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## Mutations potentially associated with decreased susceptibility to fluoroquinolones, macrolides and lincomycin in *Mycoplasma synoviae*

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### ABSTRACT

*Mycoplasma synoviae* is one of the economically most significant avian *Mycoplasma* species. It can cause great financial losses to the poultry industry by inducing respiratory diseases, infectious synovitis, or eggshell apex abnormalities. There are different approaches to control *M. synoviae* infection. Although antimicrobial therapy cannot replace long-term solutions, like eradication and vaccination, this strategy can be effective in the short term, as adequate antibiotic treatment can relieve economic losses through the attenuation of clinical signs and reduction of transmission.

Using broth microdilution method, minimal inhibitory concentration (MIC) values to fourteen antibiotics related to eight antimicrobial groups were determined in 96 *M. synoviae* strains. Whole genome sequencing and sequence analysis revealed mutations potentially associated with decreased susceptibility to fluoroquinolones, macrolides and lincomycin.

Molecular markers responsible for the high MICs to fluoroquinolones were found in the *gyrA*, *gyrB*, *parC* and *parE* genes. Besides, single nucleotide polymorphisms identified in genes encoding the 23S rRNA were found to be responsible for high MICs to the 50S inhibitor macrolides and lincomycin, while amino acid change in the 50S ribosomal protein L22 could be associated with decreased susceptibility to macrolides.

The revealed mutations can contribute to the extension of knowledge about the genetic background of antibiotic resistance in *M. synoviae*. Moreover, the explored potentially resistance-related mutations may serve as targets for molecular biological assays providing data of antibiotic susceptibility prior to the laborious and time-consuming isolation of *M. synoviae* strains.

### 1. Introduction

*Mycoplasma synoviae* is a widespread facultative pathogen bacterium and one of the most economically significant *Mycoplasma* species in chicken and turkey industry. *M. synoviae* infection usually induce infectious synovitis or rarely respiratory signs and it can be related to eggshell apex abnormalities in chickens as well. Reduced feed intake,

weight gain, egg production and hatchability can be observed in the affected flocks leading to great economic losses (Landman, 2014).

The control programs for *M. synoviae* are primarily based on eradication of the pathogen. As vertical transmission is a significant route of infection, prevention is mainly performed by maintaining commercial breeder stocks free of infection. However, due to many difficulties, aims of elimination programs are complicated to fulfil in many poultry farms.

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In these cases, vaccination also provides an effective measure of long-term disease control (Landman, 2014). Although antimicrobial therapy is not a long-term solution, this strategy can be very effective in the short term, as adequate antibiotic treatment relieves *M. synoviae* infection induced economic losses through the attenuation of clinical signs and reduction of transmission (Hong et al., 2015).

Antibiotics affecting RNA-, DNA- or protein synthesis, or impairing cell membranes, such as fluoroquinolones, tetracyclines, aminoglycosides, macrolides (except for 14-membered lactone macrolides), lincosamides, fenicolos and pleuromutilins have been shown to possess *in vitro* activity against avian mycoplasmas (Gautier-Bouchardon, 2018). However, susceptibility profiles of certain *M. synoviae* strains can be very different (Landman et al., 2008). Moreover, resistance against various antimicrobial agents is reported more frequently in the recent years. Several studies found high minimal inhibitory concentration (MIC) values of macrolides and lincosamides against *M. synoviae* isolates (Lysnyansky et al., 2015; Kreizinger et al., 2017; Catania et al., 2019). Likewise, susceptibility of *M. synoviae* strains to fluoroquinolones decreased over the last few decades (Le Carrou et al., 2006; Gerchman et al., 2008; Lysnyansky et al., 2013; Kreizinger et al., 2017; Catania et al., 2019). These data are particularly troublesome as the use of fluoroquinolones is critical in the therapy of humans.

Determination of antibiotic susceptibility in mycoplasmas by broth or agar microdilution is very labour-intensive and time-consuming method, as it requires previous isolation and pure culture of the bacterium (Hannan, 2000). Interpretation of the results is difficult as well, because standard breakpoints of susceptible, intermediate and resistant categories to antimicrobial agents concerning avian *Mycoplasma* species have not been defined yet. In the lack of official breakpoints, the MIC data can be evaluated based on breakpoints of other avian pathogens determined by the Clinical and Laboratory Standards Institute (Wayne, 2013). The MIC values also can be compared to the results of previous publications taking into account that there are no internationally harmonised and accepted testing conditions for avian mycoplasmas. Moreover, the results of *in vitro* antibiotic susceptibility tests can only predict the expected *in vivo* efficacy of the antibiotics (Hildebrand, 1985).

There is an increasing need for rapid antimicrobial susceptibility tests in order to guide antibiotic therapy more effectively. Mechanisms of antibiotic resistance are mediated by genetic alterations, thus, results of broth or agar microdilution tests can be supported by investigating the antimicrobial susceptibility at the molecular level as well. A rapid and cost-effective method is the detection of resistance-associated mutations by molecular biological assays. These assays are most commonly based on real-time polymerase chain reaction (PCR) techniques, but conventional PCR also can be applied (Sulyok et al., 2018).

The aim of this study was to investigate the genetic background of decreased susceptibility to fourteen antibiotics of eight antimicrobial groups in *M. synoviae* and to identify potentially resistance-related mutations which can be targeted by rapid molecular biological assays.

## 2. Materials and methods

### 2.1. *M. synoviae* strains used in this study

In total, 96 *M. synoviae* strains, including the *M. synoviae* type strain NCTC 10124 (GenBank accession number: CP011096), the MS-H (Vaxsafe® MS, Bioproperties Pty Ltd., Ringwood, Australia; GenBank accession number: KP704286) and MS1 (Nobilis® MS Live, MSD Animal Health Hungary, Budapest, Hungary) vaccine strains and 93 field isolates were investigated in the present study. Samples were selected to provide a diverse *M. synoviae* strain collection for the analysis concerning the geographical location and date of isolation, however, antibiotic susceptibility profile was the primary consideration when samples were chosen for whole genome sequencing. The isolation year of the 93 field strains were between 1982 and 2019, but the majority of these

samples (n = 84) was collected during the past decade (2010-2019). The samples originated from chickens (n = 65) and turkeys (n = 28) and from 18 different countries (Hungary, n = 25; Italy, n = 22; the Netherlands, n = 9; Israel, n = 4; Spain, n = 4; Austria, n = 3; Czech Republic, n = 3; Russia, n = 3; Slovenia, n = 3; Ukraine, n = 3; USA, n = 3; Jordan, n = 2; Korea, n = 2; Lebanon, n = 2; Tunisia, n = 2; China, n = 1; Serbia, n = 1; Taiwan, n = 1).

The whole genome sequence of the *M. synoviae* strain MS53 (GenBank accession number: AE017245) was also used for this study, as reference genome. Background information of the used *M. synoviae* strains are provided in Table S1.

### 2.2. Sample processing

*M. synoviae* field strains were isolated and cultured according to the following protocol: Swab samples were taken from the choana or trachea of live birds. In case of post mortem sampling, tracheal or lung tissue was collected. Ethical approval and specific permission were not required for the study as all samples were collected during routine diagnostic examinations or necropsies with the consent of the owners. The collected samples were placed into liquid Frey's media (Frey et al., 1968) (Sigma-Aldrich Inc., St. Louis, USA) immediately and transported to the laboratory for incubation at 37 °C in an atmosphere of 5 % CO<sub>2</sub>. Following colour change (red to yellow shift) of the phenol red due to the metabolic activity of mycoplasmas, the culture was inoculated onto solid Frey's media (Frey et al., 1968) (Sigma-Aldrich Inc.) and incubated at 37 °C in an atmosphere of 5 % CO<sub>2</sub> until visible colonies appeared. Filter cloning was performed to gain pure cultures from the isolates. When it was possible, cultures were filter cloned only once to minimize *in vitro* mutations of the isolates.

DNA extraction from 200 µl pure *M. synoviae* logarithmic-phase broth culture was performed using the ReliaPrep™ gDNA Tissue Miniprep System (Promega Inc., Madison, USA) according to the manufacturers' instructions for Gram-negative bacteria. In order to confirm the *M. synoviae* positivity of the samples, DNAs were submitted to *M. synoviae*-specific PCR (Raviv and Kleven, 2009). The presence of other, contaminant mycoplasmas (i.e. *M. gallisepticum*) was excluded by a universal *Mycoplasma* PCR system targeting the 16S/23S rRNA intergenic spacer region of the Mollicutes (Lauerman et al., 1995). The PCR products were subjected to Sanger sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, USA) and sequences were submitted to BLAST search in order to identify the *Mycoplasma* species (<http://www.ncbi.nlm.nih.gov/BLAST>).

### 2.3. Broth microdilution method

Antibiotic susceptibility profiles of *M. synoviae* strains were determined by broth microdilution method according to the standard guidelines and recommendations of Hannan (2000). The number of microorganisms used for the MIC determination was standardized in order to obtain comparable results. Accepted numbers of microorganisms for the MIC tests were 10<sup>4</sup>-10<sup>5</sup> colour changing unit (CCU/ml). Determination of this number was performed with a 10-fold dilution series of *Mycoplasma* suspension in liquid Frey's media (Frey et al., 1968) (Sigma-Aldrich Inc.) containing phenol red. The highest dilution, which still resulted in a colour change was regarded to contain 10<sup>0</sup> bacterium.

The following antimicrobial agents were examined during the broth microdilution tests: two fluoroquinolones: enrofloxacin and difloxacin; one aminocyclitol: spectinomycin; one aminoglycoside: neomycin; three tetracyclines: doxycycline, oxytetracycline and chlortetracycline; three macrolides: tylosin, tilmicosin and tylvalosin; one lincosamide: lincomycin; two pleuromutilins: tiamulin and valnemulin; and one phenicol: florfenicol; all products originated from VETANAL (Sigma-Aldrich Chemie GmbH., Taufkirchen, Germany) except for tylvalosin (Aivlosin), which was purchased from ECO Animal Health Ltd. (London, UK). The

antibiotics were diluted and stored according to the recommendations of Hannan (2000). 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 and stored at  $-70^{\circ}\text{C}$ . Freshly prepared two-fold dilutions were used in each microtest after checking the thawed antibiotic solutions for any visible changes in their consistency. Although official MIC breakpoints for *M. synoviae* have not been determined yet, the examined concentration range of the antibiotics was selected to represent previously suggested high and low MIC values as well (Gautier-Bouchardon et al., 2002; Gerchman et al., 2008; Landman et al., 2008; Kreizinger et al., 2017; Gautier-Bouchardon, 2018) (Table 1 and Table S2).

The 96-well microtiter plates were designed to contain the twofold dilution series of the antibiotic, a growth control (Frey broth without antibiotic), a sterility control (Frey broth without antibiotic and *Mycoplasma inoculum*) and a pH control (Frey broth adjusted to pH 6.8). The duplicates of maximum three clinical isolates and the duplicate of the *M. synoviae* type strain NCTC 10124 were tested on each plate. The reference strain was included in the test to confirm the validity of the results. The microtiter plates were sealed with adhesive film and incubated at a temperature of  $37^{\circ}\text{C}$ . The MIC value against each isolate was defined as the lowest concentration of the antibiotic that completely inhibited the growth in the broth, i.e. no colour change has been observed. The MICs were read daily and recorded as soon as the growth controls changed colour.

The MIC values were interpreted based on the paper of Kempf et al. (1989), Gautier-Bouchardon et al. (2002), Behbahan et al. (2008), Gerchman et al. (2008), Landman et al. (2008), and van Duijkeren et al. (2014). MIC values belonged to susceptible strains according to these publications were considered as low MIC values in this study, while MIC values of resistant strains reported in these papers were considered as high (elevated) MIC values. Intermediate MICs were classified as elevated MIC values, except in the case of fluoroquinolones, in order to divide the examined population into comparable groups based on susceptibility for each antibiotic.

#### 2.4. Whole genome sequencing and sequence analysis

Genomic DNAs of the pure *M. synoviae* cultures were extracted from 10 ml of logarithmic-phase broth cultures using QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany). The DNA was quantified fluorometrically on Qubit 2.0 equipment using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific Inc., Waltham, USA).

Next-generation sequencing of 77 *M. synoviae* field isolates and the

vaccine strain MS1 was performed on Ion Torrent platform (New England BioLabs, Hitchin, UK). DNA was subjected to enzymatic fragmentation using the reagents supplied in the NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent Kit (New England BioLabs). The library DNA was clonally amplified by Ion PGM Template Kit on Ion OneTouch 2 system (Thermo Fisher Scientific Inc.). Whole genome sequencing of an additional 16 *M. synoviae* field isolates was performed on Illumina next-generation sequencing platform (Illumina Inc., San Diego, USA) with NextSeq 500/550 High Output Kit v2.5 (Illumina Inc.). DNA libraries were prepared with the Nextera Mate Pair Library Preparation Kit (Illumina Inc.).

The quality of the short reads were checked with FastQC software version 0.11.8 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) (Babraham Bioinformatics, The Babraham Institute, Babraham, UK). Reads were mapped to *M. synoviae* strain MS53 as reference genome and annotated by Geneious software version 10.2.3. (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012). Genomic regions which have been previously linked to antibiotic resistance in several *Mycoplasma* species were investigated: *gyrA* and *gyrB* genes encoding the two subunits (GyrA and GyrB) of the DNA gyrase (topoisomerase II) enzyme and *parC* and *parE* genes encoding the two subunits (ParC and ParE) of the topoisomerase IV enzymes for fluoroquinolones (Le Carrou et al., 2006; Lysnyansky et al., 2013; Sulyok et al., 2017); 16S rRNA coding genes (*rrsA* and *rrsB*) for aminoglycosides and tetracyclines (Amram et al., 2015; Sulyok et al., 2017); 23S rRNA coding genes (*rrlA* and *rrlB*) and 50S ribosomal proteins L3, L4 and L22 for macrolides, pleuromutilins, lincosamides and phenicols (Lysnyansky et al., 2015; Ammar et al., 2016; Sulyok et al., 2017).

These genes of *M. synoviae* strains were aligned to detect SNPs by Geneious software (Biomatters Ltd.) (Kearse et al., 2012). In case of protein coding genes (*gyrA*, *gyrB*, *parC*, *parE*, *rplC*, *rplD*, *rplV*) only non-synonymous mutations were included in the study, while all mutations found in the 16S and 23S rRNA coding genes (*rrsA*, *rrsB*, *rrlA*, *rrlB*) were investigated. Numbering of nucleotide and amino acid positions according to *Escherichia coli* strain K-12 substrain MG1655 (GenBank accession number: U00096) was determined to enable the comparison of our results with literature data and indicated where it was necessary. However, nucleotide and amino acid positions referred throughout the text were numbered based on the individual genes and proteins of *M. synoviae* strain MS53 in order to avoid misunderstandings due to gaps generated in the alignment of the corresponding genes of *M. synoviae* and *E. coli*.

For the identification of potentially resistance-related SNPs, the correlation between the MIC values and the occurrence of several mutations were analysed. To this end, the examined *M. synoviae* strains

**Table 1**

Details and results of the minimal inhibitory concentration (MIC) determination by broth microdilution method.

Antimicrobial agents	Tested concentration range ( $\mu\text{g/ml}$ )*	Strains tested	MIC range ( $\mu\text{g/ml}$ )	Strains with decreased susceptibility	Elevated MIC above ( $\mu\text{g/ml}$ )
enrofloxacin	0.031 - 64	n = 95	0.156 - >10	n = 63	1.25
difloxacin	0.039 - 10	n = 74	0.312 - >10	n = 52	1.25
oxytetracycline	0.25 - 64	n = 84	$\leq 0.25$ - 8	n = 4	4
chlortetracycline	0.25 - 64	n = 84	$\leq 0.25$ - >8	n = 12	4
doxycycline	0.039 - 16	n = 83	$\leq 0.039$ - 1.25	n = 0	4
spectinomycin	0.25 - 64	n = 73	$\leq 0.25$ - 8	n = 11	2
neomycin	0.25 - 64	n = 78	4 - >64	n = 78	2
tilmicosin	0.016 - 64	n = 87	$\leq 0.25$ - >64	n = 25	8
tylosin	0.008 - 64	n = 87	$\leq 0.25$ - 64	n = 11	1
tylvalosin	0.005 - 64	n = 82	$\leq 0.25$ - 4	n = 4	0.5
lincomycin	0.031 - 64	n = 84	$\leq 0.25$ - >64	n = 20	2
florfenicol	0.125 - 32	n = 92	<0.5 - 8	n = 56	2
tiamulin	0.008 - 32	n = 91	$\leq 0.039$ - 2.5	n = 0	8
valnemulin	0.039 - 10	n = 70	$\leq 0.039$	n = 0	0.125

Elevated MIC values for each antibiotics indicating decreased susceptibility of *M. synoviae* strains were determined based on previous studies (Kempf et al., 1989; Gautier-Bouchardon et al., 2002; Behbahan et al., 2008; Gerchman et al., 2008; Landman et al., 2008; van Duijkeren et al., 2014). \*Data indicate the minimum and maximum concentrations of the antibiotics used in this study, exact range of the tested concentrations can differ in each strain (Table S2).

were sorted by their MIC values for each antibiotics and mutations detected in the strains more frequently as MIC values increased or occurred exclusively in isolates with high MIC values were investigated individually. Neighbouring or closely located mutations have been evaluated together. Mutations, which have been detected in a large number of strains with high MIC values (at least 33.33 %) or identified at positions previously linked to antibiotic resistance in several *Mycoplasma* species were considered as potentially resistance-related mutations and presented in the study.

## 2.5. Molecular phylogenetic analysis

Molecular phylogenetic analysis of the examined 96 *M. synoviae* strains was performed in order to investigate their genetic diversity and present their phylogenetic relationships and the distribution of strains with elevated and low MIC values for the tested antibiotics in terms of their location on a phylogenetic tree. Concatenated sequences of seven loci were analysed with multi-locus sequence typing (El-Gazzar et al., 2017) by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) with standard error estimated through 1000 bootstrap replicates in MEGA-X software.

## 2.6. Differentiation of the *rrlA* and *rrlB* genes and determination of the nucleotide at position 2054

As whole genome sequencing was not able to distinguish between reads belonging to *rrlA* and *rrlB* genes, relevant positions (nucleotide position 2054) with different nucleotides in the two *rrl* genes (MYCS-51, MYCS-60, MYCS-63, MYCS-73, MYCS-76, MYCS-77) had to be investigated with additional PCR systems. To this end, a PCR was developed for the specific amplification of the partial sequences of 23S rRNA genes *rrlA* and *rrlB*. A common reverse primer was designed targeting the internal sequence of the 23S rRNA genes. Forward primers were developed to bind to the conserved regions of predicted genes encoding hypothetical proteins located close to the *rrlA* and *rrlB* genes. The primer design and the examination of the primers' general suitability were performed by using the NetPrimer software (Premier Biosoft International, Palo Alto, USA) (<http://www.premierbiosoft.com/netprimer>). The specificity of the primers was analysed *in silico* using BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>). The PCR was carried out in 25 µl total volume, containing 1 µl target DNA, 5 µl of 5X Colour-less GoTaq Flexi Buffer (Promega Inc.), 2.5 µl MgCl<sub>2</sub> (25 mM; Promega Inc.), 0.5 µl dNTP (10 mM; Fermentas, Waltham, USA), 1 µl of each primer (10 pmol/µl), 0.25 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega Inc.) and nuclease free water. The PCRs consisted of initial denaturation for 5 minutes at 95 °C followed by 35 amplification cycles of denaturation for 30 seconds at 95 °C, primer annealing at 54 °C for 30 seconds, and extension at 72 °C for 1 minute. The final extension step was performed for 5 minutes at 72 °C. The conventional PCRs were performed using Bio-Rad C1000 Touch (Bio-Rad Laboratories Inc., Hercules, USA) thermal cyclers throughout the study.

The amplified PCR products were submitted to a MAMA test (Birdsell et al., 2012). Primer design was performed by Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse et al., 2012). The primer set consisted of a consensus reverse primer and two competing forward primers designed to specifically target the questionable nucleotide (adenine or guanine) at position 2054. At the allele-specific 3' end of the competing primers, a single antepenultimate destabilizing mismatch was inserted to enhance the discriminative capacity of the assay. The primer specific for guanine at the 3' end was marked with an additional 14 base pair (bp) long GC-clamp at the 5' end to increase the size and melting temperature of the amplicon. The primers were constructed to limit amplicon lengths of ≤100 bp. The general suitability of the designed primer set was calculated by using NetPrimer software (Premier Biosoft International) (<http://www.premierbiosoft.com/netprimer>). The

specificity of the primers was analysed *in silico* using BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>). The melt-MAMA PCR mixture consisted of 2 µl 5X Colour-less GoTaq Flexi Buffer (Promega Inc.), 1 µl MgCl<sub>2</sub> (25 mM; Promega Inc.), 0.3 µl dNTP (10 mM; Fermentas), 0.5 µl EvaGreen (20X, Biotium Inc., Hayward, USA), 0.15 µl of each primer (10 pmol/µl), 0.08 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; Promega Inc.), nuclease-free water and 1 µl DNA template with a final volume of 10 µl. Thermocycling parameters were 95 °C for 10 minutes, followed by 30 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. PCR products were subjected to melt analysis using a dissociation protocol comprising 95 °C for 15 seconds, followed by 0.3 °C incremental temperature ramping from 60 °C to 95 °C. The real-time PCRs were performed using Applied Biosystems Step-One Plus real-time PCR system with StepOne Software version 2.3 (Thermo Fisher Scientific Inc.). EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature. *M. synoviae* strains with analogous gene duplicates concerning nucleotide position 2054 (adenine in both genes or guanine in both genes) were used as positive controls, while nuclease free water was utilised as negative control.

Sequences of primers used for the conventional PCRs and the subsequent melt-MAMA tests are indicated in Table 2.

## 2.7. Investigating the presence of different *tet* genes

In order to investigate *in silico* the possible presence of *tet* genes in *M. synoviae* strains with high MIC values to tetracyclines, reads of these strains were submitted to analysis by SPAdes software version 3.11.1. (Center for Algorithmic Biotechnology, Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg, Russia) (Bankevich et al., 2012). Sequences of primers designed for the detection of the tetracycline resistance genes *tet(L)* (Amram et al., 2015), *tet(M)* (Blanchard et al., 1992; Dégrange et al., 2008; Filioussis et al., 2014; Amram et al., 2015), *tet(O)* (Filioussis et al., 2014; Amram et al., 2015), *tet(S)* (Filioussis et al., 2014), and the *tet(R)* regulator gene (Breton et al., 2010) in different *Mycoplasma* species and sequences of publicly available *tet(M)* genes of *Mycoplasma* and *Ureaplasma* species (*Mycoplasma gallisepticum* strain GDMT09 *tet(M)* gene, 398 bp, GenBank accession number: GQ424446; *Mycoplasma hominis* strain Sprott complete genome *tet(M)* gene, 1,920 bp, GenBank accession number: CP011538; *Ureaplasma parvum* strain Ply157 *tet(M)* gene, 1,563 bp, GenBank accession number: KT267561; *Ureaplasma urealyticum tet(M)* gene, 4,793 bp, GenBank accession number: U08812) were mapped to the draft genomes of these strains using Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse et al., 2012).

Beside these *in silico* examinations, *tet(M)* positivity of the *M. synoviae* strains with high MIC values to tetracyclines were investigated with a conventional PCR according to Shahid et al. (Shahid et al., 2014).

## 3. Results

The MIC values of the tested antibiotics against each examined *M. synoviae* strain including previously published MIC data (Kreizinger et al., 2017) are presented in Table S2. The MIC ranges obtained for each tested antimicrobial agent are shown in Table 1. The numbers of the tested strains and the isolates with elevated MIC values for each tested antibiotics are also included in Table 1. As none of the examined *M. synoviae* strains were found to show high MIC values against doxycycline, tiamulin and valnemulin, relatedness of the MIC values for these antibiotic agents with the detected mutations were not evaluated in the study. In case of neomycin, further investigations were omitted due to the lack of sensitive strains.

Whole genome sequencing of *M. synoviae* strains resulted on average 144,599 and 2,535,433 reads with 157.36 and 152.08 bp length, and the mean sequencing depth of the whole genomes generated on IonTorrent and Illumina platform was 28.41X and 408.42X, respectively (Table S3).



**Table 2**Data of amplicons and primers used for the differentiation of the two *rrl* genes and the determination of the nucleotide at position 2054.

PCR systems	Position in the genome (bp) <sup>a</sup>	Primers	Primer sequences (5'-3')	Tm (°C)	Product size
conventional PCR	661693-661720	rrlA-F	TTGGTCTTGATCTAATTTAACTTCTTT	82.2 ± 0.3	1138 bp
	774826-774851	rrlB-F	GCCAAAGAATTTAGTTTCATTATTG		1118 bp
	662808-662830; 775921-775943	rrl-R	CGCAATGATCTCTCAACTGTCTC		
melt-MAMA	662749-662768; 775862-775881	rrl-2054-G	ggggcggggcggggGGTACCCGCATCAAGACCAAG	76.9 ± 0.6	88 bp
		rrl-2054-A	GGTACCCGCATCAAGACAAA		74 bp
	775805-775828; 662692-662715	rrl-2054-con	CACATGTTAGGCCAAATTTCAATA		

Two primer sets (the forward primer rrlA-F or rrlB-F with the common reverse primer rrl-R) were used for the conventional polymerase chain reaction (PCR), while one primer set (competing rrl-2054-G and rrl-2054-A primers with the consensus rrl-2054-con primer) was used for the melt analysis of mismatch amplification mutation assay (melt-MAMA); <sup>a</sup>according to *M. synoviae* strain MS53 (GenBank accession number: AE017245); bp: base pair; Tm: melting temperature of the amplicons.

The average Phred score of the short reads were found to be over 23, or 99.5 % base call accuracy. The raw nucleotide sequence reads of the *M. synoviae* strains were submitted to the Sequence Read Archive (SRA) database of National Center for Biotechnology Information (NCBI) (BioProject accession numbers: PRJNA634246; PRJNA634252).

Molecular phylogenetic analysis revealed great genetic diversity of the 96 examined *M. synoviae* strains as they were classified into 42 sequence types (STs) by multi-locus sequence typing (El-Gazzar et al., 2017). Phylogenetic relationships of the strains are presented by a neighbor-joining tree, demonstrating that the antibiotic susceptibility profile of the strains can differ within the same ST or even in case of identical origin, verifying the inclusion of all these isolates in the study (Figure S1).

### 3.1. Fluoroquinolones

Aligning the corresponding genes in the reference genome of the *M. synoviae* strain MS53 and the sequences of the tested 95 *M. synoviae* strains revealed several non-synonymous mutations in the examined genes. Consecutive amino acid substitutions were found in numerous different positions of the GyrA (n = 42), GyrB (n = 18), ParC (n = 56) and ParE (n = 35) proteins. Besides, a SNP resulting amino acid deletion in the GyrA protein was also detected (data not shown). Investigating these alterations in context with the MIC values of the *M. synoviae* strains, several mutations were found to be potentially resistance-related (Table 3). These amino acid substitutions are indicated in Table S4 in parallel with the MIC values for the two tested fluoroquinolones of each examined *M. synoviae* strain.

**Table 3**Potentially resistance-related mutations identified in *M. synoviae* strains.

Antibiotics	Genes	SNP <sup>a</sup>	AA subst. <sup>a</sup>	Strains with lower MIC values possessing the mutation <sup>b</sup>	Strains with higher MIC values possessing the mutation <sup>c</sup>	
FLUOROQUINOLONES	<i>gyrA</i>	G28A	Glu10Lys	-	n = 33	46.48 %
		<u>A428G</u>	Asn143Ser	-	n = 2	2.82 %
		A566G	Glu189Gly	-	n = 35	49.3 %
		T1360A	Ser454Thr	-	n = 36	50.7 %
		C1361A	Ser454Tyr	-	n = 48	67.61 %
		G1651A	Asp551Asn	n = 1	5 %	n = 31
	<i>gyrB</i>	C446T	Ala149Val	-	n = 36	50.7 %
		<u>C1247A</u>	Ser416Tyr	-	n = 36	50.7 %
		<u>G1250A</u>	Ser417Asn	-	n = 61	85.92 %
	<i>parC</i>	<u>A253G</u>	Thr85Ala	-	n = 46	64.79 %
		<u>C254T</u>	Thr85Ile	-	n = 45	63.38 %
		<u>T256C</u>	Ser86Pro	-	n = 46	64.79 %
		<u>G265C</u>	Asp89His	-	n = 27	38.03 %
		<u>G265T</u>	Asp89Tyr	-	n = 27	38.03 %
		G1354A	Glu452Lys	-	n = 46	64.79 %
<i>parE</i>	G1798A	Val600Ile	-	n = 45	63.38 %	
	C2442A	Asn814Lys	-	n = 46	64.79 %	
	C260T	Ser87Phe	n = 1	5 %	n = 27	38.03 %
	<b>Total number and percentage of strains with high MIC values for fluoroquinolones containing at least one of the listed mutations</b>				<b>n=63</b>	<b>88.73 %</b>
MACROLIDES	<i>rrlA</i> and/or <i>rrlB</i>	<u>A2054G</u>	n.a.	n = 2	3.45 %	
		<u>A2055G</u>	n.a.	n = 19	76 %	
	<i>rplV</i>	<u>A276C/T</u>	Gln90His	-	n = 6	24 %
	<b>Total number and percentage of strains with high MIC values for macrolides containing at least one of the listed mutations</b>				<b>n = 25</b>	<b>100 %</b>
LINCOMYCIN	<i>rrlA</i> and/or <i>rrlB</i>	<u>A2054G</u>	n.a.	n = 1	1.56 %	
		<u>A2055G</u>	n.a.	n = 20	100 %	
<b>Total number and percentage of strains with high MIC values for lincomycin containing at least one of the listed mutations</b>				<b>n = 20</b>	<b>100 %</b>	

Mutations underlined were previously associated with fluoroquinolone or macrolide resistance in *M. synoviae* (Le Carrou et al., 2006; Lysnyansky et al., 2013; Lysnyansky et al., 2015).

<sup>a</sup> numbering according to *M. synoviae* strain MS53 (GenBank accession number: AE017245).

<sup>b</sup> in case of fluoroquinolones: strains with MIC values of ≤1.25 µg/ml for enrofloxacin and difloxacin (n = 20; isolates with missing MIC data (n = 4) are excluded from the evaluation); in case of macrolides: strains with MIC values of ≤8 µg/ml for tilmicosin and ≤1 µg/ml for tylosin and ≤0.5 µg/ml for tylvalosin (n = 58; isolates with missing MIC data (n = 4) are excluded from the evaluation); in case of lincomycin: strains with MIC values of ≤2 µg/ml (n = 64).

<sup>c</sup> in case of fluoroquinolones: strains with MIC values of >1.25 µg/ml for enrofloxacin and/or difloxacin (n = 71); in case of macrolides: strains with MIC values of >8 µg/ml for tilmicosin and/or >1 µg/ml for tylosin and/or >0.5 µg/ml for tylvalosin (n = 25); in case of lincomycin: strains with MIC values of >2 µg/ml (n = 20); SNP: single nucleotide polymorphism; AA subst.: amino acid substitution; n.a.: not applicable.

In the *gyrA* gene, the most frequently occurring SNP has been found at position 1651 (G1651A) and resulted in an Asp551Asn amino acid change in 48/71 *M. synoviae* strains with higher MIC values ( $>1.25$  µg/ml) for enrofloxacin and/or difloxacin. A mutation at nucleotide position 1360 (T1360A) of the *gyrA* gene resulted in a Ser454Thr amino acid change in 35 *M. synoviae* strains with higher MIC values, while another SNP in the neighbouring nucleotide position (C1361A) resulted in an amino acid substitution at the same position (Ser454Tyr) in one additional *M. synoviae* strain. These two SNPs together affected 36/71 *M. synoviae* strains with decreased susceptibility to fluoroquinolones. A non-synonymous mutation has been detected at nucleotide position 28 of the *gyrA* gene (G28A) resulted in a Glu10Lys substitution in 33/71 *M. synoviae* strains with higher MIC values for enrofloxacin and/or difloxacin. We identified a mutation at nucleotide position 566 (A566G) as well, which resulted in a Glu189Gly amino acid change in the same 33 *M. synoviae* strains and two additional *M. synoviae* isolates with higher MIC values for fluoroquinolones. A SNP in 2/71 *M. synoviae* strains with higher MIC values for enrofloxacin and/or difloxacin has been found as well at position 428 (A428G) of the *gyrA* gene resulting in an Asn143Ser amino acid substitution.

In the *gyrB* gene, non-synonymous mutations at nucleotide positions 446 (C446T) and 1247 (C1247A) resulted in Ala149Val and Ser416Tyr amino acid substitutions, respectively, in the same 31/71 *M. synoviae* strains with higher MIC values ( $>1.25$  µg/ml) for enrofloxacin and/or difloxacin. In five *M. synoviae* strains with higher MIC values for fluoroquinolones, a mutation at position 1250 (G1250A) of the *gyrB* gene resulted an amino acid substitution (Ser417Asn) at the neighbouring position of the Ser416Tyr. These two mutations together affected 36/71 *M. synoviae* strains with decreased susceptibility to fluoroquinolones.

In the *parC* gene, a mutation at nucleotide position 254 (C254T) resulted in a Thr85Ile amino acid change in 56 *M. synoviae* strains with higher MIC values ( $>1.25$  µg/ml) for enrofloxacin and/or difloxacin, while another SNP in the neighbouring nucleotide position (A253G) resulted in an amino acid substitution at the same position (Thr85Ala) in two additional *M. synoviae* strains. Besides, a mutation at nucleotide position 256 (T256C) resulted in a Ser86Pro amino acid change ( $n = 1$ ), while at nucleotide position 265, polymorphisms G265C and G265T resulted in amino acid substitutions Asp89His ( $n = 1$ ) and Asp89Tyr ( $n = 1$ ), respectively. Mutations at nucleotide positions coding the region 85-89 amino acids of the ParC together affected 61/71 *M. synoviae* strains with higher MIC values for fluoroquinolones. A mutation at nucleotide position 1798 (G1798A) of the *parC* gene resulting in a Val600Ile amino acid change was also identified. This mutation was found in 45/71 strains with higher MIC values for enrofloxacin and/or difloxacin. Non-synonymous mutation at nucleotide position 1354 (G1354A) resulted in an amino acid change Glu452Lys in the same 45 *M. synoviae* strains and one additional isolate (MYCS-78) with higher MIC value. Similarly, the mutation at position 2442 (C2442A) resulting in an Asn814Lys amino acid change was detected in the same 45 *M. synoviae* strains and one additional isolate (MYCS-92) with higher MIC value.

In the *parE* gene, a non-synonymous mutation was identified at nucleotide position 260 (C260T) resulting in a Ser87Phe amino acid change in 27/71 *M. synoviae* strains with higher MIC values ( $>1.25$  µg/ml) for enrofloxacin and/or difloxacin.

At least one of the described mutations were carried by 88.73 % of the *M. synoviae* strains with higher MIC values for fluoroquinolones ( $>1.25$  µg/ml for enrofloxacin and/or difloxacin). Interestingly, potentially resistance-related mutations could be detected in six *M. synoviae* strains with lower MIC values ( $\leq 1.25$  µg/ml) for enrofloxacin and/or difloxacin, however, four of these strains were not examined with both antibiotics. Besides, eight strains were found to have higher MIC values ( $>1.25$  µg/ml) for enrofloxacin and/or difloxacin without any potentially resistance-associated mutations found in the examined genes, however, six of these strains had lower MIC values ( $\leq 1.25$  µg/ml) or missing MIC data for one of the tested antibiotics.

### 3.2. 30S inhibitors

Aligning the corresponding genes of the *M. synoviae* strain MS53 reference genome and the sequences of the tested *M. synoviae* strains ( $n = 92$  for tetracyclines;  $n = 73$  for spectinomycin), mutations in 38 different positions of the *rrsA* and/or *rrsB* genes have been detected in this study (data not shown). None of these mutations could be related to the decreased susceptibility to tetracyclines (MIC values of  $>4$  µg/ml for oxytetracycline and/or chlortetracycline,  $n = 12$ ) or spectinomycin (MIC values of  $>2$  µg/ml;  $n = 11$ ). Investigating *in silico* the 12 *M. synoviae* strains with higher MIC values ( $>4$  µg/ml) for tetracyclines, none of the tested *tet* primers or partial gene sequences (*tet(L)*, *tet(M)*, *tet(O)*, *tet(S)*, *tet(R)*) could be mapped suitably to the scaffolds of the strains. Likewise, no amplification of specific gene sequences could be detected by PCR (Shahid et al., 2014) when the DNAs of the 12 *M. synoviae* strains with higher MIC values for tetracyclines were tested (data not shown).

### 3.3. 50S inhibitors

Aligning the corresponding genes of the *M. synoviae* strain MS53 reference genome and the sequences of the tested *M. synoviae* strains ( $n = 87$  for macrolides;  $n = 84$  for lincomycin;  $n = 92$  for florfenicol), mutations in 79 different positions of the *rrlA* and/or *rrlB* were identified in this study. Besides, non-synonymous mutations of the *rplC*, *rplD* and *rplV* genes resulted amino acid substitutions in 9, 17 and 6 different positions of the 50S ribosomal protein L3, L4 and L22, respectively (data not shown). None of these mutations could be related to the decreased susceptibility to florfenicol (MIC values of  $>2$  µg/ml;  $n = 56$ ) and no resistance-related mutation could be identified in the *rplC* and *rplD* genes. However, potentially resistance-associated mutations have been identified in the *rrl* genes in case of macrolides and lincomycin, and *rplV* gene in case of macrolides (Table 3). These SNPs are indicated in Table S5 in parallel with the MIC values for the three tested macrolides and lincomycin of each examined *M. synoviae* strain.

In 20 *M. synoviae* strains, a mutation has been found at position 2054 (A2054G) in the *rrlA/B* genes. Out of these, 14 strains were found to possess this SNP simultaneously in both *rrl* genes, while six strains were found to be heterozygous concerning this position. The PCR designed for the differentiation of the two *rrl* genes followed by the developed melt-MAMA test revealed that MYCS-60 carried this mutation in the *rrlB* gene, while the rest of these strains possessed this SNP in the *rrlA* gene (MYCS-51, MYCS-63, MYCS-73, MYCS-76, MYCS-77). Besides, a SNP at the adjacent position (A2055G) of both *rrl* genes has been observed as well in one *M. synoviae* strain. These two SNPs together affected 19/25 *M. synoviae* strains with high MIC values for macrolides ( $>8$  µg/ml for tilmicosin and/or  $>1$  µg/ml for tylosin and/or  $>0.5$  µg/ml for tylvalosin) and all of the strains (20/20) with MIC values of  $>2$  µg/ml to lincomycin. In the *rplV* gene, a mutation at nucleotide position 276 (A276C/T) resulting in a Gln92His amino acid change of the L22 protein was also identified in 6/25 *M. synoviae* strains with high MIC values for macrolides. All *M. synoviae* strains with higher MIC values for these 50S inhibitors ( $>8$  µg/ml for tilmicosin and/or  $>1$  µg/ml for tylosin and/or  $>0.5$  µg/ml for tylvalosin and/or  $>2$  µg/ml for lincomycin) have been found to carry exactly one of the described mutations (considering the same position at *rrlA* and *rrlB* as one), while no occurrence could be observed in *M. synoviae* strains with low MIC values for all of these antibiotics, except one case: A2054G could be detected in the *rrlA* gene of the MYCS-76 isolate. In this strain, unique amino acid changes were seen in the 50S ribosomal protein L3 and L22. The mutations in the *rplC* (G752A) and *rplV* (C229T) genes resulted in Arg251Lys and His77Tyr amino acid changes of the L3 and L22 protein, respectively. Besides, a mutation in the *rplV* gene (C124T) resulting in a Pro42Ser amino acid change of the L22 protein which seems to be specific for sensitive strains ( $\leq 0.25$  µg/ml for macrolides;  $\leq 2$  µg/ml for lincomycin;  $n = 8$ ) could be observed in the isolate MYCS-76 as well (data not shown).

#### 4. Discussion

Prudent use of antibiotics in the management of *M. synoviae* infection is improved by the determination of antibiotic susceptibility prior to the treatment, however, the most commonly performed broth and agar microdilution tests are very labour-intensive and time-consuming methods (Hannan, 2000). There is an increasing need for exploring resistance-related mutations in the bacterial genomes which can be targeted by rapid molecular biological tests in order to guide antimicrobial therapy more effectively. The aim of this study was to identify mutations potentially associated with decreased antibiotic susceptibility in *M. synoviae* strains.

Interpretation of the results were challenging, as SNPs may have cumulative or opposite effect, and their impact can be modified by unexplored mutations or other unknown factors as well. Moreover, small differences between the elevated MIC values according to this study and the MIC values related to certain strains further complicated the evaluation. In some cases, discrepancies between the MIC values and the presence of the identified mutations may be due to this phenomenon, as there is only one dilution step difference between the concentrations regarded as low or high MIC values. Considering these, all data have been examined individually and in context as well, and evaluated cautiously when SNPs were identified as potentially resistance-associated mutations.

Numerous potentially resistance-related mutations could be detected in the *gyrA*, *gyrB*, *parC* and *parE* genes of *M. synoviae* strains with higher MIC values for fluoroquinolones, however, not all of them were located in the quinolone resistance determining region (QRDR) of these genes. Previous studies suggest that the primary target of fluoroquinolones in *M. synoviae* is the ParC subunit of the topoisomerase IV enzyme (Le Carrou et al., 2006; Lysnyansky et al., 2013). In this study, a hot spot region could be identified in the QRDR of the *parC* gene located at nucleotide positions 253-265 resulted in alterations at the amino acid positions 85-89 (or 80-84 amino acids according to *E. coli* numbering) of the DNA topoisomerase IV A subunit (ParC). SNPs at the same or adjacent positions have already been mentioned in several studies as resistance-associated mutations. Le Carrou et al. (2006) reported a 2-4-fold increase of the enrofloxacin MIC value in *M. synoviae* strains which had a Ser to Pro substitution at position 81 in ParC. Lysnyansky et al. (2013) described full correlation between decreased susceptibility of *M. synoviae* to enrofloxacin and the amino acid substitutions at positions 79-81 and 84 in ParC. This region seems to have a principal role according to our study as well, as mutations located here were possessed by most *M. synoviae* strains (85.92 %) with MIC values of  $>1.25$  µg/ml and all of the isolates with MIC values of  $\geq 5$  µg/ml for enrofloxacin and/or difloxacin. The most frequently occurring mutation of this hot spot region was C254T resulting in a Thr85Ile amino acid substitution in the ParC. This mutation could be related to decreased susceptibility to both fluoroquinolones, while the SNP A253G affecting the same amino acid position (Thr85Ala) could be detected for the first time in *M. synoviae* strains resistant against difloxacin but not enrofloxacin. However, additional isolates carrying this mutation should be investigated in order to confirm association between this genotype and the increased difloxacin MIC values. Outside of the hot spot region in the *parC* gene, three potentially resistance-related SNPs were detected for the first time. However, fluoroquinolone resistant strains carrying these mutations were all affected by a mutation of the hot spot region as well. Therefore, impact of new detected mutations is difficult to assess.

Resistance-related alterations of the GyrA found in our study have not been mentioned in the literature before, except for the mutation in the QRDR of the *gyrA* gene displaying an Asn to Ser amino acid change at position 143 (or 87 according to *E. coli* numbering) of the GyrA (Le Carrou et al., 2006; Lysnyansky et al., 2013). The mutations in the QRDR of the *gyrB* gene resulting in amino acid changes at positions 416 (Ser to Tyr) and 417 (Ser to Asn) in GyrB (or 401 and 402 according to *E. coli* numbering) have been also reported before in the paper of Lysnyansky

et al. (2013), however their role in decreased fluoroquinolone susceptibility was not clarified in that study. On the other hand, no literature data have been found concerning the mutations detected at positions 149 (Ala to Val) in GyrB and 87 (Ser to Phe) in ParE proteins.

The SNPs identified for the first time as potentially resistance-associated mutations in the *gyrA*, *gyrB* and *parE* genes mainly occurred simultaneously with the mutations of the hot spot region in the fluoroquinolone resistant strains. However, in two strains with increased MIC values, mutations were only detected outside of the hot spot region: MYCS-60 carried all novel SNPs in the *gyrA* and *gyrB* genes, while MYCS-7 had the SNP in the *parE* gene only. These data support the presumption, that beside the importance of the *parC* hot spot region, these mutations may also play a role in the development of fluoroquinolone resistance.

Mutations in the central loop of the domain V (peptidyl transferase region) of 23S rRNA confer resistance to 50S inhibitors in many bacteria. In *M. gallisepticum*, mutations in positions 2057-2059 (according to *E. coli* numbering) can lead to a disruption of the rRNA structure, thus alterations in this area can prevent the attachment of the antimicrobial agents to their binding site (Ammar et al., 2016). Mutations A2058G and A2059G have been previously associated with decreased susceptibility for macrolides and lincomycin in *M. synoviae* as well (Lysnyansky et al., 2015).

In our study, all *M. synoviae* strains with higher MIC values for lincomycin possessed the mutation A2054G (or A2058G according to *E. coli* numbering) in the domain V of the *rrlA* and/or *rrlB* genes, except one strain (MYCS-72), which carried the A2055G (or A2059G according to *E. coli* numbering) SNP in the adjacent position of the *rrlA* and *rrlB* gene as well. The same mutations were found to affect the susceptibility of macrolides.

It seems that in *M. synoviae*, resistance to lincomycin and tilmicosin does not require the A2054G mutation in both *rrl* genes, as the presence of this mutation in the *rrlA* gene only was enough to increase the MIC values above 64 µg/ml, while a single isolate carrying the mutation in the *rrlB* gene only (MYCS-60) showed higher but not extremely high MIC values for lincomycin (4 µg/ml) and tilmicosin (16 µg/ml). *M. synoviae* strains which showed high MIC values for all tested macrolide antibiotics and lincomycin as well carried this mutation in both *rrl* genes. Nevertheless, based on these data, differences between the two heterozygous or between hetero- and homozygous resistant genotypes concerning macrolide and lincomycin susceptibility are difficult to assess.

Beside the SNPs A2054G and A2055G of the *rrlA/B* genes, non-synonymous mutations revealed in the *rplV* gene were found to decrease the susceptibility for macrolides, especially in case of tilmicosin. The SNP A276C, as well as A276T resulted in a glutamine-histidine amino acid change at the position 92 (or 90 according to *E. coli* numbering) of the 50S ribosomal protein L22. This amino acid change in the L22 protein has been already described previously in *M. synoviae* strains (Lysnyansky et al., 2015), although its role in macrolide resistance was not suggested in that study. According to our results, a mutation can occur in the closely located position (C274A) resulting in a glutamine-lysine change in the same amino acid position as well, however, it does not seem to affect the susceptibility for macrolides (data not shown). All *M. synoviae* strains with higher MIC values for lincomycin and/or macrolides have been found to possess one of the above mentioned mutations (considering the same position at *rrlA* and *rrlB* as one) indicating their significance. However, other SNPs might play a role as well, for example mutations detected in the *rplC* (G752A) and *rplV* (C124T, C229T) genes of a sensitive strain (MYCS-76) may also have an impact on the susceptibility of this isolate modulating the effect of the mutation A2054G in the *rrlA*.

No potentially resistance-related mutations could be identified in case of tetracyclines, spectinomycin and florfenicol based on the data of this study. Moreover, no *tet* genes could be detected in the DNAs of the 12 *M. synoviae* strains with higher MIC values for tetracyclines (data not shown). In case of these antibiotics, it is plausible that other resistance



mechanisms play a role. For example, decreased susceptibility of bacterial strains may be induced by increased efflux of the antimicrobial agent leading to lower intracellular concentrations. Active efflux systems might occur in *M. synoviae*, as it has been already described for the human pathogen *M. hominis* (Raheison et al., 2002).

The revealed mutations can contribute to the extension of knowledge about the genetic background of antibiotic resistance in *M. synoviae*. Moreover, the explored potentially resistance-related nucleotide positions can be investigated by molecular biological assays (Sulyok et al., 2018). Targeting the most frequently occurring C254T mutation in the *parC* gene by MAMA or analysing the whole hot spot region by HRM could provide feasible options for the rapid detection of fluoroquinolone resistance. However, simultaneous detection of several mutations in different genes could enhance the reliability of the method. Beside a previously reported SNP of the *gyrB* gene (C1247A), mutations reported here for the first time in the *gyrA* (A566G and T1360A), *gyrB* (C446T) and *parE* (C260T) genes could serve also as appropriate targets for molecular biological assays, based on their frequent occurrence in resistant isolates and low prevalence in sensitive strains. In case of lincomycin, molecular detection of the described A2054G mutation in the *rrlA/B* genes might be able to identify almost all resistant strains according to our results. The MAMA test developed in this study for the determination of the nucleotide at this position could be applied for this purpose as well. Beside this mutation, targeting the nucleotide position 276 of the *rplV* gene should be also appropriate to reveal macrolide resistance according to our data. However, these findings could be further strengthened by investigating more strains.

Supporting the results of conventional *in vitro* sensitivity tests, molecular biological assays could provide excellent guidance for antibiotic therapy, especially when susceptibility data are required quickly or when isolation of *Mycoplasma* fails. Reducing the detection time of antibiotic susceptibility, the use of these methods could contribute to achieve therapeutic success, thereby significantly reduce economic losses. Furthermore, data provided by these assays could support prudent antibiotic usage instead of empirical treatment. This trend could help to reduce the impact of antibiotic resistance and preserve critically important antibiotics for human medicine.

## 5. Conclusion

In the present study, molecular markers of decreased susceptibility to fluoroquinolones, macrolides and lincomycin have been identified in the genes encoding the subunits of DNA gyrase and topoisomerase IV, the 23S rRNA and the 50S ribosomal protein L22. The revealed mutations can contribute to the extension of knowledge about the genetic background of antibiotic resistance in *M. synoviae*. Moreover, the explored genetic markers may serve as targets for molecular biological assays providing data of antibiotic susceptibility much faster than conventional methods.

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## Declaration of Competing Interest

The authors declare that they have no competing interests.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: <https://doi.org/10.1016/j.vetmic.2020.108818>.

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