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Short communication

Identification and detection of mutations potentially associated with decreased susceptibility to macrolides and lincomycin in *Mycoplasma anserisalpingitidis* isolates

Dénes Grózner [a,b], Katinka Bekö [a,b], Áron Botond Kovács [a,b], Alexa Mitter [a,b], Veronika Hrivnák [a,b], Anna Sawicka [c], Grzegorz Tomczyk [c], Krisztián Bányai [a,b,d], Szilárd Jánosi [e], Zsuzsa Kreizinger [a,b,f], Miklós Gyuranecz [a,b,f,*]

[a] Institute for Veterinary Medical Research, Centre for Agricultural Research, ELKH, 1143, Budapest, Hungária krt. 21., Hungary
[b] Veterinary Medical Research Institute, ELKH, 1143, Budapest, Hungária krt. 21., Hungary
[c] Department of Poultry Diseases, National Veterinary Research Institute, 24-100, Pulawy, Aleja Partyzantow 57, Poland
[d] University of Veterinary Medicine, 1078, Budapest, István u. 2., Hungary
[e] Veterinary Diagnostic Directorate, National Food Chain Safety Office, 1143, Budapest, Tábornok u. 2., Hungary
[f] MolliScience Kft., 2051, Biatorbágy, Március 15. u. 1., Hungary

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ABSTRACT

Mycoplasma anserisalpingitidis infection is associated with the inflammation of the genital tract and cloaca, embryo lethality and decreased egg production in geese, leading to serious economic losses. This bacterium has so far been described in Europe and Asia. There is no commercially available vaccine against *M. anserisalpingitidis,* thus treatment of waterfowl mycoplasmosis relies mainly on antimicrobial therapy. However, *M. anserisalpingitidis* isolates with decreased susceptibility to macrolides and lincomycin have been reported before.

The minimal inhibitory concentration (MIC) values of tilmicosin, tylosin, tylvalosin and lincomycin were determined against 82 *M. anserisalpingitidis* isolates originating from Hungary, Poland, China and Vietnam. Whole-genome sequence analyses revealed two mutations in the 23S rRNA coding regions and one mutation in the 50S ribosomal protein L22 coding gene possibly correlating with decreased susceptibility to the examined antibiotics. Mismatch amplification mutation assays coupled with melt analysis (melt-MAMAs) were designed to detect the nucleotide substitutions.

This study is the first to describe resistance-related mutations in the goose pathogen *M. anserisalpingitidis*. The developed molecular assays support targeted antibiotic usage, hence their use may help to reduce the development and spread of antibiotic resistance.

1. Introduction

Mycoplasma anserisalpingitidis (also known as *Mycoplasma* sp. 1220) is a cell-wall less bacterium infecting mainly geese. It could be part of the normal microflora of the animals; however, this *Mycoplasma* species is also associated with some pathological conditions. Inflammation of the genital tract and cloaca, embryo lethality and decreased egg production are the main symptoms in the affected flocks and infection may lead to enormous economic losses in the goose industry (Stipkovits and Szathmary, 2012; Volokhov et al., 2020). *M. anserisalpingitidis* was identified in Europe in the Czech Republic, France, Germany, Hungary, Poland, Russian Federation and Ukraine and it was recently described in China and Vietnam as well (Stipkovits et al., 1986; Sprygin et al., 2012; Gyuranecz et al., 2020; Grózner et al., 2021).

Since there is no commercially available vaccine against *M. anserisalpingitidis*, adequate housing and appropriate antibiotic treatment are promoted in the control of the infection. Literature about the medication of waterfowl mycoplasmosis or the minimal inhibitory

* Corresponding author at: Veterinary Medical Research Institute, ELKH, 1143, Budapest, Hungária krt. 21., Hungary.
E-mail addresses: groznerdenes@gmail.com (D. Grózner), bekokat@gmail.com (K. Bekö), kovcsboti@hotmail.com (Á.B. Kovács), mitteralexa@gmail.com (A. Mitter), hrivnakvera75@gmail.com (V. Hrivnák), anna.sawicka@piwet.pulawy.pl (A. Sawicka), gtomczyk@piwet.pulawy.pl (G. Tomczyk), bkrota@hotmail.com (K. Bányai), JanosiSz@nebih.gov.hu (S. Jánosi), zsuzsakreizinger@yahoo.com (Z. Kreizinger), m.gyuranecz@gmail.com (M. Gyuranecz).

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concentration (MIC) values against *M. anserisalpingitidis* isolates is scarce; however, the available data revealed that numerous isolates show decreased susceptibility against macrolides (especially tilmicosin and tylosin) and lincomycin (Stipkovits and Szathmary, 2012; Grózner et al., 2016; Gyuranecz et al., 2020). Single nucleotide polymorphisms (SNPs) can cause conformation changes of the target regions of 50S inhibitors, leading to resistance against these drug agents in *Mycoplasma* spp. (Gautier-Bouchardon, 2018). The aim of the present study was to identify macrolide and lincomycin resistance-related SNPs in the genome sequences of Hungarian, Polish and Asian *M. anserisalpingitidis* isolates and to develop rapid and cost-effective molecular biological assays for their detection.

2. Materials and methods

2.1. *M. anserisalpingitidis isolates, antimicrobial agents and susceptibility testing*

In total, 82 *M. anserisalpingitidis* isolates including the ATCC BAA-2147 type strain were analysed in the present study. The isolation method has been described previously (Grózner et al., 2021). The isolates were recovered from domestic geese, swan geese and a domestic duck between 1983–2019 originating from Hungary (n = 71), Poland (n = 8), China (n = 2) and Vietnam (n = 1). Background information of the isolates are provided in Supplementary Table 1. Ethical approval and specific permission were not required for the study as all field isolates were collected during routine diagnostic examinations or necropsies by the authors with the consent of the owners, and sampling was not performed solely for the purpose of the study.

The following antimicrobial agents were examined by broth microdilution tests: three macrolides: tilmicosin, tylosin (VETRANAL, Sigma-Aldrich Chemie GmbH., Taufkirchen, Germany) and tylvalosin (Aivlosin, ECO Animal Health Ltd., London, UK); and a lincosamide: lincomycin (VETRANAL, Sigma-Aldrich Chemie GmbH.). The antibiotics were applied in the range of 0.25–64 μg/mL. The dilution and storage of the antibiotics, the broth microdilution examinations and the determination of the MIC values were performed according to the recommendations of Hannan (2000). Out of the 82 *M. anserisalpingitidis* samples, MIC values for 31 isolates were determined previously, while susceptibility testing of 51 isolates was performed during this study using the same method (Grózner et al., 2016). The *M. anserisalpingitidis* ATCC BAA-2147 type strain was tested on each microtiter plate to confirm the validity of the results.

2.2. *Whole-genome sequencing and sequence analysis*

DNA was extracted from the 82 *M. anserisalpingitidis* isolates with the QIAamp DNA Mini Kit (Qiagen, Inc., Hilden, Germany) according to the manufacturers' instructions. Next-generation sequencing was performed on NextSeq 500 Illumina equipment (Illumina, Inc., San Diego, CA, USA), with NextSeq 500/550 High Output Kit v2.5 reagent kit (Illumina, Inc.). The obtained sequences were mapped to the reference genome of *M. anserisalpingitidis* ATCC BAA-2147 (GenBank accession number: CP042295) using the Geneious Prime 2019.2.1 software (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012).

Genomic regions which have been previously linked to macrolide and lincomycin resistance in other *Mycoplasma* species with veterinary importance were analysed: 23S rRNA coding genes (*rrl1* and *rrl2*), and the genes encoding the 50S ribosomal proteins L4 and L22 (Wu et al., 2005; Kobayashi et al., 2005; Gerchman et al., 2011; Lerner et al., 2014; Lysnyansky et al., 2015; Ammar et al., 2016; Kong et al., 2016; Prats-van der Ham et al., 2017; Sulyok et al., 2017; Khalil et al., 2017; Felde et al., 2018; Gautier-Bouchardon, 2018; Bekő et al., 2020; Földi et al., 2021). Furthermore, all other genes encoding 50S ribosomal proteins were also analysed in the study. The selected gene sequences were obtained from the genomes of the ATCC BAA-2147 type strain and the 81 *M. anserisalpingitidis* field isolates, and aligned by the Geneious Prime 2019.2.1 software (Biomatters Ltd.) (Kearse et al., 2012). For the identification of potentially antibiotic resistance-related SNPs, the correlation between the MIC values and the occurrence of several mutations in the genes were analysed. To this end, the examined *M. anserisalpingitidis* isolates were sorted by their MIC values for each antibiotics, and mutations detected in the samples more frequently as MIC values increased or occurred exclusively in isolates with high MIC values were investigated individually. All mutations found in the *rrl* genes were examined whilst only mutations causing amino acid changes were considered in the case of the 50S ribosomal proteins. Numbering of nucleotide and amino acid positions is given according to *Escherichia coli* strain K-12 substrain MG1655 (GenBank accession number: U00096) to enable the comparison of our results with other published data.

Sequences of the examined genes of 82 *M. anserisalpingitidis* isolates were deposited in GenBank under accession numbers OK166669 - OK166750 for *rrl1*, OK162137 - OK162218 for *rrl2*, and OK078532 - OK078613 for the 50S ribosomal protein L22 coding gene.

2.3. *Melt-MAMA design*

For the detection of SNPs related to decreased susceptibility of *M. anserisalpingitidis* isolates to macrolides and lincomycin, mismatch amplification mutation assays coupled with melt analysis (melt-MAMAs) were designed (Birdsell et al., 2012). Primers were constructed with the help of the Geneious Prime 2019.2.1 software (Biomatters Ltd.) (Kearse et al., 2012) and the NetPrimer software (http://www.premierbiosoft.com/netprimer).

The melt-MAMA polymerase chain reaction (PCR) was carried out in 10 μl total volume and consisted of nuclease-free water, 2 μl 5X Colourless GoTaq Flexi Buffer (Promega Inc., Madison, WI, USA), 1 μl $MgCl_2$ (25 mM; Promega Inc.), 0.3 μl dNTP (10 mM; Fermentas, Waltham, MA, USA), 0.5 μl EvaGreen (20X, Biotium Inc., Hayward, CA, USA), 0.08 μl GoTaq G2 Flexi DNA polymerase (5 U/μl; Promega Inc.), 1 μl target DNA sample and primers (10 pmol/μl) according to Table 1. The primer sets consisted of a consensus reverse and two competing forward primers, except for the MAMA-L22-279 PCR system, which contained three forward primers due to the three nucleotide variants of the target position (see in the Results section). Thermocycling parameters were 95 °C for 10 min, followed by 30 cycles of 95 °C for 15 s and 61.5 °C for 1 min. PCR products were subjected to melt analysis using a dissociation protocol comprising 95 °C for 15 s, followed by 0.3 °C incremental temperature ramping from 61.5 °C to 95.1 °C. The real-time PCRs were performed using CFX96 Touch real-time PCR detection system with CFX Maestro 2.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature. Nuclease-free water was used as negative control in all PCR assays.

2.4. *Evaluation of the assays*

Specification of the designed MAMAs was performed on the *M. anserisalpingitidis* isolates' DNA samples. Genotype calls of these strains (genotype H: genotype characterized by high MIC values for certain antibiotics; genotype L: genotype characterized by low MIC values for certain antibiotics; genotype Het: heterozygous concerning nucleotide position 748 or 2058 of the *rrl* genes) were compared with their whole genome sequences.

The PCR sensitivity was evaluated with serial tenfold dilutions (10^6–10^0) of the DNA of each genotype. Template copy numbers were calculated by an online dsDNA copy number calculator (https://cels.uri.edu/gsc/cndna.html) based on the DNA concentration measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.) and the genome size. The lowest template copy number yielding melting temperature specific to the genotype was considered as the detection limit of each assay.

Table 1
Primers and parameters of mismatch amplification mutation assays (MAMAs) designed in this study.

Gene	SNP[a]	MAMA system	Primer name[b]	Primer sequence (5' - 3')	Primer volume (µl)[c]
rrl1/rrl2	G748A	MAMA-rrl-748	rrl-748-H	ggggcggggcggggAGGACCGAACCGTAGTACGATA	0.6
			rrl-748-L	AGGACCGAACCGTAGTACGTTG	0.15
			rrl-748-con	GGAATTTCTCCACTATTCACAAGTC	0.15
rrl1/rrl2	A2058G	MAMA-rrl-2058	rrl-2058-H	ggggcggggcggggGGTACCCGCATCAAGACCAG	0.6
			rrl-2058-L	GGTACCCGCATCAAGACAAA	0.15
			rrl-2058-con	GCCAAGTTCCAATACGAAGTTGT	0.15
L22 coding gene	A270C/T (Gln90His)	MAMA-L22-270	L22-270-H-A	ggggcggggcggggCGTTTGAAAATAGAATATGCTCTTACA	0.15
			L22-270-H-G	ggggcggggcggggCGTTTGAAAATAGAATATGCTCTTACG	0.15
			L22-270-L	CGTTTGAAAATAGAATATGCTCTTTCT	0.15
			L22-270-con	AGGACCAACACTCAAAAGATTTCA	0.15

[a] Single nucleotide polymorphism (SNP) according to *E. coli* numbering (K-12 substrain MG1655, GenBank accession number: U00096).
[b] Primers specific for the genotypes of *M. anserisalpingitidis* with high or low minimal inhibitory concentration (MIC) values are labelled with "H" or "L" abbreviation, respectively, while „con" means consensus in the primer name.
[c] Primer (10 pmol/µl) volume in 10 µL reaction mixture.

The specificity of the PCR assays was assessed by testing DNA extracts from avian *Mycoplasma* type strains: *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234), *M. cloacale* (NCTC 10199), *M. columbinasale* (ATCC 33549), *M. columbinum* (ATCC 29257), *M. columborale* (ATCC 29258), *M. gallinaceum* (ATCC 33550), *M. gallinarum* (ATCC 19708), *M. gallisepticum* (ATCC 19610), *M. gallopavonis* (ATCC 33551), *M. imitans* (ATCC 51306), *M. iners* (ATCC 19705), *M. iowae* (ATCC 33552), *M. meleagridis* (NCTC 10153) and *M. synoviae* (NCTC 10124).

2.5. Differentiation of the rrl1 and rrl2 genes and determination of the SNPs at positions 748 and 2058

Certain *M. anserisalpingitidis* isolates (MYCAV 67, 76, 77 and 313) showed heterogeneous (Het) genotype (double melt curve peaks) in the MAMA-rrl-748 and/or MAMA-rrl-2058 assays targeting point mutations in the *rrl1* and *rrl2* genes. As the reads belonging to the two *rrl* genes could not be distinguished based on the whole-genome sequences, a conventional PCR was developed for the specific amplification of the partial sequences of the *rrl1* and *rrl2* genes in order to examine the nucleotide positions 748 and 2058. A common forward primer (forw-rrl1/rrl2) was designed targeting the internal sequence of the 23S rRNA genes and two reverse primers were developed: 1) to bind to the predicted gene encoding hypothetical protein following the *rrl1* gene (rev-rrl1), and 2) to bind to the tRNA-Pro coding gene following the *rrl2* gene (rev-rrl2). Primers were constructed with the aforementioned software.

The conventional PCR assays were carried out in 25 µl total volume, containing 5 µl 5X Colourless GoTaq Flexi Buffer (Promega Inc.), 2 µl $MgCl_2$ (25 mM; Promega Inc.), 0.5 µl dNTP (10 mM, Qiagen Inc.), 2 µl of the forward and one reverse primer (10 pmol/µl; forw-rrl1/rrl2: 5'-GAGCCGTAGAGAAATCGAGTCTTA-3', rev-rrl1: 5'-TTTTCAAATCTAGGAACTTCTATATTTTTAAG-3', rev-rrl2: 5'-TCTGCCAAGCTGAGCTACTTCTC-3'), 0.25 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; Promega Inc.), 2 µl target DNA sample and nuclease-free water. Thermocycling parameters were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 50 s, 61 °C for 1 min and 72 °C for 1 min, completed with a final elongation step at 72 °C for 5 min. The expected molecular size of the amplicons was confirmed by electrophoresis in agarose gel stained with ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics Co., Ltd, New Taipei City, Taiwan) followed by UV visualization. The amplified PCR products were diluted in nuclease-free water (1:1000) and were submitted to the corresponding MAMA tests.

3. Results

3.1. Antibiotic susceptibility profiles

The ATCC BAA-2147 type strain showed consistent results throughout the study. MIC ranges of the macrolides and lincomycin determined in the present study were in accordance with MIC ranges in our previous study (Grózner et al., 2016). Field isolates with elevated MIC values were found in the cases of all tested antibiotics. For detailed MIC values see Supplementary Table 1.

The MIC values for tilmicosin (TIL) were divided into two groups: ≤0.25 to 4 µg/mL (n = 25) and 64 to >64 µg/mL (n = 57). MIC values were measured at all tested tylosin (TYL) concentrations (≤0.25 to >64 µg/mL). The isolates' TIL and TYL susceptibility profile showed correlation as the 25 isolates inhibited with lower TIL concentration yielded low to moderate MIC values (≤0.25 to 4 µg/mL) for TYL, whilst the 57 isolates showing low susceptibility for TIL yielded moderate to elevated MIC values (4 to >64 µg/mL) for the latter compound. Among the examined three macrolides, tylvalosin (TLV) showed the lowest MIC range (≤0.25 to 16 µg/mL) against the isolates. The MIC values for lincomycin (LIN) were between 0.5 and 8 µg/mL except for three Hungarian (MYCAV 47, 67 and 68) and one Chinese (MYCAV 783) isolates which could not be inhibited even with the highest antibiotic concentration used in the study. All four isolates showed elevated MIC values for TIL and TYL (>64 µg/mL and ≥16 µg/mL, respectively) and two of them (MYCAV 67 and 68) yielded the highest MIC for TLV observed in the study.

3.2. Mutations correlating with decreased antibiotic susceptibility

After aligning the examined genes of the *M. anserisalpingitidis* isolates, several mutations were identified (data not shown). Among them, three mutations were found possibly resistance-associated in the case of macrolides and lincomycin: mutations at positions 748 (G748A) and 2058 (A2058G) in the *rrl1/rrl2* genes and a mutation in the gene coding the L22 ribosomal protein (A270C/T; Gln90His).

Isolates susceptible to TIL and TYL (n = 21; MIC values: ≤1 µg/mL for both compounds) did not possess any of the identified resistance-related mutations. Four isolates (MYCAV 34, 50, 179 and 512) yielding MIC values 2−4 µg/mL for TIL and 1−4 µg/mL for TYL revealed mutations either at position 748 in the *rrl* genes or showed the amino acid change in L22. The *M. anserisalpingitidis* isolates (n = 53/57) showing decreased susceptibility for these two antibiotic agents (MIC values: ≥64 µg/mL for TIL and ≥4 µg/mL for TYL) possessed both mutations (G748A and A270C/T). The G748A mutation appeared mostly in both *rrl* alleles. The four isolates (MYCAV 47, 67, 68 and 783) with decreased susceptibility to TIL, TYL and LIN (MIC values: >64 µg/mL, ≥16 µg/mL and >64 µg/mL, respectively) carried the A2058G mutation in the *rrl* genes. The two isolates (MYCAV 67 and 68) showing the highest MIC values to all examined antibiotics (MIC values: >64 µg/mL for TIL, TYL and LIN, and 16 µg/mL for TLV) possessed both the G748A and A2058G mutations in the *rrl1/rrl2* genes.

3.3. Detection of SNPs related to high MIC values

Three melt-MAMA tests were designed to detect the SNPs G748A and A2058G in the *rrl* genes and A270C/T in the gene coding the 50S ribosomal protein L22 (Table 1). Melting temperature (T_m) ranges of the amplicons are listed in Table 2. T_m values for all tested isolates are provided in Supplementary Table 1. The MAMAs clearly differentiated genotypes H and L based on their distinguishable peaks of melting curves. Besides, MAMA-rrl-748 and MAMA-rrl-2058 were able to differentiate heterozygous samples (genotype Het) as well with bimodal melting peaks at the specific melting temperatures. The MAMAs performed on the PCR amplicons of the partial sequences of differing *rrl1* and *rrl2* genes revealed that the G748A and the A2058G substitutions were located in the *rrl2* gene in the case of Het samples. Genotype calls by the designed MAMAs were congruent with the whole genome sequences for all DNAs extracted from pure cultures of *M. anserisalpingitidis* isolates.

Sensitivity of the melt-MAMAs were 10^3-10^4 template copy numbers/reaction (Table 2). In the MAMA-rrl-2058 PCR system, some DNA samples containing 10^6–10^5 template copy numbers / reaction showed a second exponential phase after the plateau phase leading to non-specific T_m value (83.4–83.6 °C). This problem can be eliminated by the dilution of the DNA samples (1:100 in nuclease-free water). Cross-reactions with other avian *Mycoplasma* species occurred as follows: with *M. anatis*, *M. anseris*, *M. columbinum*, *M. gallinaceum*, *M. gallopavonis*, *M. iners*, *M. meleagridis* and *M. synoviae* in MAMA-rrl-748; with *M. anatis*, *M. columbinum*, *M. gallinaceum*, *M. gallopavonis*, *M. iners*, *M. meleagridis* and *M. synoviae* in MAMA-rrl-2058; and with *M. anatis* and *M. iners* in MAMA-L22-270. Melting temperatures were in the same ranges as the DNA samples of *M. anserisalpingitidis*.

4. Discussion

Macrolide and lincosamide antibiotics share a similar mode of action. Modification of the ribosomal target leads to broad-spectrum resistance to both classes of antibiotics (Leclercq, 2002). Macrolides bind within the tunnel of the 50S ribosomal subunit and interact mainly with the A2058 nucleotide of the 23S rRNA (domain V) with an additional interaction with the G748 nucleotide (domain II) and with the surface proteins L4 and L22 (Gautier-Bouchardon, 2018).

The G748A nucleotide substitution of the *rrl* genes correlating with decreased susceptibility to TIL and TYL in *M. bovis* and *M. synoviae* was published before. The substitution appeared in one or both alleles (Lerner et al., 2014; Lysnyansky et al., 2015; Khalil et al., 2017; Sulyok et al., 2017; Gautier-Bouchardon, 2018). Higher MIC values of TIL, TYL and/or LIN were reported against *M. synoviae*, *M. bovis* and *M. agalactiae* isolates carrying the Gln90His/Lys amino acid substitution in the 50S ribosomal protein L22 coding gene (Khalil et al., 2017; Prats-van der Ham et al., 2017; Gautier-Bouchardon, 2018; Bekő et al., 2020). In the present study, the joint presence of the aforementioned mutations in the *rrl* genes and in the L22 coding gene was sufficient in all cases for the development of TIL resistance (MIC values ≥64 μg/mL). The *M. anserisalpingitidis* isolates showing slight decrease in the TIL and TYL susceptibility (MIC values: 2−4 μg/mL for TIL and 1−4 μg/mL for TYL) carried only one of the mutations. However, *M. anserisalpingitidis* isolates carrying these two mutations together yielded broad range of MIC values for TYL (4->64 μg/mL), which indicates the presence of additional mechanisms leading to decreased susceptibility (*e.g.* unrevealed SNPs or possibly efflux pumps).

Table 2
Results of mismatch amplification mutation assays (MAMAs) designed in this study.

MAMA system	Genotype[a]	T_m (°C)	Sensitivity (template copy numbers/reaction)
MAMA-rrl-748	H	81.0−81.9	10^3
	Het	75.9−77.0; 81.3−81.4	10^4
	L	75.3−77.0	10^4
MAMA-rrl-2058	H	82.2−82.5	10^3
	Het	76.5; 82.2	10^3
	L	76.2−77.1	10^3
MAMA-L22-270	H	78.0−79.5	10^3
	L	72.3−73.3	10^3

bp: base pair; T_m: melting temperature.
[a] Genotype H: genotype characterized by high minimal inhibitory concentration (MIC) values for certain antibiotics; genotype L: genotype characterized by low MIC values for certain antibiotics; genotype Het: heterozygous concerning nucleotide position 748 and 2058 of the *rrl* genes.

The nucleotide substitutions A2058G and/or A2059G in the domain V of the *rrl* genes were associated with decreased susceptibility to TIL, TYL, TLV and/or LIN before in *M. agalactiae*, *M. bovis*, *M. gallisepticum*, *M. hyopneumoniae*, *M. hyorhinis* and *M. synoviae* isolates (Wu et al., 2005; Kobayashi et al., 2005; Gerchman et al., 2011; Lerner et al., 2014; Lysnyansky et al., 2015; Kong et al., 2016; Prats-van der Ham et al., 2017; Sulyok et al., 2017; Khalil et al., 2017; Felde et al., 2018; Gautier-Bouchardon, 2018; Bekő et al., 2020; Földi et al., 2021). Among the examined *M. anserisalpingitidis* samples, only four isolates (MYCAV 47, 67, 68 and 783) showed resistance to LIN (MIC values >64 μg/mL) and all four isolates revealed the A2058G mutation in *rrl2* or in both *rrl* genes. While MIC values of LIN, TIL and TYL were all increased in the case of these isolates - indicating the resistance triggering effect of the A2058G mutation - isolates which carried both the G748A and A2058G substitutions (MYCAV 67 and 68) yielded the highest MIC values for TLV (16 μg/mL) also. Therefore, it could be speculated that the two mutations together could lead to decreased susceptibility to TLV; however, more *M. anserisalpingitidis* samples yielding elevated MIC values of this macrolide should be examined to assess this hypothesis.

The designed MAMA systems were able to detect and differentiate the mutations at the three nucleotide positions in the *M. anserisalpingitidis* isolates. The resistant isolates with heterozygous *rrl* genes were also identified by the MAMA-rrl-748 and MAMA-rrl-2058 assays (showing bimodal peaks), and with the help of the designed conventional PCR systems, we were able to reveal and discriminate the SNPs in the positions in question. Nevertheless, there was no difference in MIC values between hetero- and homozygous resistant isolates, thus the MAMA assays alone should be sufficient to determine susceptibility of *M. anserisalpingitidis* strains. As the MAMA tests cross-reacted with other *Mycoplasma* species due to the broad appearance of the described resistance-related SNPs in these bacteria, use of DNA samples of pure *M. anserisalpingitidis* field isolates are recommended for the assays. The three MAMAs could be run simultaneously, therefore submitting a *M. anserisalpingitidis* sample to the MAMA-rrl-748, MAMA-rrl-2058 and MAMA-L22-270 assays could reveal information about the susceptibility to TIL, TYL, TLV and LIN in a rapid and cost-effective way.

As far as we know this is the first report of mutations correlating with decreased antibiotic susceptibility in *M. anserisalpingitidis* isolates. The SNPs in the *rrl* genes and in the L22 coding gene were also identified in other *Mycoplasma* species in previous publications. The designed molecular assays could reduce time required to determine macrolide and lincomycin susceptibility and support the results of the conventional MIC tests. Therefore, the presented assays promote adequate antibiotic selection for treatment, and the prudent drug usage could help to reduce the development and spread of antibiotic resistance in *M. anserisalpingitidis*, as well as in other waterfowl pathogens.

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Declaration of Competing Interest

The authors report no declarations of interest.

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.2022.109362.

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