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Genetic diversity and antibiotic resistance of *Mycoplasma hyopneumoniae* isolates

Ph. D. thesis

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Introduction

Myoplasma hyopneumoniae is a member of the class Mollicutes causing significant economic losses to the swine industry worldwide through mycoplasma pneumonia or porcine respiratory disease complex (PRDC). The main routes of the infection are the direct contact of the animals and airborne transmission (Artiushin and Minion, 1996; Maes *et al.*, 1996, 2008). Genotyping assays can help to trace the spread of the infection during epidemiologic investigations, therefore they can contribute to the inhibition of further infections.

The most important possibilities of prevention and elimination are vaccination and antibiotic treatment of the animals (Maes *et al.* 1996). The commercially available vaccines are mostly bacterin type vaccines, which are able to moderate the clinical signs caused by *M. hyopneumoniae,* however they cannot completely inhibit the bacterial colonisation (Meyns *et al.,* 2006).

Although most of the antimicrobial agents are able to treat *M. hyopneumoniae* infections, some groups, which interfere with cell-wall synthesis or the sulphonamides, have no effect because of the natural resistance of the bacterium (Taylor-Robinson and Bébéar, 1997). However, acquired resistance was also described against fluoroquinolones, macrolides and lincosamides (Gautier-Bouchardon, 2018). For the appropriate choice of the antimicrobial agent, it is necessary to accomplish the antibiotic susceptibility testing of the strains circulating in the herds. However, the conventional microbroth dilution method in case of *M. hyopneumoniae* (Hannan, 2000) is not performed routinely because of the fastidious and time-consuming culturing process. In the practice the antibiotics are rather chosen empirically, increasing the probability of inappropriate antibiotic usage, which may lead to emerging resistance.

Fluoroquinolone type antibiotics act by binding to the DNA gyrase and topoisomerase IV enzymes (encoded by *gyrA* and *parC* genes), and mutations have been identified in these genes which show correlation with the decreased antibiotic susceptibility of *M. hyopneumoniae* strains (Le Carrou *et al.,* 2006; Vicca *et al.,* 2007). Macrolide and lincosamide type antibiotics act by binding to the 50S ribosomal subunit, and accordingly mutations in the 23S rRNA sequence were described in correlation with decreased antibiotic effectiveness (Gautier-Bouchardon, 2018). Molecular biological tests discriminating genetic markers enable the fast and cost-effective differentiation of sensitive and resistant genotypes.

Aims of the study

The aims of the study were:

Ad 1. to establish a *M. hyopneumoniae* strain collection as the bases of the examinations involved in this study;

Ad 2. to perform the genotyping of 44 *M. hyopneumoniae* isolates based on multi-locus sequence typing (MLST) and multi-locus variable number tandem repeat analysis (MLVA) assays and the analyses of gene *p146*, in order to evaluate and compare the used molecular typing systems;

Ad 3. to determine the *in vitro* susceptibility profiles of 44 *M. hyopneumoniae* isolates to 15 antibiotics;

Ad 4. to investigate the genetic background of increased MIC values of fluoroquinolone, macrolide and lincosamide type antibiotics;

Ad 5. to develop rapid and cost-effective PCR based assays for the determination of antibiotic susceptibility profile of *M. hyopneumoniae* strains in the case of fluoroquinolones, macrolides and lincosamides.

Materials and methods

M. hyopneumoniae isolates

The *M. hyopneumoniae* type strain (strain J, NCTC 10110) and 44 clinical strains were involved in the examinations. The strains originated from porcine lung samples with typical pulmonic lesions which were collected between 2015 and 2016 from different Hungarian slaughterhouses. Friis broth medium was used for the isolation (37°C, 5% CO_2) and incubated for 4-6 weeks. Species specific PCR, targeting the 16S rRNA sequence, was performed to check the presence of *M. hyopneumoniae* (Mattsson *et al.*, 1995) in the broth cultures. A universal mycoplasma PCR system, targeting the 16S/23S rRNA intergenic spacer region, was accomplished to exclude the presence of other contaminating *Mycoplasmas* (Lauermann *et al.*, 1995).

Genotyping

Multi-locus sequence typing (MLST) systems based on three (*adk, rpoB* és *tpiA*) and seven (*efp, metG, pgiB, recA, adk, rpoB* és *tpiA*) housekeeping genes were performed for the phylogenetic analysis of the *M. hyopneumoniae* isolates (Mayor *et al.*, 2008). For further resolution of the isolates different combination of four tandem repeat regions were investigated by MLVA (Locus-1, -2 and P97-RR1, -RR2) (Vranckx *et al.*, 2011; Charlebois *et al.*, 2014). The different genotyping methods were extended with the sequence analysis of an adhesion-like protein encoding gene, *p146* which also involves a serine-repeat region (Mayor *et al.*¸2007*)*.

The discriminatory power of the typing methods was determined using Simpson's index of diversity (Hunter and Gaston, 1988). The quantitative level of congruence and the interchangeability between typing methods were calculated based on the data of the isolates analysed with all methods using the adjusted Rand and Wallace coefficients (Carriço *et al.*, 2006; Severiano *et al.*, 2011).

Antibiotic susceptibility testing

Antibiotic susceptibility profiles of 44 *M. hyopneumoniae* strains and the type strain (strain J) were defined by microbroth dilution method (Hannan, 2000), for 15 members of eight antibiotic groups (fluoroquinolones (enrofloxacin, marbofloxacin); tetracyclines (oxytetracycline, doxycycline); aminocyclitols (spectinomycin); aminoglycosides (gentamicin); macrolides (tylosin, tilmicosin, tylvalosin, tulathromycin, gamithromycin); lincosamides (lincomycin); pleuromutilins (tiamulin, valnemulin) and phenicols (florfenicol).

Initial minimum inhibitory concentration (MIC) values were recorded when colour change of the growth control was visible (4-14 days after inoculation), and final MIC values were registered when no further colour change was observed. In the absence of official breakpoints, the observed MIC values were evaluated based on previously published, unofficial breakpoints.

Identification of mutations responsible for antibiotic resistance

Whole genome sequencing of the members of the strain collection was accomplished on IonTorrent platform (Rónai *et* al., 2015). Sequence analysis was performed in order to reveal the genetic background of the increased MIC values observed during the *in vitro* antibiotic susceptibility tests. The examined genes were selected according to the literature, showing correlation with decreased fluoroquinolone (*gyrA, gyrB, parC, parE*), macrolide and lincosamide (23S rRNS) susceptibility (Le Carrou *et al*., 2006; Vicca *et al*., 2007; Gautier-Bouchardon, 2018). The nucleotide sequences of strains having higher and lower MIC values were compared. The presence of mutations resulting in amino acid alterations in the examined genes were confirmed by Sanger sequencing.

Development of molecular assays

Mismatch amplification mutation assay (MAMA) and high resolution melt (HRM) assay were developed for the detection of single nucleotide polymorphisms (SNPs) resulting in increased MIC values of fluoroquinolones, macrolides and lincosamides. The designed assays are able to identify one single nucleotide alteration either by competing primers of different sizes or by detecting the different melting temperatures (Tm) of the amplicons. For the validation of the tests all members of the strain collection, with known antibiotic susceptibility, were involved. Furthermore, the specificity of the assays and the presence of cross-reactions were checked using other porcine *Mycoplasma* species.

Results

Genotyping

High variability of the studied *M. hyopneumoniae* isolates was observed by all genotyping assays. Most of the strains with common herd of origin clustered together, however exceptions were also found, when isolates from the same sampling clustered separately. The minimal MLST and the conventional scheme showed congruency. The Simpson's index of diversity of the MLST systems were the same (0.907), clustering the isolates into 27 different sequence types. The additional sequence analysis of the gene *p146* increased the discriminatory power of the MLST assays (Simpson's index: 0.977), differentiating 33 types. The MLVA and MLST assays and the sequence analyses of gene p146 generally showed low congruency, while variations and extended versions of the assays were congruent within the typing systems. The MLVA assays based on two or four loci, extended with the serine repeat analysis of gene *p146* showed the highest interchangeability with the other methods, and showed the highest discriminatory power (Simpson's index: 0.992), resulting in 40 different genotypes of the examined *M. hyopneumoniae* strains.

Antibiotic susceptibility testing

The initial MIC values are evaluated and discussed throughout the study (Hannan, 2000), and according to our results all of the studied antimicrobial agents were effective against the majority of the examined *M. hyopneumoniae* isolates. However, one strain was inhibited by significantly higher concentration of fluoroquinolones (2.5-5 µg/ml), and several strains showed increased final MIC values (1.25-10 µg/ml). Difference was also defined between the antibiotic concentrations inhibiting the growth of 50% and 90% of the isolates (≤0.039 µg/ml and 1.25-2.5 µg/ml). Low MIC values of tetracyclines (≤0,039-4 µg/ml), aminoglycosides and aminocyclitols (≤0,25-4 µg/ml), pleuromutilines (≤0,039-0,156 µg/ml) and phenicols (≤0,125-2 µg/ml) were observed. Low concentrations of the macrolides (≤0,25-8 µg/ml) and lincomycin (≤0,25-0,5 µg/ml) were effective against most of the isolates. However, one strain showed significantly increased MIC values of macrolides and lincomycin (2- >64 µg/ml).

Identification of mutations responsible for antibiotic resistance

Amino acid alterations, showing correlation with decreased fluoroquinolone susceptibility, were found in the *gyrA* (Gly81Ala, Ala83Val, Glu87Gly) and *parC* (Ser80Phe, Ser80Tyr, Asp84Asn) genes, but not in the genes *gyrB* and *parE*. Single alterations in the *parC* gene seem to have no crucial effect on fluoroquinolone susceptibility (range of initial MIC values belonging to strains with one alteration: ≤0.039-0.625 µg/ml), however it may result in the increase of the final MIC values (0.312-2.5 µg/ml). Double substitutions in the *gyrA* and *parC* genes clearly showed correlation with the increase of the initial and final MIC values of fluoroquinolones (0.625-5 µg/ml and 1.25-10 µg/ml, respectively). The nucleotide substitution A2059G in the 23S rRNA sequence was found in a *M. hyopneumoniae* strain showing extremely increased initial (macrolides: 2->64 µg/ml, lincosamides: >64 µg/ml) and final (macrolides: 8->64 µg/ml, lincosamides: >64 µg/ml) MIC values of macrolides and lincosamides.

Development of molecular assays

All of the designed MAMA and HRM assays clearly differentiated the sensitive (low MIC values) and resistant (increased MIC values) genotypes of the studied *M. hyopneumoniae* strains. Two MAMAs and an HRM assay were developed for the detection of the nucleotide substitutions (C239T/A and G250A, according to *E. coli* numbering) showing correlation with decreased fluoroquinolone susceptibility.

The sensitivity of the melt- and agarose-MAMA systems targeting the C239T/A alteration was 10^4 template copy numbers (copies) for the SNP containing genotype and 10^3 copies for the sensitive genotype. The sensitivity of the melt- and agarose-MAMA systems targeting the G250A substitution was 10^2-10^3 copies for the resistant and 10^3 copies for the sensitive genotype. A non-specific melt curve (different melting temperature compared to that of the sensitive or resistant genotypes) was observed in the presence of *M. flocculare* in case of the examination of the G250A nucleotide substitution. The HRM assay successfully differentiated the sensitive genotype and two resistant genotypes. The sensitivity of the assay was 10^5 copies for the sensitive and $10^{5\text{-}6}$ copies for the SNP containing genotypes.

Agarose- and melt-MAMA systems were designed for the detection of A2059G nucleotide substitution in the 23S rRNA sequence, showing correlation with increased MIC values of macrolides and lincosamides. The sensitivity of the agarose-MAMA was 10^3 copies for the sensitive and 10^2-10^3 copies for the resistant genotypes, while the sensitivity of the melt-MAMA was 10^4 copies for both genotypes. Cross-reaction was observed in the presence of *M. hyorhinis* and *M. flocculare*.

Discussion

Genotyping

This was the first time that the phylogenetic relationships of Hungarian *M. hyopneumoniae* isolates were examined. The molecular typing of the members of the strain collection was accomplished by MLST and MLVA systems, and by their extension with the sequence analysis of the gene *p146*, furthermore, the genotyping assays were compared. All of the assays showed high heterogeneity of the strains, in congruence with previous observations (de Castro *et al.,* 2006; Calus *et al.,* 2007). Our results confirmed, that the MLST assay based on the genes *adk*, *rpoB* and *tpiA* has the same resolution power as the seven gene-based MLST (Mayor *et al.,* 2008; Kuhnert *et al.*, 2011), therefore it is appropriate for phylogenetic analyses. The discriminatory power of the MLST assays was increased by the addition of the sequence analysis of gene *p146*, in accordance with a previous observation (Overesch and Kuhnert, 2017). The MLVA assay extended with the serine repeat analysis of the gene *p146* provided the most refined data. The method was suitable for the further resolution of isolates with common MLST sequence types and different herd of origin. Therefore, the MLVA method provides a fast and cost-effective option for epizootiologic investigations (Charlebois *et al.,* 2014).

Antibiotic susceptibility testing

Antibiotic susceptibility testing of the circulating *M. hyopneumoniae* strains is usually not part of the routine diagnostics, because it is fastidious and time-consuming (Hannan, 2000). Furthermore, official standards are available only for certain human pathogen *Mycoplasma* species, therefore the lack of official standards for *M. hyopneumoniae* makes the interpretation of the results complicated (Wayne, 2011).

Although all of the examined antibiotics were effective against the members of the strain collection, declined susceptibility was observed in the case of fluoroquinolones. Similar results were previously defined in Thailand and Belgium (Vicca *et al.,* 2004; Thongkamkoon *et al.,* 2013). The decreasing susceptibility against fluoroquinolones is a notable problem, because these agents are important antibiotics for human therapy (Collignon *et al.,* 2009).

Extremely high MIC values were defined in case of macrolides and lincosamides, which are among the most frequently used antibiotics in the swine industry (Maes *et al.,* 2008). Although macrolides and lincosamides are chemically distinct from each other, cross-resistance can be observed due to the same mode of action against bacterial metabolism (Weisblum, 1995). Similarly to our results, macrolide and lincosamide resistance was previously reported in *M. hyopneumoniae* from different countries of Europe and Asia (Vicca *et al.,* 2004; Thongkamkoon *et al.,* 2013; Tavío *et al.,* 2014). The increased MIC values support the importance of regularly accomplished antibiotic susceptibility testing, enabling appropriate antibiotic usage and moderating the emergence of resistant bacteria.

Identification of mutations responsible for antibiotic resistance

Fluoroquinolones and macrolides are among the most frequently utilised antibiotic agents to control mycoplasma pneumonia in Hungary (EMA, 2015). They play an important role in reducing clinical signs, however the inappropriate usage may lead to emerging resistance (Maes *et al.,* 1996, 2008). Fluoroquinolone type antibiotics inhibit the bacterial metabolism through binding to the DNA gyrase (*gyrA, gyrB*) and topoisomerase IV (*parC, parE*) enzymes. No amino acid substitutions, correlating with increased MIC values, were observed in the genes *gyrB* and *parE*, corroborating earlier publications (Vicca *et al.,* 2007). Although single amino acid substitutions in the *parC* gene resulted in decreased fluoroquinolone susceptibility, the decrease was more significant in the simultaneous presence of alterations in the *gyrA* gene (Le Carrou *et al.,* 2006; Vicca *et al.,* 2007; Gautier-Bouchardon, 2018). Macrolide and lincosamide type antibiotics act by binding to the 23S rRNA sequence. Increased MIC values of macrolides and lincosamides were observed in case of alteration in the nucleotide positions 2057-2059 in mycoplasmas (Kobayashi *et al.,* 2005; Stakenborg *et al.,* 2005; Sulyok *et al.,* 2017; Gautier-Bouchardon, 2018). Accordingly, an A2059G substitution of the 23S rRNA sequence of the macrolide and lincosamide resistant *M. hyopneumoniae* strain was described in our study.

Development of molecular assays

MAMA and HRM assays were developed for the detection of SNPs correlating with decreased fluoroquinolone, macrolide and lincosamide susceptibility, to complement the time-consuming and fastidious culture-based susceptibility testing (Hannan, 2000). Overlapping regions were used in the MAMA and HRM systems, which increases the reliability of the assays. Agarose-MAMA systems provide an available tool for most of the laboratories for PCR-based susceptibility testing, while HRM, in spite of the special laboratory equipment, is able to investigate multiple nucleotide positions in one single real-time PCR test. Our tests provide an opportunity for the fast and cost-effective detection of fluoroquinolone, macrolide and lincosamide resistance, supporting targeted antibiotic therapy.

Overview of the new scientific results

Ad 1. Comparative genetic analysis of the Hungarian isolates was performed. MLVA extended with the gene *p146* can successfully resolve MLST sequence types, therefore it is useful for epizootic and phylogenetic investigations.

Ad 2. Antibiotic susceptibility profiles of the Hungarian *M. hyopneumoniae* isolates was defined for 15 antimicrobial agents for the first time. Although all of the agents were effective against the examined isolates, declined susceptibility was also defined against fluoroquinolones, macrolides and lincosamides.

Ad 3. Mutations of the *parC* and *gyrA* genes were defined in *M. hyopneumoniae,* showing correlation with decreased fluoroquinolone susceptibility, while mutation in the 23S rRNA sequence was also detected showing correlation with extremely high MIC values of macrolides and lincosamides.

Ad 4. MAMA and HRM assays were designed for the rapid and cost-effective detection of single nucleotide polymorphisms correlating with decreased fluoroquinolone, macrolide and lincosamide susceptibility in *M. hyopneumoniae* isolates.

Scientific publications

In peer-reviewed journals

1. **Felde O.**, Kreizinger Z., Sulyok K. M., Hrivnák V., Kiss K., Jerzsele Á., Biksi I., Gyuranecz M.: **Antibiotic susceptibility testing of *Mycoplasma hyopneumoniae* field isolates from Central Europe for fifteen antibiotics by microbroth dilution method**, Plos ONE 13:12p e0209030, 2018
2. **Felde O.**, Kreizinger Z., Sulyok K.M., Marton S., Bányai K., Korbuly K., Kiss K., Biksi I., Gyuranecz M.: **Genotyping *Mycoplasma hyopneumoniae* isolates based on multi-locus sequence typing, multi-locus variable number tandem repeat analysis and analysing gene p146** Vet. Mic. 222. 85-90, 2018.
3. **Felde O.**, Kiss K., Biksi I., Jerzsele Á., Gyuranecz M.: **A sertések *Mycoplasma hyopneumoniae* okozta tüdőgyulladása**, Magy Állatorvosok Lapja 140. 337-348, 2018.

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8. Kiss O., **Felde O.**, Moskát C.: **A mozaikgyepek szerepe a szalakóta (Coracias garrulus) táplálkozó területeinek megőrzésében.** Természetvédelmi Közlemények. 18 pp. 276-282, 2012

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