotic in the examinations (Fig. 2l and m). In the case of florfenicol, the susceptibility profiles of most strains were similar to each other and showed the MIC_{50} and MIC_{90} value (8 μg/ml) or its two-fold lower dilution (4 μg/ml) with few exceptions (Fig. 2n).

M. sp. 1220 strains isolated year by year from the same farms and from the same tissue types (e.g. strains MYCAV 34, 53 and 67 from Szentes, strains MYCAV 50, 70, 176 and 177 from Cered, or strains MYCAV 38 and 202 from Kelebia) showed elevated MIC values from
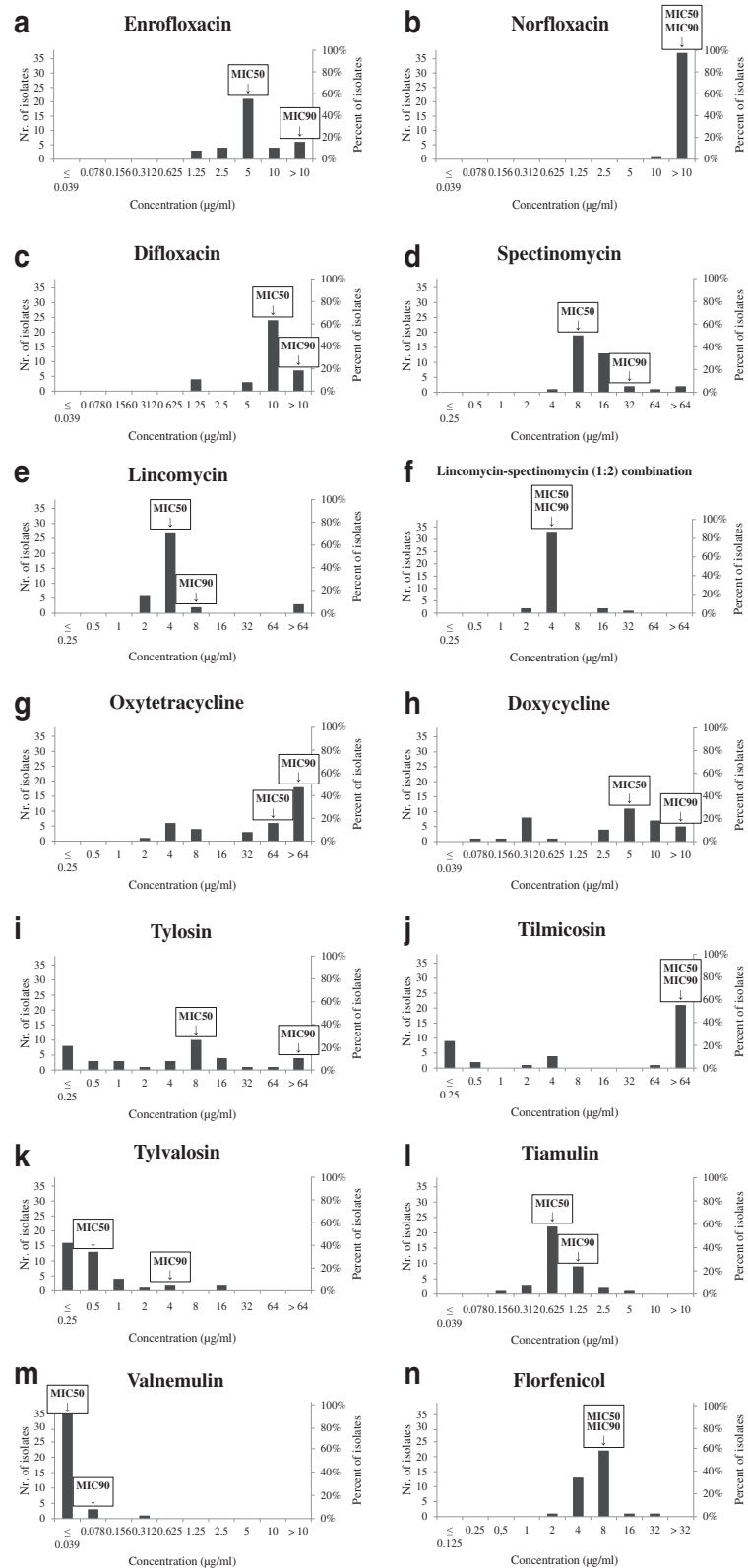
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Fig. 2 MIC distribution of test antibiotics against *Mycoplasma* sp. 1220 isolates

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year to year in the cases of certain antibiotics. Higher MIC values were detected in subsequent isolates for lincomycin, lincomycin-spectinomycin combination, tetracyclines (both oxytetracycline and doxycycline), macrolides (tylosin, tilmicosin and tylvalosin), tiamulin and for florfenicol as well.

Discussion

Information about the susceptibility of *M.* sp. 1220 strains to antimicrobials is scarce, as until to date the sole published reference concerning the antibiotic susceptibility profile of this species is a review of Stipkovits and Szathmary [3]. Stipkovits and Szathmary determined the values of enrofloxacin, tylosin, chlortetracycline, oxytetracycline, doxycycline, tiamulin and lincomycin in *Mycoplasma* species affecting waterfowl (*M. anatis*, *M. cloacale*, *M. anseris* and *M.* sp. 1220), although detailed data of their method is lacking [3]. Thus we are facing the absence of reports about the antibiotic susceptibility of *M.* sp. 1220 and also of other *Mycoplasma* species occurring in waterfowl. Therefore, the results of the current study are also compared to data of antibiotic susceptibility of the well-studied *Mycoplasma* species of poultry: *M. synoviae* and *M. gallisepticum*.

Elevated MIC values were reported previously in the case of the fluoroquinolones, especially of enrofloxacin in *M.* sp. 1220 (MIC_{50} 2 μg/ml and MIC_{90} 4 μg/ml) and other *Mycoplasma* species of poultry [3, 13, 20, 21]. In addition, the increasing occurrence of quinolone-resistant *M. synoviae* and *M. gallisepticum* field isolates were also observed [13, 22]. In the current study, the detected MIC_{50} values (5 μg/ml for enrofloxacin, 10 μg/ml for difloxacin and ≥10 μg/ml for norfloxacin) were even higher than the ones reported before [3, 13, 20–22], confirming the observation of increasing quinolone-resistance in *Mycoplasma* species. In order to save these antibiotics for human disease treatment the directive was to reduce the use of these agents in livestock. Former efforts for the prevention of the appearance of quinolone-resistant species are proved to be unsuccessful considering the observed dramatic elevations in the MIC values of these antibiotics in avian *Mycoplasma* species [13, 21, 23].

Administration of the combination of lincomycin and spectinomycin could reduce the egg infertility rates and increase the hatching rates and the egg production in *M.* sp. 1220 infected geese [11]. The lincomycin-spectinomycin therapy was proved to be effective against other *Mycoplasma* species as well; however, application of spectinomycin in monotherapy is not recommended for its insufficient effectiveness and relatively high MIC values in in vitro experiments [12]. In vitro effectiveness of lincomycin at 2 μg/ml MIC_{50} values against *M.* sp. 1220, *M. anseris* and *M. anatis* species has been reported [3]. In the present study, all isolates showed elevated MIC values for

spectinomycin, lincomycin and lincomycin-spectinomycin combination. The growth of a couple of strains was not inhibited even at the highest concentrations used (64 μg/ml) for spectinomycin and lincomycin individually. The combination of the two antibiotics improved their effectiveness, as lincomycin-spectinomycin combination could inhibit the growth of all examined strains within the concentration range used (0.25 to 64 μg/ml) and lower MIC_{90} value was observed also.

Previously, tetracyclines (chlortetracycline, doxycycline and oxytetracycline) showed 1–2 μg/ml MIC values against *M.* sp. 1220 strains. Growth of other *Mycoplasma* species isolated from waterfowl were inhibited at 2–4 μg/ml MIC_{50} values using the same antibiotics [3]. Previously *Mycoplasma* species infecting poultry were observed to be inhibited by elevated MIC values, although with exceptions, as *M. synoviae* strains showed high susceptibility to doxycycline in the Netherlands [12–14]. In the current study, although the *M.* sp. 1220 strains showed broad ranges of MIC values for oxytetracycline and doxycycline, more than 50 % of the strains were inhibited by only higher antibiotic concentrations (64 and 5 μg/ml, respectively) and MIC_{90} values exceeded the concentration ranges used for both compounds. These results show a dramatic increase of the MIC values of tetracyclines against *M.* sp. 1220 strains and reveals the presence of probably highly resistant strains in Hungary.

Macrolides, especially tylvalosin have good in vitro effectiveness against most *Mycoplasma* species infecting poultry, showing lower MIC values in previous examinations than quinolones and tetracyclines [3, 12–15]. However, *M. gallisepticum* could develop resistance rapidly to these compounds, especially to tilmicosin [24]. Earlier, the MIC_{50} values in *M.* sp. 1220, *M. anatis*, *M. anseris* and *M. cloacale* strains were defined to be 2 μg/ml for tylosin [3]. In the current study, the MIC_{50} value (8 μg/ml) of tylosin was higher than the previous observation [3], and the MIC_{90} value exceeded the concentration range used in the experiment. However, high variability was observed in the susceptibility of the strains to this compound. Similarly, wide range of the MIC values was detected for tilmicosin, highlighting the necessity of susceptibility testing before antibiotic treatments. As opposed to the diverse susceptibility profiles of the strains for tylosin, the MIC values of tilmicosin were categorized into three separate groups. The observed distribution of the MIC values is likely in association with the capability of *Mycoplasma* sp. 1220 to develop resistance more rapidly to tilmicosin (i.e. with one or two mutations) than to other macrolides. The same phenomenon was described in other *Mycoplasma* species as well [24]. Out of the three macrolides examined in the study, tylvalosin proved to be the most effective agent against *M.* sp. 1220 strains, showing lower MIC_{50} value

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(0.5 µg/ml) against the pathogen than the majority of the antibiotics tested.

Pleuromutilins showed good in vitro effectiveness against avian *Mycoplasma* species in previous studies and low tendency of the development of resistance to these agents has been reported [16–18, 21]. Tiamulin was used successfully for the treatment of mycoplasmosis and its effectiveness was similar to spectinomycin therapy in the treated geese [11]. Stipkovits and Szathmary described low MIC values (MIC_{50}: 0.06 µg/ml, MIC_{90}: 0.25 µg/ml) of tiamulin in the case of *M.* sp. 1220, and similarly low MIC_{50} values (0.125–1 µg/ml) were observed against *M. anseris*, *M. anatis* and *M. cloacale* [3]. In the present study, pleuromutilins were found to be the most effective antibiotic agents and the examined compounds, especially valnemulin showed high in vitro effectiveness against all tested isolates of the pathogen. However, it is noteworthy, that strains with elevated MIC values were detected for tiamulin (MIC: 2.5–5 µg/ml) and even for valnemulin (MIC: 0.312 µg/ml). Although the low MIC values of valnemulin against *M.* sp. 1220 strains in vitro are promising for its clinical use, it should be noted that in a previous study only a single mutation in *M. gallisepticum* could cause elevation in the MIC values of valnemulin [17]. To date, the use of pleuromutilins in humans is limited, as only one commercially available product is authorized containing this active substance. However, bacterial strains resistant to pleuromutilins have already been described and these strains also show multidrug resistance, which warrants the prudent use of these antibiotic agents [25].

Phenicols showed good in vitro activity against *Mycoplasma* species of poultry, but information about their effectiveness in waterfowl is lacking [26, 27]. In the present study, most of the *M.* sp. 1220 isolates yielded the same MIC values (4 or 8 µg/ml) for florfenicol, and only two isolates (originating from the same region) showed elevated MIC values compared to the MIC_{50} (8 µg/ml), one of them reaching the highest antibiotic concentration (32 µg/ml) used.

The elevated MIC values of several antibiotics detected in subsequent isolates from the same farms from year to year are likely in association with the inconsistent use of antibiotics, the rapid development of antibiotic resistance and highlight the importance of susceptibility testing before therapy and responsible use of antimicrobial drugs.

Conclusion

In the present examinations the antibiotic susceptibility profiles of thirty-eight *M.* sp. 1220 strains isolated in Hungary were determined. To the best of our knowledge, this is the first detailed study about the antibiotic susceptibility of *M.* sp. 1220, a pathogen which could cause significant economic losses in waterfowl flocks. Valnemulin, tiamulin and tylvalosin were found to be the most effective antibiotics in the present study. Most of the isolates showed elevated MIC values for more than one agent, but none of the strains yielded high MIC values for all the examined antibiotics. Nevertheless, our results confirmed that increasing resistance could be observed in the cases of several antibiotics. These findings highlight the consistent use of antibiotics and the need for determination of antibiotic susceptibility of *Mycoplasma* species before treatment.

Availability of data and materials
All data supporting the findings is contained within the manuscript.

Authors' contributions
All authors read and approved the final manuscript. DG, ZK and KMS analysed the data and wrote the manuscript. DG and VH performed the examinations. ZR, IT and SJ collected the samples, isolated the strains and edited the manuscript. MG designed the study, analysed the data and edited the manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The ethics committees of the Institute for Veterinary Medical Research ruled that no formal ethics approval or consent were required as the samples were collected during routine diagnostic examinations or necropsies.

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References
1. Stipkovits L, Varga Z, Czifra G, Dobos-Kovacs M. Occurrence of mycoplasmas in geese affected with inflammation of the cloaca and phallus. Avian Pathol. 1986;15:289–99.
2. Stipkovits L, Kempf I. Mycoplasmoses in poultry. Rev Sci Tech. 1996; 15:1495–525.
3. Stipkovits L, Szathmary S. Mycoplasma infection of ducks and geese. Poult Sci. 2012;91:2812–9.
4. Stipkovits L, Varga Z, Glavits R, Ratz F, Molnar E. Pathological and immunological studies on goose embryos and one-day-old goslings experimentally infected with a Mycoplasma strain of goose origin. Avian Pathol. 1987;16:453–68.
5. Dobos-Kovacs M, Varga Z, Czifra G, Stipkovits L. Salpingitis in geese associated with Mycoplasma sp. strain 1220. Avian Pathol. 2009;38:239–43.
6. Hinz K-H, Pfützner H, Behr K-P. Isolation of mycoplasmas from clinically healthy adult breeding geese in germany. J Vet Med B. 1994;41:145–7.
7. Jordan FT, Gilbert S, Knight DL, Yavari C a. Effects of baytril, tylosin and tiamulin on avian mycoplasmas. Avian Pathol. 1989;18:659–73.
8. Stipkovits L, Glavits R, Ivanics E, Szabo E. Additional data on Mycoplasma disease of goslings. Avian Pathol. 1993;22:171–6.

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9. Hannan PCT. Guidelines and recommendations for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary mycoplasma species. Vet Res. 2000;31:373–95.

10. Stipkovits L, Varga Z, Dobos-Kovacs M, Santha M. Biochemical and serological examination of some Mycoplasma strains of goose origin. Acta Vet Hung. 1984;32:117–25.

11. Czifra G, Varga Z, Dobos-Kovacs M, Stipkovits L. Medication of inflammation of the phallus in geese. Acta Vet Hung. 1986;34:211–23.

12. Behbahan NGG, Asasi K, Afsharifar AR, Pourbakhsh SA. Susceptibilities of Mycoplasma gallispeticum and Mycoplasma synoviae Isolates to Antimicrobial Agents in vitro. Int J Poult Sci. 2008;7:1058–1064.

13. Landman WJ, Mevius DJ, Veldman KT, Feberwee A. In vitro antibiotic susceptibility of Dutch Mycoplasma synoviae field isolates originating from joint lesions and the respiratory tract of commercial poultry. Avian Pathol. 2008;37:415–20.

14. Wang C, Ewing M, Aarabi SY. In vitro susceptibility of avian mycoplasmas to enrofloxacin, sarafloxacin, tylosin, and oxytetracycline. Avian Dis. 2001;45:456–60.

15. Forrester CA, Bradbury JM, Dare CM, Domangue RJ, Windsor H, Tasker JB, Mockett APA. Mycoplasma gallisepticum in pheasants and the efficacy of tylvalosin to treat the disease. Avian Pathol. 2011;40:581–6.

16. Drews J, Georgopoulos A, Laber G, Shütze E, Unger J. Antimicrobial Activities of 81.723 hfu, a New Pleuromutilin Derivative. Antimicrob Agent Chemother. 1975;7:507–516.

17. Li B-B, Shen J-Z, Cao X-Y, Wang Y, Dai L, Huang S-Y, Wu C-M. Mutations in 23S rRNA gene associated with decreased susceptibility to tiamulin and valnemulin in Mycoplasma gallisepticum. FEMS Microbiol Lett. 2010;308:144–9.

18. Xiao X, Sun J, Chen Y, Zou M, Zhao D-H, Liu Y-H. Ex vivo pharmacokinetic and pharmacodynamic analysis of valnemulin against Mycoplasma gallisepticum S6 in Mycoplasma gallisepticum and Escherichia coli co-infected chickens. Vet J. 2015;204:54–9.

19. Lauerman LH, Chilina AR, Closser JA, Johansen D. Avian mycoplasma identification using polymerase chain reaction amplicon and restriction fragment length polymorphism analysis. Avian Dis. 1995;39:804–11.

20. Lysnyansky I, Gerchman I, Mikula I, Gobbo F, Catania S, Levisohn S. Molecular characterization of acquired enrofloxacin resistance in Mycoplasma synoviae field isolates. Antimicrob Agents Chemother. 2013;57:3072–7.

21. Gautier-Bouchardon AV, Reinhardt AK, Kobisch M, Kempf I. In vitro development of resistance to enrofloxacin, erythromycin, tylosin, tiamulin and oxytetracycline in Mycoplasma gallisepticum, Mycoplasma iowae and Mycoplasma synoviae. Vet Microbiol. 2002;88:47–58.

22. Gerchman I, Lysnyansky I, Perk S, Levisohn S. In vitro susceptibilities to fluoroquinolones in current and archived Mycoplasma gallisepticum and Mycoplasma synoviae isolates from meat-type turkeys. Vet Microbiol. 2008;131:266–76.

23. European Medicines Agency, EMA/186029/2010. [http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/quinolones_35/WC500094630.pdf]. Accessed 11 Nov 2015.

24. Wu CM, Wu H, Ning Y, Wang J, Du X, Shen J. Induction of macrolide resistance in Mycoplasma gallisepticum in vitro and its resistance-related mutations within domain V of 23S rRNA. FEMS Microbiol Lett. 2005;247:199–205.

25. van Duijkeren E, Greko C, Pringle M, Baptiste KE, Catry B, Jukes H, Moreno MA, Pomba MCMF, Pyorala S, Rantala M, Ru auskas M, Sanders P, Teale C, Threlfall EJ, Torren-Edo J, Torneke K. Pleuromutilins: use in food-producing animals in the European Union, development of resistance and impact on human and animal health. J Antimicrob Chemother. 2014;69:2022–31.

26. Gharaibeh S, Al-Rashdan M. Change in antimicrobial susceptibility of Mycoplasma gallisepticum field isolates. Vet Microbiol. 2011;150:379–83.

27. Lin MY. In vitro comparison of the activity of various antibiotics and drugs against New taiwan isolates and standard strains of avian mycoplasma. Avian Dis. 1987;31:705–12.

28. Hungary outline. [http://d-maps.com/carte.php?num_car=23243&lang=en]. Accessed 10 Aug 2015.