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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 MIC₅₀ 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 MIC₅₀ values with spectinomycin, tylosin and florfenicol (8 µg/ml), while enrofloxacin (MIC₅₀ 5 µg/ml), doxycycline (MIC₅₀ 5 µg/ml), lincomycin (MIC₅₀ 4 µg/ml) and lincomycin-spectinomycin (1:2) combination (MIC₅₀ 4 µg/ml) inhibited the growth of the bacteria with lower concentrations. Tylvalosin (MIC₅₀ 0.5 µg/ml) and two pleuromutilins (tiamulin MIC₅₀ 0.625 µg/ml; valnemulin MIC₅₀ ≤ 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, follicles 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 % CO₂ 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 % CO₂ 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 *Mycoplasma* 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 10⁴–10⁵ 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. MIC₅₀ and MIC₉₀ 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

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

| Sample ID | Sample source | Place | Animal | Date | MIC values ($\mu\text{g/ml}$) | | | | | |
|-----------|---------------|------------------|--------|------|---------------------------------|-------------|------------|----------------|-------------|--|
| | | | | | 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 | Tiszababolna | 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 | Tiszababolna | 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 |

| Sample ID | MIC values ($\mu\text{g/ml}$) | | | | | | | |
|-----------|---------------------------------|-------------|-------------|-------------|-------------|-----------------|--------------|-------------|
| | Tetracyclines | | Macrolides | | | Pleuromutilines | | Phenicol |
| | 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 $\mu\text{g/ml}$), while all strains had very high MIC values for norfloxacin (≥ 10 $\mu\text{g/ml}$) (Fig. 2a, b and c). The MIC₅₀ was 8 $\mu\text{g/ml}$ for spectinomycin and most of the strains yielded the MIC₅₀ or higher MIC values (Fig. 2d). The MICs for lincomycin clustered around the MIC₅₀

value (4 $\mu\text{g/ml}$) as well, but high MIC values (>64 $\mu\text{g/ml}$) were yielded in the case of three isolates (Fig. 2e). The MIC₅₀ and the MIC₉₀ values (4 $\mu\text{g/ml}$) for lincomycin-spectinomycin (1:2) combination was the same as the MIC₅₀ value for lincomycin. In the case of lincomycin-

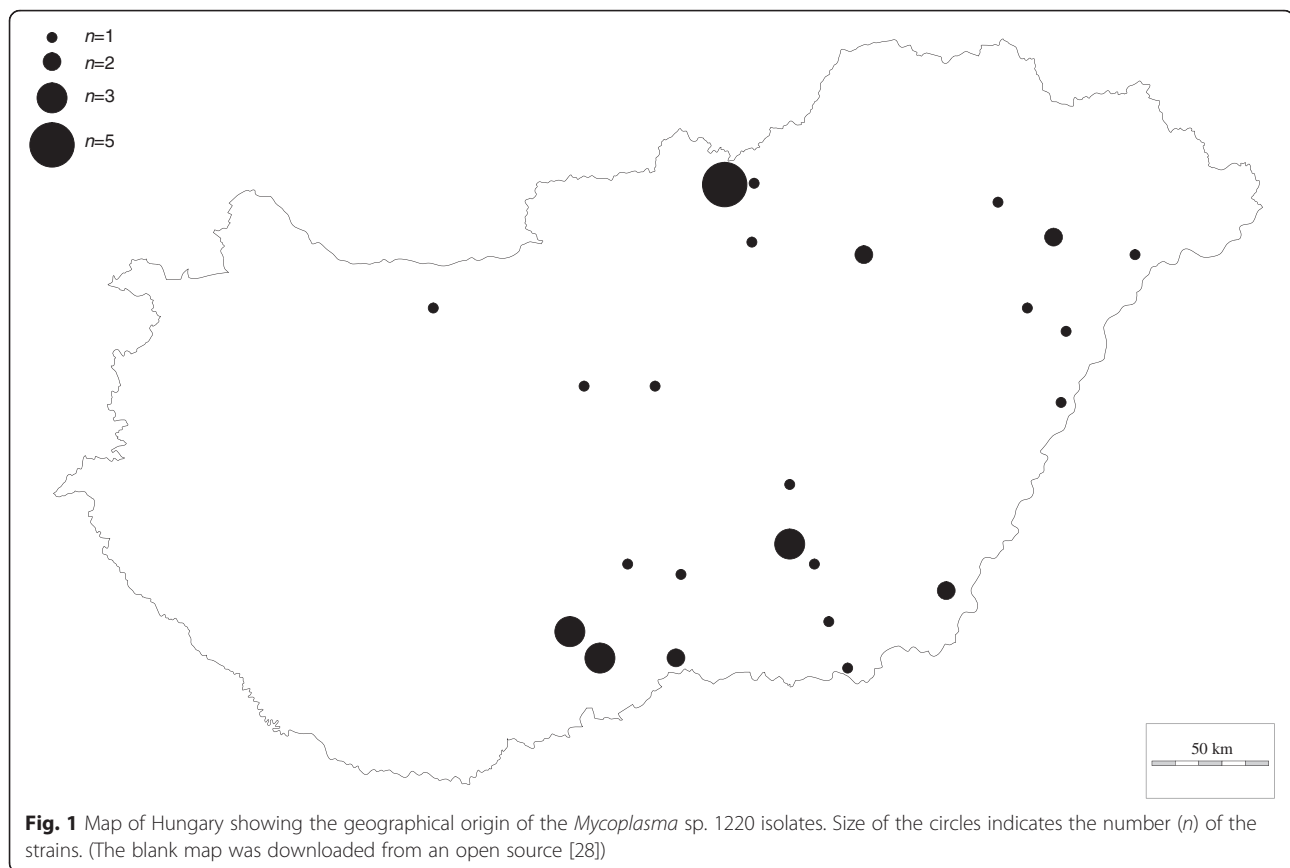


Table 2 Summary of MIC range, MIC₅₀ and MIC₉₀ values of the isolated *Mycoplasma* sp. 1220 strains

| 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 MIC₅₀ and MIC₉₀ 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 MIC₅₀ and MIC₉₀ 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 MIC₅₀ 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 MIC₅₀ and MIC₉₀ 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

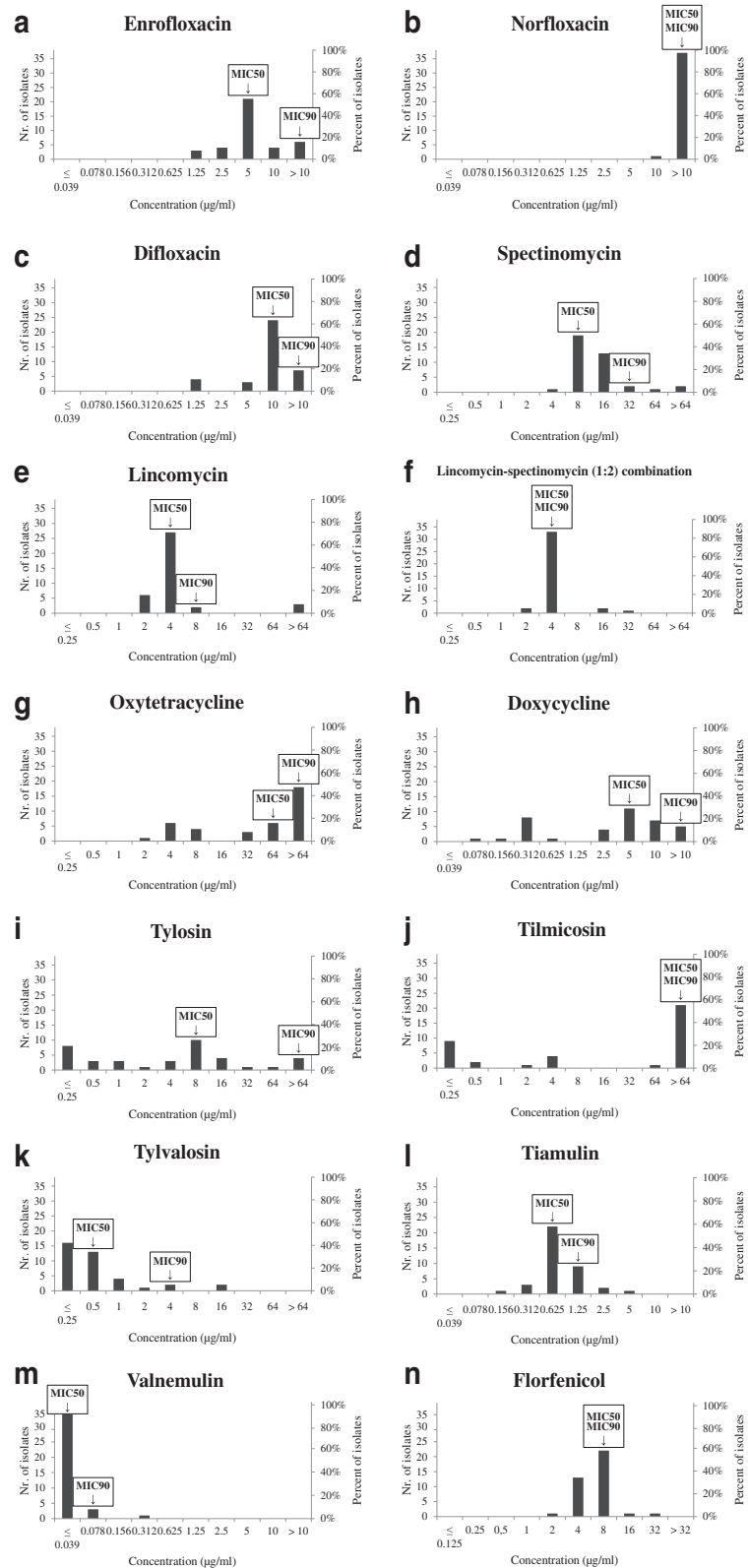


Fig. 2 MIC distribution of test antibiotics against *Mycoplasma* sp. 1220 isolates

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 Szathmáry [3]. Stipkovits and Szathmáry 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* (MIC₅₀ 2 µg/ml and MIC₉₀ 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 MIC₅₀ 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 MIC₅₀ 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 MIC₉₀ 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 MIC₅₀ 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 MIC₉₀ 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 MIC₅₀ 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 MIC₅₀ value (8 µg/ml) of tylosin was higher than the previous observation [3], and the MIC₉₀ 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 MIC₅₀ value

(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 Szathmáry described low MIC values (MIC₅₀: 0.06 µg/ml, MIC₉₀: 0.25 µg/ml) of tiamulin in the case of *M. sp.* 1220, and similarly low MIC₅₀ 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].

Phenicol 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 MIC₅₀ (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.

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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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