Summary of Ph.D. thesis

ASSESSMENT OF THE ANTIBIOTIC SENSITIVITY AND GENOMIC STUDIES ON WATERFOWL *MYCOPLASMA* SPECIES

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Introduction

Mycoplasma anserisalpingitidis, *M. anatis*, *M. anseris* and *M. cloacale* colonise geese and ducks. All four *Mycoplasma* species could be a part of the normal microflora of the animals, which do not demonstrate any clinical signs of *Mycoplasma* infection; however, clinically manifested mycoplasmosis can occur in waterfowl during excessive stress. The main symptoms in the affected flocks are inflammation of the cloaca and genital tracts, decreased egg production and increased embryo lethality, and could be associated with infections of respiratory and nervous systems. Infection of geese and ducks with these mycoplasmas could cause significant production and economic losses to the waterfowl industry worldwide, and *M. anserisalpingitidis* causes the most damages in the goose farming business in Hungary.

Since there is no commercially available vaccine against these *Mycoplasma* species, adequate housing and appropriate antibiotic treatment are promoted in the control of the disease. Quinolones, tetracyclines, macrolides and pleuromutilins are the drugs of choice for the therapy of mycoplasmosis; however, there are big variations in antibiotic sensitivity among strains of one species. Information about the susceptibility of waterfowl

Mycoplasma field isolates to antimicrobials was scarce, as only one paper revealed data concerning the antibiotic susceptibility of these species.

Comprehensive molecular biology and bioinformatics research require the knowledge of the genetic background of the studied organism. At the time of our study, the complete genomes were not available for any of the examined waterfowl *Mycoplasma* species, only gene fragments and shotgun sequencing projects were accessible in the GenBank. However, the handling of these sequences is complicated. Publicly available complete genome sequences could provide the basis for novel investigations.

Co-occurrence of the waterfowl *Mycoplasma* species is frequently detected in the animals and identification of these mycoplasmas is difficult due to their similar morphological, cultural and biochemical properties. The identification of these bacteria to the species level is possible by the sequence analysis of the product of a genus-specific polymerase chain reaction (PCR) assay; however, this method is not always feasible at regular veterinary laboratories and this kind of identification is impossible from birds co-infected with different mycoplasmas. Species-specific PCR assays could

improve the robust detection of these mycoplasmas; however, no *M. anatis*-, *M. anseris*- or *M. cloacale*-specific PCR assays have been published previously, and a not comprehensively established study has been published for the detection of *M. anserisalpingitidis*.

M. anserisalpingitidis is frequently detected in Central and Eastern European countries, and the occurrence of this species has been recently described in China. Information about the spatial and temporal diversity of *M. anserisalpingitidis* is limited, and tools for its better understanding are lacking. Multilocus sequence typing (MLST) can provide a valuable insight into the phylogeny and molecular epidemiology of bacterial pathogens, by analysing the genetic relationship between strains and monitoring the temporal and geographical distribution of bacterial spread, identifying infection and transmission routes.

Aims of the study

The aims of the study were:

Ad 1. to determine the *in vitro* susceptibility of Hungarian *M. anserisalpingitidis* field isolates to thirteen different antibiotics and an antibiotic combination.

Ad 2. to sequence *de novo* the complete genomes of three isolates (type strain and two field isolates) of *M. anserisalpingitidis*, and the type strains of *M. anatis*, *M. anseris*, and *M. cloacale*.

Ad 3. to develop an effective and robust molecular test for the identification of *M. anserisalpingitidis*, *M. anatis*, *M. anseris*, and *M. cloacale* in avian clinical specimens.

Ad 4. to develop a reproducible and useful MLST assay that can be used to analyse the phylogenetic relationships between *M. anserisalpingitidis* strains.

Materials and methods

Antibiotic susceptibility testing of *M. anserisalpingitidis*

In order to determine the antibiotic susceptibility of *M. anserisalpingitidis*, 38 field isolates from geese and a duck originating from different parts of Hungary were tested in the study. The samples were collected between 2011 and 2015, from several organs of the animals.

The following antimicrobial agents were examined during the broth microdilution tests: the fluoroquinolones: enrofloxacin, difloxacin and norfloxacin; the aminoglycoside: spectinomycin; the lincosamide: lincomycin; the tetracyclines: doxycycline and oxytetracycline; the macrolides: tilmicosin, tylosin and tylvalosin; the pleuromutilins: tiamulin and valnemulin; and the phenicol: florfenicol. Lincomycin and spectinomycin were also applied in combination.

The minimum inhibitory concentration (MIC) values were determined from the lowest concentration of the antibiotics where no pH and colour change of the broth was detected, meaning that the growth of the bacteria was completely inhibited. MIC_{50} and MIC_{90} values were defined as the lowest concentrations that inhibit 50% and 90% of the isolates.

De novo sequencing of waterfowl *Mycoplasma* strains/isolates

DNA samples of the *M. anserisalpingitidis* (ATCC BAA-2147, MYCAV 93, MYCAV 177), *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234), and *M. cloacale* (NCTC 10199) type strains/isolates were sequenced on Illumina MiSeq and/or NextSeq 500 equipments (Illumina, Inc., San Diego, CA, USA). Contigs were assembled from the output data using the SPAdes Genome Assembler 3.11. Pieces of online available software were used to annotate all six genomes.

Whole genome comparisons of the waterfowl *Mycoplasma* type strains were performed using Mauve version 2.3.1 software.

Development of species-specific PCR assays

In order to design species-specific primers for the detection of *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale*, several housekeeping genes were randomly selected from the *Mycoplasma* minimal genome set, which are presented in the genomes of the examined species. The genes were manually analysed in the Geneious software and the primer pairs were designed according to the following acceptance criteria: (i) the gene of interest should have at least two regions containing as

many species-specific nucleotide substitutions as possible, (ii) the distance between the primers' regions should be approx. 500–1000 bp, and (iii) the selected species-specific primers should be able to amplify the target gene from all tested field isolates of the same species but not from others.

To evaluate the species-specific PCR assays, 18 *M. anserisalpingitidis*, eight *M. anatis*, 13 *M anseris*, and 18 *M. cloacale* field isolates were investigated obtained from domestic geese and ducks originating mostly from Hungary. The specificity and the sensitivity of the PCR assays was assessed.

Twenty-eight clinical specimens were screened for the presence of *Mycoplasma* DNA using the genus-specific PCR and the designed species-specific PCR assays.

Direct DNA sequencing was performed on selected amplicons obtained from type strains, field isolates and clinical specimens using the *Mycoplasma* species-specific or genus-specific PCR systems to confirm their species identity.

MLST study on *M. anserisalpingitidis*

Eighty-nine *M. anserisalpingitidis* strains/isolates and clinical specimens were collected from domestic geese, swan geese and a domestic duck between 1983-2019

originating from Hungary, Poland, Ukraine, China and Vietnam. Among these samples, 36 field isolates were collected from flocks of a Hungarian livestock integration. Some field isolates originated from the same flock or same animal's different organs. *M. anatis* strains/isolates were examined in the study as an outgroup.

Next-generation sequencing was performed on the DNA samples of the isolates with NextSeq 500 Illumina equipment. The obtained sequences were mapped to the appropriate reference genome using the Geneious software.

Forty housekeeping genes were selected for analyses based on previous MLST studies and random selection. The sequences of the genes were obtained from the genomes of the *M. anserisalpingitidis* and *M. anatis* strains/isolates. Criteria for the selection of the genes were in accordance with previous publications: (i) the selected genes are present in all *M. anserisalpingitidis* genomes, (ii) the selected genes possess highly diverse internal fragments surrounded by conserved regions, (iii) the selected fragments show high Simpson's index of diversity, (iv) the amplicon sizes are between 300–600 bp, (v) the genes are evenly distributed in the genome, and (vi) preferably PCR primers are species-specific.

The samples were grouped into sequence types (STs) according to the allelic numbers of the loci. Phylogenetic analysis of the concatenated sequences was performed using MEGA X 10.0.5. The evolutionary history with the *M. anatis* outgroup was inferred using the Maximum Likelihood method.

Results

Antibiotic susceptibility profile of *M. anserisalpingitidis*

The MIC values of enrofloxacin and difloxacin showed a wide range (1.25 to >10 µg/ml), while all field isolates had very high MIC values for norfloxacin (≥10 µg/ml). The MICs for spectinomycin and lincomycin clustered around the MIC_{50} values (8 µg/ml and 4 µg/ml, respectively). The MIC_{50} and the MIC_{90} values were 4 µg/ml for lincomycin-spectinomycin (1:2) combination. Broad ranges of the MIC values were observed for tetracyclines (2 to >64 µg/ml for oxytetracycline and 0.078 to >10 µg/ml for doxycycline) with high MIC_{50} and MIC_{90} values (>64 µg/ml and >10 µg/ml, respectively). The broadest ranges of MIC values were detected for tylosin and tilmicosin (≤0.25 to >64 µg/ml) with high MIC_{50} and MIC_{90} values. Among the examined macrolides, tylvalosin showed the lowest MIC_{50} value (0.5 µg/ml) against the isolates. The MIC values of tiamulin (MIC_{50}: 0.625 µg/ml; MIC_{90}: 1.25 µg/ml) were higher than those of valnemulin, and the latter compound was found to be the most active antibiotic in the examinations (MIC_{50}: ≤0.039 µg/ml; MIC_{90}: 0.078 µg/ml). In the case of florfenicol, the susceptibility profiles of most field isolates showed the MIC_{50} and MIC_{90} value (8 µg/ml).

M. anserisalpingitidis samples isolated year by year from the same farms showed elevated MIC values in the cases of several antibiotics.

De novo genomes of waterfowl *Mycoplasma* strains/isolates

The total genome sizes were approximately 910,000-960,000 bp for the *M. anserisalpingitidis* and *M. anatis* type strains and isolates, 750,000 bp for the *M. anseris*, and 660,000 bp for the *M. cloacale* type strains. G+C content was 26.4-27.0%. The raw read data and the annotated genome sequences (accession numbers CP042295, CP041663, CP041664, CP030141, CP030140, and CP030103) were deposited in GenBank.

The annotated genes associated with amino acid and carbohydrate metabolisms were present at similar percentages in the genomes of the *M. anserisalpingitidis* and *M. anatis*, and likewise in the *M. anseris* and *M. cloacale* type strains.

Whole genome alignment of the type strains of *M. anserisalpingitidis* and *M. anatis* revealed longer locally collinear blocks compared to the *M. anseris* and *M. cloacale* alignment.

Species-specific PCR assays

Eight mycoplasma housekeeping genes were found suitable for the design of species-specific primers, and 17 primer combinations were tested in the study. PCR assays, which did not demonstrate any cross-amplification with other tested *Mycoplasma* strains and showed superior sensitivity, were selected as the final assays. The *rpoB* gene-based PCR assay was suitable for precise identification of *M. anserisalpingitidis* (sensitivity of 10^2 genomic equivalents (GE) per reaction), the *dnaX* gene-based PCR assays were the most suitable for precise identification of *M. anatis* and *M. cloacale* (sensitivity: 10^2 GE for both reactions), and the *pcrA* gene-based PCR assay was accepted for species identification of *M. anseris* (sensitivity: 10^1 GE). The presence of multiple waterfowl *Mycoplasma* DNAs did not have any effect on the assays' performance.

The *Mycoplasma* genus-specific PCR revealed two amplicons at the corresponding molecular weights when *M. anserisalpingitidis* / *M. anatis* and *M. anseris* / *M. cloacale* co-occurred in the clinical specimens; however, neither the number of PCR products nor the DNA sequence chromatograms indicated mixed infection in several samples. The designed species-

specific PCR assays identified the mycoplasmas to species level in these samples.

MLST study on *M. anserisalpingitidis*

According to the acceptance criteria for the housekeeping genes, the loci of the *atpG*, *fusA*, *pgiB*, *plsY*, and *uvrA* genes were selected for the finally MLST scheme.

The 89 *M. anserisalpingitidis* samples yielded 76 unique STs with a 0.994 Simpson's index of diversity. A 'PubMLST' database has been set up for this MLST scheme (https://pubmlst.org/manserisalpingitidis/). Phylogenetic tree constructed from the concatenated nucleotide sequences of all five loci of *M. anserisalpingitidis* samples showed highly congruent topology with the samples' background data and revealed three clades (clade A-C), and six subclades within clade C.

Sequence analysis revealed that field isolates in clade A showed 100% sequence similarity with the *M. anatis* samples for the *plsY* locus, and these samples were located closest to the *M. anatis* outgroup. Clade B and subclade 1C contained Hungarian isolates mostly from Eastern Hungary. The Ukrainian sample and a Hungarian ST formed subclade 2C. The ATCC BAA-2147 type strain was placed into subclade 3C. Subclade 4C comprised field

isolates originating from several parts of Hungary. Subclade 5C comprised three Hungarian, two Chinese, and a Vietnamese *M. anserisalpingitidis* isolates. In the phylogenetic tree, this subclade showed the highest genetic distance among the subclades of clade C. Subclade 6C contained all Polish samples, and a Hungarian ST. The examined Hungarian livestock integration's samples were divided into three (sub)clades (A, B and 1C).

Discussion

Antibiotic susceptibility profile of *M. anserisalpingitidis*

The detected MIC values were higher than the ones reported in the previous paper for fluoroquinolones, confirming the observation of increasing quinolone-resistance in *Mycoplasma* species. All field isolates showed elevated MIC values for spectinomycin and lincomycin. The combination of the two antibiotics improved their effectiveness. Although the *M. anserisalpingitidis* field isolates showed broad ranges of MIC values for oxytetracycline and doxycycline, MIC_{90} values exceeded the highest concentration ranges. These results revealed the presence of probably highly tetracycline-resistant field isolates in Hungary. The MIC_{50} of tylosin was higher than the value reported in the previous study. High variability was observed in the susceptibility of the field isolates to tylosin and tilmicosin; however, the MIC_{90} values exceeded the concentration ranges used in the experiment. Out of the three macrolides, tylvalosin proved to be the most effective agent against *M. anserisalpingitidis* field isolates. In the present study, pleuromutilins were found to be the most effective antibiotic agents among the examined compounds, especially

valnemulin showed high *in vitro* effectiveness against all tested isolates. Most of the *M. anserisalpingitidis* field isolates yielded the same MIC values for florfenicol.

The elevated MIC values of several antibiotics detected in subsequent isolates from the same farms are likely in association with the inconsistent use of antibiotics and the rapid development of antibiotic resistance. Our results highlight the consistent use of antibiotics and the need for determination of antibiotic susceptibility of *M. anserisalpingitidis* before treatment.

De novo **genomes of waterfowl** ***Mycoplasma*** **strains/isolates**

The size and the low G+C content of the genomes of the *M. anserisalpingitidis*, *M. anatis*, *M. anseris*, and *M. cloacale* type strains/isolates was characteristic to mycoplasmas.

The number of genes associated with amino acid and carbohydrate metabolisms was in accordance with the glucose-splitting metabolism of *M. anserisalpingitidis* and *M. anatis*, and with the arginine utilization of *M. anseris* and *M. cloacale*.

The observed high coverage between the genomes of the *M. anserisalpingitidis* and *M. anatis* type strains supports the theory that these mycoplasmas may have

separated from a common ancestor and they are closely related. The genomes of the *M. anseris* and *M. cloacale* type strains also share homologous regions; however, their intense rearrangements also suggest that these species are more distinct and separated from each other.

These publicly available records for *M. anserisalpingitidis*, *M. anatis*, *M. anseris*, and *M. cloacale* could be the basis for further scientific projects with worldwide relevance and interest.

Species-specific PCR assays

In contrast to the results of the genus-specific PCR assay, we were able to detect and identify all concerned waterfowl *Mycoplasma* species in mixed clinical specimens.

The PCR assays developed in this study were able to confirm and/or identify these *Mycoplasma* species in new or rarely-observed waterfowl hosts as well. According to the available literature, *M. anserisalpingitidis* has been isolated only from geese before; however, we described this species from a domestic duck for the first time. *M. anatis* normally colonises ducks and it was rarely detected in geese; however, *M. anatis* isolates originated from geese were confirmed in our sample collection.

The developed PCR assays showed high specificity and sensitivity, enabling rapid, precise and reliable identification of *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale*, and therefore proved to be a suitable and cost-effective method in routine veterinary laboratory diagnostics.

MLST study on *M. anserisalpingitidis*

In the *M. anserisalpingitidis* MLST study, *M. anatis* was used as an outgroup because of the observed genome similarity between these bacteria. The observation, that some *M. anserisalpingitidis* field isolates' *plsY* locus is highly similar to the *plsY* locus of *M. anatis* is in accordance with the theory of a common ancestor.

The novel MLST scheme distinguished numerous STs, and the detected high variability is in accordance with the observation, that mycoplasmas are some of the fastest evolving organisms. Nevertheless, despite the high number of unique STs, samples originating from the same geographical locations (e.g. isolates from Poland, or Hungarian farms not part of the examined integration) showed close genetic relationship and clustered together in the phylogenetic tree. The high variability observed among the Hungarian livestock integration's samples and their separation into different (sub)clades could be

explained by the horizontal transmission of this infectious agent.

Subclade 5C comprised the Asian field isolates, and interestingly, a few Hungarian samples. The similarity between the Chinese and the Hungarian isolates was also confirmed in a comprehensive genomic study by our research group. As *M. anatis* and *M. cloacale* were already described in wild ducks, it could not be precluded that *M. anserisalpingitidis* may spread by animal migration as well.

The novel MLST scheme was found to be an adequate method to differentiate *M. anserisalpingitidis* samples, and it can be a useful genotyping tool for phylogenetic studies and future epidemiological investigations.

Overview of the new scientific results

Ad 1. Detailed antibiotic susceptibility profiles of *M. anserisalpingitidis* strains/field isolates were defined for the first time. Based on our *in vitro* examinations of thirteen antibiotics and a combination, tiamulin and valnemulin from the pleuromutilins family and the macrolide type tylvalosin proved to be the most effective drugs for the therapy of *M. anserisalpingitidis* infections in Hungary. However, isolates with elevated MIC values were detected, highlighting the importance of the susceptibility testing before treatment.

Ad 2. The type strain of *M. anserisalpingitidis* (ATCC BAA-2147) and two field isolates (MYCAV 93 and MYCAV 177), and the type strains of *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234) and *M. cloacale* (NCTC 10199) were *de novo* sequenced. The records of the complete genomes were deposited in GenBank, promoting the research of waterfowl mycoplasmosis as these were the first available complete genomes for these species.

Ad 3. Species-specific PCR assays were designed for the most common waterfowl *Mycoplasma* species. These assays were the first published for *M. anatis*, *M. anseris* and *M. cloacale*, and the first established concerning the specificity and sensitivity for *M. anserisalpingitidis*. The

tests promote the rapid, single and reliable identification of the species even from DNA samples extracted from clinical specimens without the need of prior *Mycoplasma* culture. With the help of the *M. anserisalpingitidis*-specific assay, this species was detected from a duck, a previously unpublished host animal.

Ad 4. We reported seventy-nine *M. anserisalpingitidis* draft assembly from the whole genome sequencing datasets, and the previously published three complete genomes were also included in a phylogenetic study. After comprehensive *in silico* and *in vitro* examination of housekeeping genes, an MLST assay was firstly designed for *M. anserisalpingitidis*. The five loci based assay divided altogether 89 *M. anserisalpingitidis* samples into 76 STs, showing wide diversity for this species. A web-accessible phylogenetic database was established for this *Mycoplasma* species by our research group. The developed MLST scheme constitutes a universal tool for global, long-term screening of dissemination for *M. anserisalpingitidis*, promoting the phylogenetic and epidemiological investigation of this species.

Scientific publications

Publications in the topic of the dissertation in peer-reviewed journals:

Grózner, D., Kovács, Á.B., Wehmann, E., Kreizinger, Z., Bekő, K., Mitter, A., Sawicka, A., Jánosi, S., Tomczyk, G., Morrow, C.J., Bányai, K., Gyuranecz, M.: **Multilocus sequence typing of the goose pathogen *Mycoplasma anserisalpingitidis***, Vet. Microbiol. 254, 108972, 2021.

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