University of Veterinary Medicine
Doctoral School of Veterinary Science

Genetic diversity and antibiotic resistance of *Mycoplasma hyopneumoniae* isolates

Ph. D. thesis

Stammné Felde Orsolya

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Supervisor and consultants:

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Miklós Gyuranecz, Ph.D.
Institute for Veterinary Medical Research
Centre for Agricultural Research
Hungarian Academy of Sciences
Supervisor

Ádám Dán, Ph.D.
Veterinary Diagnostic Directorate
National Food Chain Safety Office
Consultant

Krisztián Bányai, Ph.D.
Institute for Veterinary Medical Research
Centre for Agricultural Research
Hungarian Academy of Sciences
Consultant

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Stammné Felde Orsolya

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Abbreviations

AFLP	amplified fragment length polymorphism
ATCC	American Type Culture Collection
bp	base pair
CI	confidence interval
CCU	colour changing unit
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ELISA	enzyme-linked immunosorbent assay
gyrA	gene of DNA gyrase, A subunit
gyrB	gene of DNA gyrase, B subunit
HRM	high resolution melt
MAMA	mismatch amplification mutation assay
MIC	minimum inhibitory concentration
MIC50	minimum inhibitory concentration that inhibits 50% of the isolates
MIC90	minimum inhibitory concentration that inhibits 90% of the isolates
MLST	multi-locus sequence typing
MLVA	multi-locus variable-number tandem repeat analysis
parC	gene of DNA topoisomerase IV, A subunit
parE	gene of DNA topoisomerase IV, B subunit
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PRDC	porcine respiratory disease complex
QRDR	quinolone resistance-determining region
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SNP	single nucleotide polymorphism
ST	sequence type
T_{melt}	melting temperature
VNTR	variable number tandem repeat

1. Summary

Mycoplasma hyopneumoniae is prevalent worldwide, causing significant economic losses through induction of enzootic pneumonia or porcine respiratory disease complex (PRDC). The major ways of the spread of the bacterium are direct contact of the animals, contaminated vehicles shuttling between certain swine herds, and the airborne transmission.

A *M. hyopneumoniae* strain collection was established between 2015 and 2016 including 44 isolates originating from lung samples collected in Hungarian slaughterhouses. Molecular biological methods used for epizootiologic investigations can help to moderate the spread of the pathogen by revealing the route of the infection. Multi-locus sequence typing (MLST) using housekeeping genes, multi-locus variable-number tandem repeat analysis (MLVA) utilising tandem repeat regions and analysis of the nucleotide sequence of the adhesin-like protein encoding *p146* gene are all capable assays to study the relationship of the highly heterogeneous *M. hyopneumoniae* strains. Genotyping of the Hungarian *M. hyopneumoniae* isolates and comparison of genotyping assays of the literature has not been accomplished so far. Therefore, the 44 members of the *M. hyopneumoniae* strain collection were genotyped by MLST, MLVA and by the sequence analysis of the *p146* gene, then the data were applied for the comparison of the utilised assays and their combinations. All of the assays described high heterogeneity of the isolates. While most of the isolates with the same herd of origin were genetically the same or closely related, within herd differences were also found. MLVA extended with the analysis of the nucleotide sequence of the gene *p146* had the highest discriminatory power, and proved to be suitable for epizootiologic purposes. Furthermore, our results support the use of three genes-based MLST extended with the analysis of gene *p146* for phylogenetic investigations.

Antibiotic treatment of infected animals plays an important role in moderating the signs of porcine mycoplasma pneumonia. Determination of the minimum inhibitory concentration (MIC) can improve targeted antibiotic treatment. Since isolation of *M. hyopneumoniae* is time-consuming and fastidious, the choice of the antimicrobial agent is usually not supported by susceptibility testing, but is made empirically. Consequentially, inappropriate usage of antimicrobials can lead to emerging resistance. According to our knowledge, this was the first study describing the *in vitro* susceptibility profile of Hungarian *M. hyopneumoniae* isolates against 15 members of eight antibiotic groups. Although all of the examined agents were effective against the isolates, increased MIC values of fluoroquinolones, macrolides and lincosamides were defined. The regularly accomplished antibiotic susceptibility testing of the swine herds should enable appropriate antibiotic use during treatment.

Revealing the genetic background of antibiotic resistance enables the development of polymerase chain reaction (PCR) based assays targeting resistance-associated genetic

markers. These assays would represent rapid and cost-effective alternatives for the time-consuming microbroth dilution method by avoiding the isolation process. In this study nucleotide sequence analyses of the genes described in the literature to be in correlation with fluoroquinolone, macrolide and lincosamide resistance were accomplished. Sequence data from whole genome sequencing of the 44 *M. hyopneumoniae* isolates previously submitted for MIC determination were examined. Single nucleotide polymorphisms (SNPs) in the DNA gyrase and topoisomerase IV subunit A (*gyrA* and *parC*) responsible for decreased susceptibility to fluoroquinolones were found. Furthermore, point mutation in the 23S rRNA sequence, correlating with macrolide and lincosamide resistance was observed in the sequence of a multi-resistant strain. Mismatch amplification mutation assays (MAMAs) and high resolution melt (HRM) analysis were developed for the determination of point mutations in the *parC* gene and 23S rRNA sequence, correlating with raised MIC values of fluoroquinolones, macrolides and lincosamides. Since the MAMA method can be performed both on basic real-time PCR platform and in conventional gel electrophoresis based assays, it can be part of routine diagnostics in laboratories with basic equipment.

Összefoglalás

A *Mycoplasma hyopneumoniae* egy világszerte elterjedt baktérium, mely súlyos gazdasági károkat okozhat több légzőszervi kórkép, így a mycoplasma pneumonia vagy a sertések légzőszervi betegség komplexének (PRDC) kialakítása révén. A baktérium terjedésének főbb módjai az állatok közvetlen érintkezése mellett, az egyes telepek között ingázó kontaminált járművek, illetve a baktérium levegő útján való terjedése is lehet.

Magyarországi vágóhidakon gyűjtött tüdő minták segítségével saját *M. hyopneumoniae* törzsgyűjteményt állítottunk fel 2015 és 2016 években, mely negyvennégy izolátumot foglal magába. A járványtani vizsgálatok során alkalmazott molekuláris biológiai módszerek elősegíthetik a járvány megállítását a kórokozó terjedési útvonalának feltárásán keresztül. A nagy változatosságot mutató *M. hyopneumoniae* törzsek rokonsági kapcsolatainak megismerésére a háztartási géneket vizsgáló multi-lókusz szekvencia tipizálás (MLST), a tandem ismétlődő régiókat vizsgáló módszer (multi-locus variable-number tandem repeat analysis, MLVA) és a sejtfelszíni adhezin-szerű fehérjét kódoló *p146* gén szekvenciájának vizsgálata egyaránt alkalmas. Mivel a hazai *M. hyopneumoniae* populáció genetikai sokféleségéről nem rendelkeztünk adatokkal, ezért munkánk során célul tűztük ki a hazai izolátumok összehasonlító genetikai vizsgálatát, továbbá a szakirodalmi adatok alapján fellelhető genotipizáló módszerek felbontóképességének egybevetését. A törzsgyűjtemény 44 tagját tipizáltuk MLST és MLVA rendszerekkel, valamint a *p146* gén szekvenciájának felhasználásával. Az így kapott adatokat továbbá felhasználtuk az egyes rendszerek és kombinációik összehasonlítására is. Mindegyik módszer nagy genetikai változatosságot mutatott ki. Míg az azonos állományból származó törzsek általában azonos vagy közeli rokon típusokba kerültek, egyes esetekben állományon belüli eltérést is leírtunk. A legnagyobb felbontással a *p146* gén szekvencia elemzésével kiegészített MLVA rendszer rendelkezett, mely megfelelő lehet járványtani nyomozásokhoz. Továbbá, eredményeink alátámasztják azt a korábbi felvetést, miszerint három háztartási gén vizsgálatán alapuló MLST rendszer, kiegészítve a *p146* gén vizsgálatával megfelelő lehet filogenetikai vizsgálatokra.

A fertőzött állatok antibiotikumos kezelése fontos szerepet játszik a sertések mycoplasma pneumóniája során kialakuló tünetek enyhítésében. A minimális gátló koncentráció (MIC) meghatározása hozzájárulhat a célzott antibiotikumos gyógykezeléshez. Mivel a *M. hyopneumoniae* izolálása idő- és anyagigényes, a kezelést általában nem előzi meg antibiotikum érzékenységi vizsgálat és a gyógyszer kiválasztása legtöbbször tapasztalati úton történik. A nem megfelelő antibiotikum használat azonban elősegítheti a rezisztens törzsek megjelenését. Vizsgálatunk során elsőként határoztuk meg a hazánkban jelenlévő *M. hyopneumoniae* törzsek *in vitro* antibiotikum érzékenységét nyolc antibiotikum csoport tizenöt tagjával szemben. Habár az összes vizsgált antibiotikum hatékonynak bizonyult az

izolátumokkal szemben, emelkedett MIC értékeket tapasztaltunk a fluorokinolonokkal, makrolidokkal és linkózamidokkal szemben. A sertés telepeken végzett rendszeres antibiotikum érzékenységi vizsgálat elősegítheti az állomány sikeres kezelését.

Az antibiotikum rezisztencia genetikai hátterének feltárása lehetőséget nyújt a polimeráz láncreakció (PCR) alapú antibiotikum érzékenységi tesztek fejlesztésére. Ezek a módszerek gyors és olcsó alternatívát nyújtanak a hosszadalmas, tenyésztést igénylő mikroleves hígításos módszerrel szemben. Kutatásunk során a szakirodalomban leírt flurokinolon, makrolid és linkózamid rezisztenciával összefüggésbe hozott gének szekvenciáit elemeztük. A vizsgálathoz a 44 *M. hyopneumoniae* izolátum teljes genom szekvenálásból származó szekvenciáit használtuk, melyek antibiotikum érzékenységi vizsgálatát korábban elvégeztük. Csökkent fluorokinolon érzékenységért felelős pontmutációkat találtunk a DNS-giráz és a topoizomeráz IV A alegységein (*gyrA* és *parC*). Továbbá a 23S rRNS szekvenciában leírt, makrolid és linkózamid rezisztenciával összefüggést mutató pontmutációt is megfigyeltünk egy többszörösen rezisztens törzs szekvenciájában. Mismatch amplification mutation assay (MAMA) és high resolution melt (HRM) rendszereket fejlesztettünk a *parC* génen és 23S rRNS szekvencián található, csökkent fluorokinolon, makrolid és linkózamid érzékenységgel összefüggésbe hozható pontmutációk gyors kimutatására. Mivel a MAMA vizsgálatokat úgy terveztük, hogy hagyományos PCR vizsgálatként gélelektroforézis segítségével is kiértékelhetőek legyenek, hozzájárulhatnak a széles körben elvégezhető rutin diagnosztikai vizsgálatokhoz.

2. Introduction

2.1. History and taxonomy

Mycoplasmas are the smallest procaryotes capable of self-replication, belonging to the family *Mycoplasmataceae* in the class Mollicutes (Tully *et al.*, 1993; Artiushin and Minion, 1996). *M. hyopneumoniae* usually have important role in the development of the porcine respiratory disease complex (PRDC) (Halbur, 1996) or mycoplasma pneumonia (enzootic pneumonia), a chronic respiratory disease, by the coinfection of secondary pathogens (Artiushin *et al.*, 1993; DeBey and Ross, 1994; Thacker and Minion, 2012). The disease has already been investigated in 1948 (Pullar, 1948) but the causative agent remained unknown until the 1960's, when it was successfully grown in tissue culture by Goodwin and Whittlestone (1963). In 1965 two bacterial strains, representing the causative agent of porcine mycoplasmal pneumonia, were independently deposited in the American Type Culture Collection (ATCC). One of them, strain 11 (ATCC 25617) was cultured by Switzer as *M. hyopneumoniae* (Maré and Switzer, 1965), the other, strain J (ATCC 25943) was cultured by Goodwin as *M. suipneumoniae* (Goodwin *et al*., 1965). The originally deposited *M. hyopneumoniae* strain with the description was lost, and had to be replaced by another culture from Switzer, therefore the description of the deposited strain became doubtful. As the two originally deposited strains of Switzer and Goodwin were serologically and biologically indistinguishable, strain J was proposed as the neotype for *M. hyopneumoniae* (Rose *et al*., 1979).
Other mycoplasmas found in swine are the pathogenic *M. hyorhinis* inducing polyserositis and arthritis, *M. hyosynoviae* also causing arthritis in grow-finishing pigs and the non-pathogenic *M. flocculare* (Thacker and Minion, 2012).

2.2. Aetiology

Mollicutes are prokaryotes of gram-positive lineage, that evolved from *Clostridium*-like bacteria by gene deletion (Caswell and Archambault, 2007). *Mycoplasmas* are the smallest, wall-less bacterial cells able to propagate in cell-free medium. *M. hyopneumoniae* has a small genome (897 405 base pairs) with a relatively low G+C content (28%) and a limited number of genes, lacking several biosynthetic pathways (Weisburg *et al.*, 1989; Pollack *et al*., 1997; Vasconcelos *et al.*, 2005; Thacker and Minion, 2012; Maes *et al.*, 2017). As a consequence of the absence of certain pathways, animal pathogen mycoplasmas obtain for example amino acids, purines, pyrimidines and membrane components from the host (Thacker and Minion, 2012). Glucose is oxidised during the metabolism of *M. hyopneumoniae*. As a typical feature of mycoplasmas, *M. hyopneumoniae* utilises UGA (a stop codon in other eubacteria) as a tryptophan codon, and

contains a minimal set of tRNA (Muto and Ushida, 2002; Minion *et al.*, 2004). In the absence of cell-wall, *Mycoplasmas* are sensitive to environmental conditions and inherently resistant to antibiotics which interfere with cell-wall synthesis (Maes *et al.*, 1996). The morphology of the bacterial cells is pleomorphic, mostly round or oval, with a medium diameter of 0.2 µm, bound by a simple, about 10 nm thick plasma membrane (Kobisch and Friis, 1996; Caswell and Archambault, 2007). The growth of *M. hyopneumoniae* is very slow (from 4-15 days up to 4-6 weeks), compared to that of other porcine mycoplasmas. Colonies on solid medium have a size up to approximately 0.5 mm, without the typical mycoplasmal central dark area (Kobisch and Friis, 1996) (Figure 1).

Figure 1. *M. hyopneumoniae* colonies on solid media
(The size range of a single colony is about 0-0.5 mm)

2.3. Geographic distribution

M. hyopneumoniae infection is prevalent almost all over the world and is responsible for tremendous economic losses (Kobisch and Friis, 1996; Maes *et al.*, 2008, 2017; Holst *et al*., 2015). Reporting of *M. hyopneumoniae* infections is not obligatory (Maes *et al.*, 2017); nevertheless, eradication of the pathogen has been aimed in several countries. Scandinavian countries seem to have controlled successfully the spread of the bacterium (Rautiainen *et al.*, 2001a; Rautiainen *et al.*, 2001b; Heinonen *et al.*, 2011), and the number of cases per year remained below 10 since 2015 (Overesch and Kuhnert, 2017). The prevalence of the bacterium was similar in the last decades in different parts of Europe (26.6% in Spain (Closa-Sebastià *et al.*, 2011), 30% in Italy (Chiari *et al.*, 2014), 21% in Slovenian (Vengust *et al*., 2006) and 33% at Gran Canaria (Assunção *et al.*, 2005), similar to earlier descriptions made in New Zealand (Macpherson and Hodges, 1985) and Canada (Baroch *et al*., 2015). Hungarian pig herds were found to be mostly infected, even though symptoms were not always observable (Stipkovits, 1997).

2.4. Epidemiology

According to our recent knowledge, domestic pigs and wild boars are the only hosts of *M. hyopneumoniae* (Thacker and Minion, 2012; Maes *et al.*, 2017). Although new born pigs are considered free from the pathogen, mostly growing and finishing age groups are concerned, but pigs of all ages are susceptible to the infection (Maes *et al.*, 1996, 2017; Calsamiglia and Pijoan, 2000; Sibila *et al.*, 2009; Nathues *et al.*, 2013). According to the literature, the infection can spread prior to the onset of coughing (Pieters *et al.*, 2009). Sows and piglets in the breeding herds have a reservoir role for *M. hyopneumoniae* in the production system, facilitating the circulation of the pathogen (Calsamiglia and Pijoan, 2000; Fano *et al*., 2005). The main route of transmission is direct contact, for example during the lactation period, when piglets are in contact with the sows shedding the pathogen (Maes *et al.*, 1996, 2008; Calsamiglia and Pijoan, 2000; Marois *et al.*, 2007), or between infected suckling pigs and susceptible litter- or pen mates (Maes *et al.*, 1996; Thacker and Minion, 2012). Nevertheless, several studies supported the hypothesis of airborne transmission of *M. hyopneumoniae* (Goodwin, 1985; Maes *et al.*, 1996; Stärk *et al*., 1998; Cardona *et al*., 2005; Fano *et al*., 2005; Otake *et al.*, 2010). Transmission via contaminated objects is not typical, but in certain conditions, the bacterium can survive for 10-17 days outside the host (Maes *et al.*, 1996; Stipkovits, 1997).

Stressful conditions and several risk factors (floor and air temperature, relative humidity, proximity of other farms and transport parking sites or frequent purchase of animals) can

facilitate the incidence of outbreaks. However, appropriate environmental conditions and management practice such as minimizing cross-fostering, limiting the length of lactation period or controlling the number of pen mates can moderate the colonization of piglets (Maes *et al.*, 1996, 2008; Hege *et al.*, 2002; Nathues *et al.*, 2013; Charlebois *et al.*, 2014; Pieters *et al.*, 2014; Vangroenweghe *et al.*, 2015). The development of the immunity through veterinary treatments like grinding piglets' teeth for better colostrum and milk uptake or avoiding anaemia at weaning time may help to evading the infection (Nathues *et al.*, 2013).

2.5. Pathogenesis

2.5.1. Adherence of *M. hyopneumoniae*

M. hyopneumoniae is primarily located on the mucosal surface of the trachea, bronchi and bronchioles (Blanchard *et al.*, 1992), but extraordinary re-isolations were achieved from brain (Friis, 1974), spleen, liver, kidneys and lymph nodes (Le Carrou *et al.*, 2006; Marois *et al.*, 2007). Electron microscopy confirmed the microcolony formation of *M. hyopneumoniae* at the tip of the cilia, in the interciliary space and in contact with the microvilli in the respiratory tract (Jacques *et al.*, 1992; Kobisch and Friis, 1996). Mycoplasmas stimulate the proliferation of lymphoid cells, resulting in a growing pressure of the lymphoid tissue and causing the obliteration of the lumen of bronchioles and the collapse of alveoli (Baskerville, 1981) (Figure 2). Exfoliation of epithelial cells and infiltration of the peribronchiolar and perivascular tissues by macrophages, B and T lymphocytes are characteristic consequences of the colonisation (Blanchard *et al.*, 1992; Kobisch *et al*., 1993; Kobisch and Friis, 1996; Maes *et al.*, 1996; Sarradell *et al.*, 2003).

No classical virulence factors are known to be responsible for cilia destruction by *M. hyopneumoniae*, toxic metabolites like H_2O_2 may be responsible instead (Maes *et al.*, 1996, 2017). Comparative genomic and proteomic analyses revealed some major differences between commensal and pathogenic porcine mycoplasmas and between pathogenic and attenuated *M. hyopneumoniae* strains (Pinto *et al.*, 2009; Liu *et al.*, 2013; Paes *et al.*, 2017), which is supported by the observation that the adherence of *M. flocculare* causes no or less damage to the epithelium compared to *M. hyopneumoniae* (Young *et al.*, 2000). However, the factors responsible for virulence and pathogenesis are still unknown (Maes *et al.*, 2017).

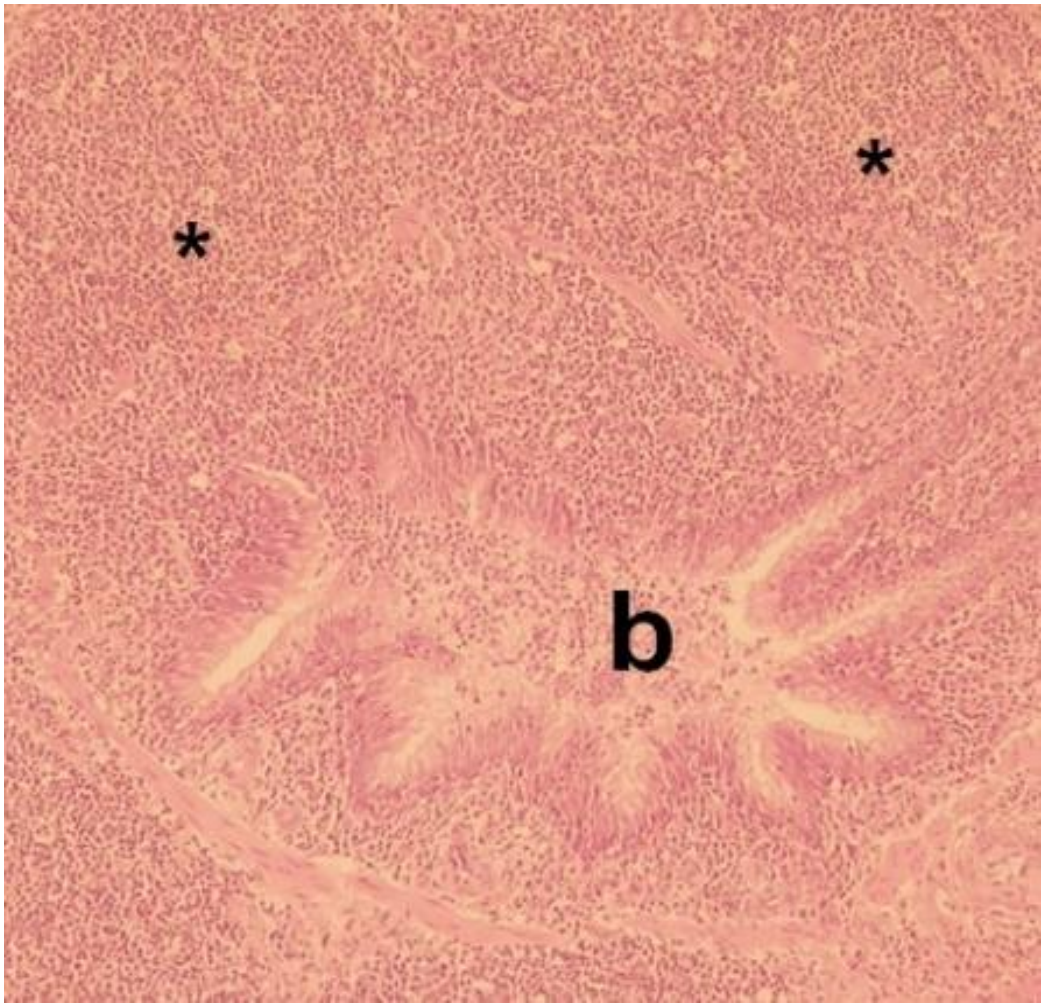

Figure 2. Histopathology of *M. hyopneumoniae* infected lung tissue
Abbreviations are: b-bronchioles, *-follicular BALT-hyperplasia, HE 40X (origin: Imre Biksi)

The adhesin P97 is presumably one of the factors responsible for adherence, but other proteins may also contribute cilium binding (Hsu and Minion, 1998a). The gene P97 is part of a two-gene genetic structure, where the downstream gene is encoding a 102 kDa protein, namely P102. Since accessory proteins are often found in multigene operons and protein P102 is detectable during enzootic pneumonia, a potential role of P102 in cilium adherence is also presumably (Hsu and Minion, 1998b; Adams *et al*., 2005). Numerous regions in the genome of *M. hyopneumoniae* related to adherence in the host contain variable number of tandem repeats (VNTRs) (Michiels *et al.*, 2017), being prone to recombination events and slipped strand mispairing, resulting in different size of the translated proteins (Torres-Cruz and van der Woude, 2003). These regions are suitable to be a basis of molecular typing (Mayor *et al.*, 2007).

2.5.2. Immune responses to *Mycoplasma hyopneumoniae* infection

The systemic spread of *M. hyopneumoniae* is inhibited by the immune system, but colonisation of the airways and the production of proinflammatory cytokines stimulating inflammation and tissue injury in the lungs can be observed (Choi *et al.*, 2006; Thacker and Minion, 2012). The immune response is affected on both the humoral and cellular levels. The humoral immune response is suppressed due to decreased antibody production, while cellular immune response is suppressed by the inhibition of macrophage-mediated phagocytosis (Maes *et al.*, 1996; Sarradell *et al.*, 2003). Suppression of the immune responses is most pronounced at the early stage of the infection, but it can continue for several weeks (Maes *et al.*, 1996).

The adaptive immune system provides a specific response including the production of pathogen-specific antibody proteins (Goedbloed *et al.*, 2015). Although the exact role of antibodies in the protection against *M. hyopneumoniae* infection is not well known so far (Maes *et al.*, 1996; Sarradell *et al.*, 2003), increased number of immunoglobulin A and G (IgA and IgG) producing cells can be defined in the lung of infected pigs (Redondo *et al.*, 2009). A higher amount of IgA can be observed in the trachea, where it can help to clear inhaled foreign material by prevention of epithelial adherence, penetration and toxin neutralisation. By contrast, phagocytosis is the primary defence mechanism in the lower respiratory tract, enhanced by a higher amount of IgG through its opsonic activity (Sheldrake *et al*., 1993; Walker *et al.*, 1996). Seropositivity can be observed in pigs, experimentally infected with *M. hyopneumoniae*, 3-4 weeks post infection, with a peak registered 10-12 weeks after challenge (Kobisch *et al*., 1993).

2.6. Clinical signs and pathology

Enzootic pneumonia is characterised by catarrhal bronchopneumonia, high morbidity but low mortality (Kobisch and Friis, 1996). The mean incubation period of *M. hyopneumoniae* infection is 10-16 days. There is a considerable difference between the progression of the disease in uncomplicated infections and co- or subsequent presence of secondary pathogens, however, environmental stressors can also exacerbate the symptoms (Maes *et al.*, 1996).

In the absence of secondary agents, either a subclinical course of the infection can be observed, or non-specific symptoms such as slight fever with anorexia and non-productive cough may occur (Maes *et al.*, 1996). Non-productive cough is one of the typical signs observed in affected pigs mostly from 4-8 weeks post infection, depending on the immune status of the animals and on herd management (Kobisch and Friis, 1996; Maes *et al.*, 1996; Garcia-Morante *et al.*, 2016). The approximate date of the infection can be determined according to the condition of the lung lesions. The consolidated areas usually affect the apical and middle lobes and eventually the cranial part of diaphragmatic lobes (Maes *et al.*, 2008;

Garcia-Morante *et al.*, 2016). In subclinical cases, tissue damages can heal or disappear from 8-10 weeks post infection (Maes *et al.*, 1996; Pieters *et al.*, 2009). Collapsed alveoli and/or alveolar emphysema as well as extensive hyperplastic lymphoid nodules and fibrosis are common in recovering lesions (Kobisch *et al*., 1993; Maes *et al.*, 1996; Thacker and Minion, 2012; Charlebois *et al.*, 2014). If clinical symptoms appear, purple or grey consolidations are observed generally in the apical and cardiac lobes, the accessory lobe and the cranial portion of the caudal lobes of the lung (Kobisch and Friis, 1996; Thacker and Minion, 2012) (Figure 3). Complete loss of ciliary activity can be observed 9 days following infection (Jacques *et al.*, 1992; Kobisch *et al*., 1993). Lung lesions are the most pronounced 2 to 4 weeks post infection, when coughing appears.

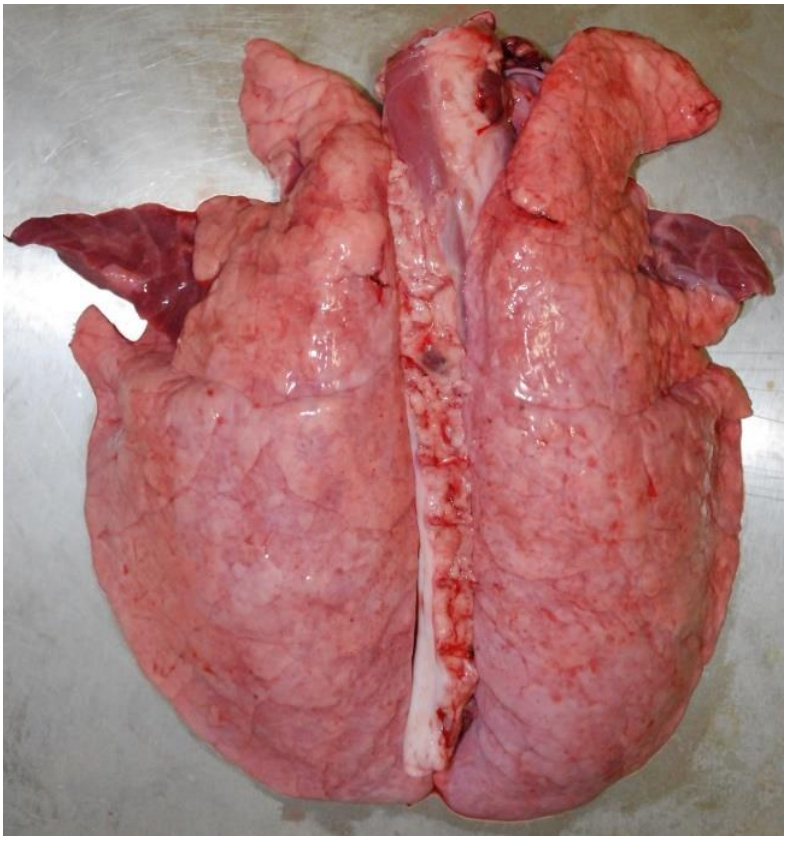

Figure 3. *M. hyopneumoniae* induced pneumonia in the cranial lobes of porcine lung (origin: Miklós Gyuranecz)

The reduction in the efficiency of the mucociliary apparatus, the suppression of innate and acquired pulmonary immunity and the presence of thick, viscous mucus allows commensal bacterial pathogens as *Pasteurella multocida*, *Streptococcus suis*, *Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* to invade and contribute to the disease or potentiate diseases caused by viral pathogens like porcine respiratory and reproductive syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) (Calsamiglia *et al*., 1999; Sorensen *et al*., 2006; Thacker and Minion, 2012; Maes *et al.*, 2017). The presence of other respiratory pathogens or poor air quality usually results in clinical disease characterised by severe symptoms like

productive cough, high fever, anorexia, laboured breathing, prostration and an increased mortality rate (Kobisch and Friis, 1996; Maes *et al.*, 1996, 2017; Michiels *et al.*, 2015). Chronic lesions consist generally of interlobular scarring, with grey-to-white mucopurulent exudate in the airways (Thacker and Minion, 2012; Garcia-Morante *et al.*, 2016; Maes *et al.*, 2017). The simultaneous or subsequent infection with more than one strain might be responsible for more pronounced lung lesions (Villarreal *et al.*, 2009; Vranckx *et al.*, 2011; Michiels *et al.*, 2017), although conflicting data are also published (Charlebois *et al.*, 2014).

2.7. Diagnosis

2.7.1. Sampling and cultivation

Reliable diagnosis in the early stages of *M. hyopneumoniae* infection is essential for developing cost-effective prevention and control programs, limiting the effects of enzootic pneumonia and monitoring the status of the herd (Kurth *et al.*, 2002; Pieters *et al*., 2017). As clinical signs (e.g. coughing and fever) and pathological signs (e.g. lung lesions) are characteristic but not specific for *M. hyopneumoniae* infections, laboratory diagnosis is based on the isolation of the agent and/or demonstration of its presence by molecular (polymerase chain reaction; PCR) and/or immunological methods (enzyme-linked immunosorbent assay (ELISA), immunohistochemistry) (Maes *et al.*, 1996, 2017).

The sensitivity of certain sampling methods is suspected to vary during the course of the infection (Mattsson *et al.*, 1995), however, laryngeal swabs are reliable samples for early diagnosis of *M. hyopneumoniae* infection by real-time PCR, including situations where exposure may be suspected but not clinically apparent (Pieters *et al*., 2017). Oral or nasal fluid samples and mucosal scrapings are less reliable compared to other sampling types (Mattsson *et al.*, 1995; Kurth *et al.*, 2002; Pieters *et al*., 2017). Furthermore, tracheo-bronchial swabbing proved to be more sensitive sampling method in live animals than nasal swabbing (Fablet *et al.*, 2010). Samples from the lower respiratory tract exhibit in general higher sensitivity compared to upper respiratory tract samples (Maes *et al.*, 2017; Pieters, Daniels and Rovira, 2017). Furthermore, lung tissue samples are usually utilised for the detection and culture of *M. hyopneumoniae*. Detection of *M. hyopneumoniae* can also be accomplished with immunohistochemistry (Opriessnig *et al.*, 2004) or *in situ* hybridisation (Boye *et al.*, 2001), allowing the detection of *M. hyopneumoniae* in the target tissue.

Although culturing *M. hyopneumoniae* is the "gold standard" method for diagnostic purposes, it is not accomplished routinely, because of its slow and fastidious growth characteristics and the potential overgrowth by other porcine mycoplasmas, like *M. hyorhinis* or *M. flocculare* (Maes *et al.*, 1996, 2017). The medium described by Friis (Friis, 1975) and novel culture medium from Mycoplasma Experience Ltd. can also be utilised (Maes *et al.*, 1996; Cook *et al.*,

2016). The media should always contain selective agent (β-lactam antibiotics), amino acid source (yeast extract), sterol source (serum), energy source (glucose) and pH indicator (phenol red) for the detection of bacterial metabolism (Friis, 1975; Cook *et al.*, 2016). Acidic colour shift in the broth can be seen 4 to 15 days after inoculation (Kobisch and Friis, 1996), however, isolation from clinical material needs 4 to 6 weeks incubation. Several passages in liquid medium might be necessary before successful inoculation on solid medium (Maes *et al.*, 1996). The last step of producing pure cultures is the filter-cloning, which is the passage of one bacterium colony into sterile broth media.

2.7.2. Immunological methods

Accuracy and sensitivity of antigen-based assays are highly dependent on sample quality and collection timing (Kurth *et al.*, 2002; Pieters *et al*., 2017). Detection of the pathogen from tissue samples is possible by immunofluorescent microscopy, immunohistochemistry and *in situ* hybridisation technique (Amanfu *et al.*, 1984; Boye *et al.*, 2001), although these methods are effective also mostly in the acute stage of the infection (Maes *et al.*, 1996; Calsamiglia *et al*., 1999; Thacker and Minion, 2012).

Serological tests are based on the developing immune response of the animals to *M. hyopneumoniae*, which can vary considerably (3-8 weeks) among pigs (Maes *et al.*, 1996; Kurth *et al.*, 2002; Strait *et al.*, 2008; Thacker and Minion, 2012). However, it should also be noted, that levels of the antibodies decrease in the chronic phase of infection, and can no longer been detected (Maes *et al.*, 2017). Several ELISA-kits are commercially available to analyse the humoral immune response of the animals against *M. hyopneumoniae* infections (Marois *et al.*, 2007; Giacomini *et al.*, 2016; Michiels *et al.*, 2017). Although serology is routinely used to detect the presence of the bacterium in live animals, the assays should be interpreted at herd level, and it must be considered that cross-reactions with other porcine *Mycoplasmas* may happen (Freeman *et al.*, 1984; Maes *et al.*, 1996; Calsamiglia, Pijoan and Trigo, 1999). ELISA tests are usually applied for defining vaccination status, but are not able to distinguish infected and vaccinated animals. Furthermore, these tests are not suitable to determine protection against the infection (Maes *et al.*, 1996, 2008, 2017; Pieters *et al*., 2017).

2.7.3. Molecular identification

Although DNA probes can be used for the diagnosis of *M. hyopneumoniae*, DNA-DNA hybridization assays are generally not regarded as reliable diagnostic tools, especially on individual level, because of cross-reactions and low sensitivity (Bölske *et al.*, 1987; Stemke, 1989; Artiushin *et al*., 1993; Maes *et al.*, 1996). PCR technique is a useful cost-effective tool for the diagnosis of *M. hyopneumoniae* infection, which can be performed directly on clinical

samples, avoiding the isolation procedure of the pathogen. Furthermore, antibiotic treatment of the animals or co-infective organisms do not affect the success of the reaction (Maes *et al.*, 1996; Pieters *et al*., 2017). Several species-specific PCR systems have been developed for detecting *M. hyopneumoniae*, targeting the 16S rRNA region, multidrug resistance protein-encoding gene, porcine β2 microglobulin or the *mhp165* gene (Mattsson *et al.*, 1995; Blanchard *et al.*, 1996; Kurth *et al.*, 2002; Strait *et al.*, 2008). The real-time PCR assays using laryngeal swab samples are able to detect *M. hyopneumoniae* 5 days post infection, preceding the serum IgM response, which makes them ideal in early infection stages instead of serologic testing (Pieters et al., 2017).

2.7.4. Molecular typing

The molecular typing methods differentiating bacterial field isolates are valuable tools in epidemiological investigations. Numerous methods are available for molecular typing of *M. hyopneumoniae*, like random amplified polymorphic DNA (RAPD) analysis (Artiushin and Minion, 1996), amplified fragment length polymorphism (AFLP) (Kokotovic *et al.*, 1999), pulsed-field gel electrophoresis (PFGE) (Stakenborg *et al.*, 2005b), restriction fragment length polymorphism (RFLP) of the gene *p146* or analysis of variable number of tandem repeats (VNTR) of the gene *P97* (Stakenborg *et al.*, 2006), however, most of these assays are expensive, time-consuming or difficult to reproduce. RAPD analysis has a satisfactory discriminatory power, but as a consequence of the weak reproducibility, all isolates of interest should be examined in one single experiment at the same time (Stakenborg *et al.*, 2006; Nathues *et al.*, 2011; Charlebois *et al.*, 2014). Most important advantages of AFLP and PFGE are the reproducibility between laboratories and the high discriminatory power, but these assays are fastidious (Stakenborg *et al.*, 2006; Charlebois *et al.*, 2014).

Results of the PCR-based typing assays must be handled with care, as omitting filter-cloning, the assays may not differentiate mixed strains within a sample (Nathues *et al.*, 2011). Recently used PCR-based genotyping assays applicable directly on clinical materials, are based on the sequence analysis of housekeeping genes (multi-locus sequence typing, MLST) or on the VNTR analysis in highly variable adhesin or surface protein coding genes (de Castro et al., 2006; Mayor et al., 2007, Vranckx et al., 2011, Charlebois et al., 2014; Mayor et al., 2008).

MLST technique is dedicated to compare evolutionary relationships of strains on intermediate-level (Keim *et al*., 2007; Sulyok *et al.*, 2014). An important advantage of this technique is that MLST results are easily comparable between laboratories through online databases (e.g. https://pubmlst.org/bigsdb?db=pubmlst_mhyopneumoniae_seqdef).

Multiple-locus VNTR analysis (MLVA) is a rapid and feasible assay for epidemiological investigations and genetic characterisation of *M. hyopneumoniae* strains, without prior

cultivation (dos Santos *et al.*, 2015). The number of infecting strains in a host and the number of VNTRs in certain MLVA loci in a strain might be associated with the pathogenicity of *M. hyopneumoniae* (de Castro *et al.*, 2006; Villarreal *et al.*, 2009; Vranckx *et al.*, 2011; Michiels *et al.*, 2017). Disadvantages of this assay are the lack of standardised target regions and the rarely available sequence data or repeat numbers of previous investigations, which make the inter-laboratory comparisons difficult.

Another typing method is the analysis of the *p146* gene, which is an adhesin-like protein encoding gene containing a serine-repeat region. The gene *p146* can be examined either by VNTR analysis (Nathues et al., 2011; Vranckx et al., 2011; dos Santos et al., 2015; Michiels et al., 2017) or by sequence analysis (Mayor *et al.*, 2007).

2.8. Economic impact and control of the disease

2.8.1. Economic impact

M. hyopneumoniae infection is prevalent almost all over the world and is responsible for tremendous economic losses through increased costs (either medication or prevention) and decreased performance of the animals (Kobisch and Friis, 1996; Maes *et al.*, 2008, 2017; Holst *et al*., 2015). Infected animals are predisposed to secondary bacterial (e.g. *A. pleuropneumoniae*, *H. parasuis*, *P. multocida*) or viral (Swine Influenza Virus, PRRSV, PCV2, Porcine Respiratory Coronavirus) infections, which also increase the losses of swine industry (Calsamiglia *et al*., 1999; Sorensen *et al*., 2006; Thacker and Minion, 2012; Pieters and Sibila, 2017). Economic losses due to *M. hyopneumoniae* infection can be defined numerically by comparing the average daily gain and the feed conversion ratio of pneumonic pigs and healthy pigs, but it should be considered that environmental and management conditions highly affect the severity of pneumonia (Maes *et al.*, 1996). Also, the quantification of the severity of lung lesions during slaughter by scoring technique is an additional tool to determine the damage associated with the infection (Maes *et al.*, 1996).

2.8.2. Vaccination

Vaccination is widely applied to control *M. hyopneumoniae* infections in pig herds (Maes, 2017). The majority of the commercially available vaccines are inactivated, adjuvanted whole-cell preparations, administered intramuscularly (Maes 2008), but most recently a soluble antigen-based vaccine (Suvaxyn Circo+MH RTU, Zoetis, New Jersey, USA) and a temperature sensitive mutant-based vaccine (VaxSafe MHYO, Avimex, Granjas Esmeralda, Mexico) have been introduced (Maes, 2017). The use of recombinant proteins of *M. hyopneumoniae* is a

possible way of protection, but only a few recombinant vaccines have been tested in swine yet (Maes, 2017).

The degree of beneficial effects of vaccination can depend on the infection level of the herd, the diversity of the involved strains, the presence of co-infective agents and on the storage conditions and administration of the vaccine (Maes, 2017). Although recently available bacterin type vaccines do not provide complete protection against lung lesions neither prevent colonisation or *M. hyopneumoniae* transmission (Meyns *et al.*, 2004, 2006; Stakenborg *et al.*, 2006; Villarreal *et al.*, 2009), it can considerably improve performances of animals through mitigating clinical signs, moderating the severity of lung lesions and reducing the number of bacteria in the respiratory tract (Maes *et al.*, 1999, 2017; Meyns *et al.*, 2006; Vranckx *et al.*, 2012; Woolley *et al.*, 2013).

Traditional vaccination protocols consist of two occasions, although the use of one-shot vaccines is increasing (Baccaro *et al.*, 2006; Sibila *et al.*, 2007). The correlation between the stress caused by the weaning process and the effectiveness of vaccination is still questionable, however, vaccination of piglets a few days before weaning may be more gentle from animal welfare aspects (Arsenakis *et al.*, 2016, 2017). Vaccination of sows does not directly affect the colonisation of the piglets, but a decreased number of bacterial agents can be registered in the respiratory tract of piglets at weaning (Arsenakis *et al.*, 2017) and an increased number of seropositive animals and moderate lung lesion scores can be observed (Sibila *et al.*, 2008).

2.8.3. Antibiotic treatment

In the absence of cell-wall, mycoplasmas are naturally resistant against antibiotics such as β-lactams, glycopeptides or fosfomycin that target cell-wall synthesis, and are also unaffected by rifampicin, polymixins, sulphonamides, first-generation quinolones and trimethoprim (Taylor-Robinson and Bébéar, 1997; Chernova *et al.*, 2016; Gautier-Bouchardon, 2018). However, several antibiotics are active against *M. hyopneumoniae*, such as tetracyclines, 15- and 16-membered ring macrolides, lincosamides, pleuromutilines, fluoroquinolones, florfenicol, aminoglycosides and aminocyclitols (Maes *et al.*, 1996, 2008, 2017; Hannan *et al.*, 1997a). *M. hyopneuomoniae* infection is often complicated by secondary respiratory bacterial pathogens, which make the selection of the appropriate antibiotic difficult. Quinolones and tetracycline type antibiotics are effective in complicated infections (Hannan *et al.*, 1989), while combination of antibiotics can also be indicated (Maes *et al.*, 1996, 2008).

Tetracyclines and aminoglycosides inhibit bacterial protein synthesis through binding to the 30S subunit of the bacterial ribosome (Noah *et al.*, 1999). Doxycycline is a member of the class tetracyclines, showing a very good pharmacokinetic effect, therefore it is widely used to control *M. hyopneumoniae* infections in swine herds. Aminoglycosides like gentamicin or

spectinomycin, are effective against *M. hyopneumoniae* and the combination of spectinomycin with lincomycin has a synergistic effect. However, the combination is effective only as injection, because spectinomycin has weak biological effect administered *per os*.

Antimicrobial agents, inhibiting the bacterial protein synthesis through binding to the 50S ribosomal subunit include lincosamides, macrolides, pleuromutilins and phenicols. Lincomycin is a member of the class lincosamides, showing a good distribution administered *per os*. It is permitted to use for the treatment of porcine mycoplasmosis in the European Union (Pyörälä *et al.*, 2014). Despite the same mode of action, macrolides are chemically distinct from lincosamides. Macrolide type antibiotics in general have bacteriostatic activity, but can also be bactericidal depending on the applied drug concentration (Jain and Danziger, 2004). They reversibly bind to 23S ribosomal RNA in the 50S subunit of bacterial ribosomes, inhibiting protein synthesis (Menninger, 1985). The 16-membered ring macrolides, tylosin and tilmicosin are the earliest antibiotics of the class, being used only for veterinary purposes (Vicca *et al.*, 2005). Tylvalosin is a tylosin derivate able to significantly reduce the mycoplasmatic lesions, and increase weight-gain of the animals (Pallarés *et al.*, 2015). Gamithromycin and tulathromycin are 15-membered semi-synthetic macrolides of the azalide subclass. While gamithromycin is originally developed for the treatment and prevention against bovine respiratory disease (Huang *et al.*, 2010), tulathromycin is developed for one-time treatment of respiratory diseases of both cattle and swine. According to the good distribution of tulathromycin, high concentration is developed in the lung tissue and in the macrophages (Benchaoui *et al.*, 2004). Tiamulin and valnemulin are the most widespread pleuromutilin type agents, playing an important role in the treatment of *M. hyopneumoniae* infections (Poulsen *et al.*, 2001). Florfenicol, which is a member of the class phenicols, is used exclusively in veterinary medicine, and it is frequently applied for the treatment of respiratory diseases of bacterial origin in cattle and swine (Priebe and Schwarz, 2003).

Most of the agents used for the treatment of mycoplasma-pneumonia have a mycoplasmastatic effect, while the greatest mycoplasmacidal activity is exhibited by the quinolone type antibiotics (Taylor-Robinson and Bébéar, 1997). The members of the class fluoroquinolones act through binding to topoisomerase type enzymes inhibiting the bacterial DNA replication process. Enrofloxacin and marbofloxacin are the most widely used members of fluoroquinolones, able to treat enzootic pneumonia of swine (Le Carrou *et al.*, 2006). Since fluoroquinolones are very important antibiotics in human therapy, the usage of these agents is restricted to justified cases.

Antibiotic medication has been used since the 1940s in the European swine industry (Goedbloed *et al.*, 2015) especially after clinical outbreaks (Maes *et al.*, 2017). Antimicrobial treatment cannot absolutely eliminate the bacterium from the respiratory tract; however, it is able to reduce clinical symptoms and moderate the mortality rate caused by

M. hyopneumoniae (Maes *et al.*, 1996). An apparently healthy herd can also contain infected animals without any conspicuous signs of the disease; thus antibiotic treatment can be used as metaphylaxis in certain cases (Aarestrup *et al*., 2008; Mathers *et al*., 2011). One of the most successful method among elimination processes is the Swiss method, which also includes medication and partial depopulation besides a farrowing pause (Maes *et al.*, 2017). Although treatment during risk periods like replacement or weaning may decrease bacterial spreading, cessation of the medication is often followed by the reappearance of the symptoms (Maes *et al.*, 1996, 2008). Furthermore, medication during extended periods of time can lead to emerging resistance, and increase of the withholding period (Maes *et al.*, 2008).

2.8.4. Mutations responsible for increased minimum inhibitory concentration values

Although antibiotic therapy is successfully applied to moderate clinical signs, the inappropriate use of antimicrobial agents contributes to the emergence of resistant bacterial strains. Single nucleotide polymorphisms (SNPs) are point mutations with biological significance (Birdsell *et al.*, 2012), affecting for example the antibiotic susceptibility via conformation changes of the target regions of certain antibiotics.

The targets of the fluoroquinolone type antibiotics are DNA gyrase and topoisomerase IV (Le Carrou *et al.*, 2006; Vicca *et al.*, 2007). Emerging resistance to fluoroquinolones in mycoplasmas is usually due to transitions in the quinolone resistance-determining regions (QRDR) in genes encoding subunits of the topoisomerase enzymes (*gyrA*, *gyrB*, *parC*, *parE*) (Hooper, 2000; Gautier-Bouchardon, 2018), however, no fluoroquinolone resistance-related mutations were detected in *parE* gene of resistant *M. bovis* strains (Sulyok *et al.*, 2017). The majority of the substitutions, causing amino acid changes and therefore increased MIC values against *M. hyopneumoniae*, are observed in the *parC* gene (e.g. Ser80Phe, Ser80Tyr, Asp84Asn and Ala116Glu, according to *Escherichia coli* numbering) (Le Carrou *et al.*, 2006; Vicca *et al.*, 2007). The amino acid change Ala83Val in the *gyrA* gene of *M. hyopneumoniae* showed a correlation with decreased susceptibility to enrofloxacin (Vicca *et al.*, 2007). Further amino acid substitutions observed in the *gyrA* gene of other *Mycoplasma* species were for example Gly81Ala or Glu87Gly (Reinhardt *et al*., 2002; Sulyok *et al.*, 2017).

Tetracyclines and the aminocyclitol antibiotic spectinomycin attach to the 30S ribosomal subunit. The non-synonymous substitutions in positions 965 and 967 of the *rrs* genes inhibit the bond of oxytetracyclin, thus increased MIC values of oxytetracycline against *M. bovis* can be observed (Khalil *et al*., 2017; Sulyok *et al.*, 2017). C1192A/T mutations of the *rrs1* gene and the C335T within the *rrs1* and *rrs2* alleles were defined in *M. bovis* strain showing increased MIC values of spectinomycin (Sulyok *et al.*, 2017).

According to the literature, 14-membered macrolides show low MIC against *M. hyopneumoniae* due to a G2057A transition in the 23S rRNA sequence (Stakenborg *et al.*, 2005a). In addition, A2058T transition in the same region is frequently observed in association with increased resistance to 15- and 16-membered macrolides and lincosamides (Vester and Douthwaite, 2001; Stakenborg *et al.*, 2005a). According to the literature, the nucleotide alteration A2059G can also be involved in macrolide and lincosamide resistance in case of *M. agalactiae*, *M. bovis*, *M. gallisepticum* and *M. synoviae* (Sulyok *et al.*, 2017; Gautier-Bouchardon, 2018). Although mutations in the ribosomal proteins L4 and L22 show correlation with macrolide resistance in *Escherichia coli* and *Streptococcus pneumonia* (Pereyre *et al.*, 2002), no SNP was detected in *M. hyopneumoniae* so far (Stakenborg *et al.*, 2005a).

Point mutations in the 23S rRNA gene and L3 protein are associated with decreased susceptibility to pleuromutilins in several bacteria (Gautier-Bouchardon, 2018). The substitutions C2035A, A2060G, G2062T and C2500A, closely associated with the pleuromutilin binding sites of the 23S rRNA gene, were described in artificially resistant *M. bovis* isolates (Sulyok *et al.*, 2017). Florfenicol resistant *M. bovis* strains showed a correlation with the nucleotide substitutions G2062T or A2063T in the 23S rRNA gene (Sulyok *et al.*, 2017).

3. Aims of the study

The aims of the study were to apply, compare or develop conventional and molecular biological methods used in the control of enzootic pneumonia, accordingly:

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 MLST and 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 frequently utilised in Hungary.

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.

4. Materials and methods

4.1. *M. hyopneumoniae* strains

4.1.1. Isolation method

Porcine lung samples with typical pulmonary lesions were collected between 2015 and 2016 from different Hungarian slaughterhouses with the permission of the owners, from the carcasses being processed, thus no ethical approval was required. 0,5×0,5 cm large pieces of the reddish, congested apical lung lobe were washed into Friis broth (Friis, 1975). The Friis broth medium consisted of 0.0214 g/ml Friis Mycoplasma base (Teknova Inc., Hollister, CA), 20% porcine serum, 5% rabbit serum, 200 IU/ml penicillin G, 1% glucose, 1% pyruvate and distilled water. The broth sample was filtered through a 0.45 µm filter and its 30-fold dilution was incubated for 4 weeks or until colour change of phenol red indicator (from red to yellow) at 37 °C. After the incubation period, a 10-fold serial dilution was prepared, and incubated until colour change (Etheridge, Cottew and Lloyd, 1979). When colour change of the broth media occurred cultures were inoculated onto solid media and incubated at 37 °C and 5% CO_2 for 4-10 days until visible colonies appeared. *Mycoplasma* strains were once filter-cloned, and aliquots of purified cultures were stored at -70 °C until use. The origins of the studied Hungarian *M. hyopneumoniae* isolates were presented on a map (Figure 4), and the detailed background data are collected in Table 1.

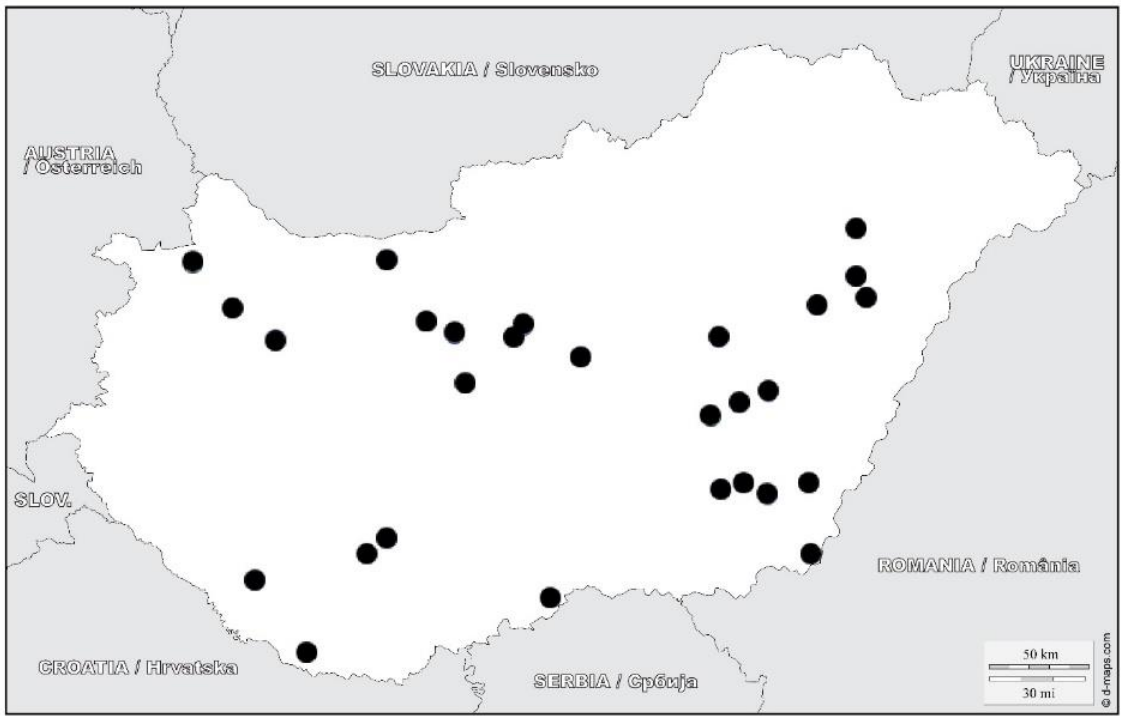

Figure 4. Geographical origin of the *M. hyopneumoniae* isolates from Hungary

Table 1. Background data of the examined *M. hyopneumoniae* isolates

Number	Sample ID	Herd of origin	County	Country	Date of isolation
1	MycSu1	Hajdúszoboszló	Hajdú-Bihar	Hungary	2015
2	MycSu2	Hajdúszoboszló	Hajdú-Bihar	Hungary	2015
3	MycSu3	Nagyhegyes	Hajdú-Bihar	Hungary	2015
4	MycSu4	Nagyhegyes	Hajdú-Bihar	Hungary	2015
5	MycSu5	Tata	Komárom-Esztergom	Hungary	2015
6	MycSu6	Tata	Komárom-Esztergom	Hungary	2015
7	MycSu7	Mezőtúr	Jász-Nagykun-Szolnok	Hungary	2015
8	MycSu8	Mezőtúr	Jász-Nagykun-Szolnok	Hungary	2015
9	MycSu9	Hajdúnánás	Hajdú-Bihar	Hungary	2015
10	MycSu10	Békéscsaba	Békés	Hungary	2015
11	MycSu11	Békéscsaba	Békés	Hungary	2015
12	MycSu12	Dombegyház	Békés	Hungary	2015
13	MycSu13	Rábaszentandrás	Győr-Moson-Sopron	Hungary	2015
14	MycSu14	Csikóstöttös	Tolna	Hungary	2015
15	MycSu15	Bácsalmas	Bács-Kiskun	Hungary	2015
16	MycSu16	Bácsalmas	Bács-Kiskun	Hungary	2015
17	MycSu17	Pálhalma	Fejér	Hungary	2016
18	MycSu18	Bácsalmas	Bács-Kiskun	Hungary	2016
19	MycSu19	Lábod	Somogy	Hungary	2016
20	MycSu20	no data	no data	Czech	2016
21	MycSu33	Lábod	Somogy	Hungary	2016
22	MycSu34	Oroshaza	Békés	Hungary	2016
23	MycSu37	Csemő	Pest	Hungary	2016
24	MycSu39	Sellye	Baranya	Hungary	2016
25	MycSu40	Felsőbabád	Pest	Hungary	2016
26	MycSu41	Mezőtúr	Jász-Nagykun-Szolnok	Hungary	2016
27	MycSu42	Mesterszállás	Jász-Nagykun-Szolnok	Hungary	2016
28	MycSu43	no data	no data	Slovakia	2016
29	MycSu44	no data	no data	Slovakia	2016
30	MycSu45	Döbrököz	Tolna	Hungary	2016
31	MycSu46	Szentes	Csongrád	Hungary	2016
32	MycSu47	Lovasberény	Fejér	Hungary	2016
33	MycSu49	Felsőbabád	Pest	Hungary	2016
34	MycSu50	Fegyvernek	Jász-Nagykun-Szolnok	Hungary	2016
35	MycSu52	Baracska	Fejér	Hungary	2016
36	MycSu53	Ócsa	Pest	Hungary	2016
37	MycSu70	Fábiánsebestyén	Csongrád	Hungary	2016
38	MycSu79	Pápa	Veszprém	Hungary	2016
39	MycSu80	Nádudvar	Hajdú-Bihar	Hungary	2016
40	MycSu81	Nádudvar	Hajdú-Bihar	Hungary	2016
41	MycSu82	Nádudvar	Hajdú-Bihar	Hungary	2016
42	MycSu83	no data	no data	Slovakia	2016
43	MycSu84	Osli	Győr-Moson-Sopron	Hungary	2016
44	MycSu85	Dévaványa	Békés	Hungary	2016

All isolates originated from porcine lung samples.

4.1.2. Molecular identification

DNA extraction was performed on 200 µl of broth culture using QIAamp DNA mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions for Gram-negative bacteria. All isolates were identified by PCR amplifying a 649 bp fragment of the 16S ribosomal RNA gene of *M. hyopneumoniae* according to Mattsson *et al*. (1995), utilising the primers listed in Table 2, and using the PCR conditions described in Table 3. The purity of the cultures (i.e. to exclude other contaminating Mycoplasmas) was confirmed by a universal mycoplasma PCR system targeting the 16S/23S rRNA intergenic spacer region in Mollicutes according to Lauermann *et al*. (1995), using the primers listed in Table 2, and using the PCR conditions described in Table 3.

Table 2. Primer sequences used for identification of *M. hyopneumoniae* isolates

Primer	Primer sequence (5'-3')	Amplicon (bp)	Reference
16S rRNA			
forward	GAGCCTTCAAGCTTCACCAAGA	649	(Mattson *et al*., 1995)
reverse	TGTGTTAGTGACTTTTGCCACC		
16S/23S rRNA intergenic region			
forward	ACACCATGGGAGCTGGTAAT	680	(Lauermann *et al*., 1995)
reverse	CTTCATCGACTTTCAGACCCAAGGCAT		

All conventional PCRs were carried out in a Bio-Rad C1000 Touch™ Thermal Cyclers (Bio-Rad Laboratories Inc., C.A.). After amplification, 5 µl of each sample was loaded in 1% agarose gel (SeaKem LE Agarose Lonza Inc., Switzerland) containing ECO Safe nucleic acid staining solution (Pacific Image Electronics Co, Ltd., New Taipei City, Taiwan) for electrophoresis. Stained gels were visualised by UV light and photographically documented with the help of Kodak MI SE software package (Kodak Inc., Rochester, NY).

Amplicons of universal mycoplasma PCR were sequenced on ABI 3130XL genetic analyser (Applied Biosystems, Foster City, CA). Reading errors were corrected with SeqMan software (Lasergene package, DNASTAR Inc., Madison, WI) and sequences were submitted for nucleic acid database search using the BLASTN program in GenBank.

Table 3. Reaction mixtures and conditions used for identification of *M. hyopneumoniae* isolates

Reaction mixture (volumes/µl/1 sample)	16S rRNA[a]	16S/23S rRNA intergenic region[b]
Target DNA (10 to 100 ng)	1	1
5×Green GoTaq Flexi Buffer (Promega, Inc., Madison, WI)	5	5
$MgCl_2$ (25 mM; Promega, Inc.)	2	2.5
dNTP (10 mM; Qiagen Inc.)	0.75	0.5
Primers (10 pmol/µl)	2	1
GoTaq Flexi DNA polymerase (5 U/µl; Promega Inc.)	0.2	0.25
PCR program		
Initial denaturation	95 °C - 5 min	95 °C - 2 min
Number of cycles	30	40
Denaturation	95 °C - 30 s	95 °C - 30 s
Annealing	54 °C - 30 s	55 °C - 30 s
Extension	72 °C - 60 s	72 °C - 60 s
Final extension	72 °C - 5 min	72 °C - 5 min

Abbreviations: [a] - Mattson *et al.*, 1995; [b] - Lauermann *et al.*, 1995

4.2. Genotyping

All members of the *M. hyopneumoniae* strain collection were genotyped with MLST methods (using the nucleotide sequences of housekeeping genes), MLVA systems (utilising non-coding tandem repeat regions) and using the extension of the mentioned analyses with the nucleotide sequence data of the gene *p146*. The discriminatory power, congruency and interchangeability of the different genotyping assays were evaluated and an appropriate application either for phylogenetic or epizootiologic usage was examined.

Publicly available complete genome sequences of the strains J (type strain, GenBank Accession Number: AE017243), 232 (GenBank Acc. No.: AE017332) from USA, 7448 (GenBank Acc. No.: AE017244) from Brazil and strain 168 (GenBank Acc. No.: CP002274) from China were included in the MLST and *p146* gene analyses.

4.2.1. Multi-locus sequence typing

MLST is a genotyping method based on the differences of the nucleotide sequences of the housekeeping genes *efp*, *metG*, *pgiB*, *recA*, *adk*, *rpoB* and *tpiA*. Nucleotide sequence data were collected from the whole genome sequences of the 44 *M. hyopneumoniae* field isolates. Next-generation sequencing of whole genomes was accomplished on Ion Torrent platform as described before (Rónai *et al.*, 2015). A total of 100 ng of DNA was enzymatically fragmented using the reagents supplied in the NEBNext Fast DNA Fragmentation & Library Prep Set for

Ion Torrent kit (New England Biolabs, Hitchin, United Kingdom) according to the manufacturer's instructions. The adaptor ligation was performed using reagents from the same kit. The barcoded adaptors were retrieved from the Ion Xpress Barcode Adapters (Life Technologies Inc., Waltham, MA). The barcoded library DNA samples were column purified using the Gel/PCR DNA fragments extraction kit (Geneaid Biotech Ltd., Taipei, Taiwan). 2% precast gel (Thermo Fisher Scientific Inc., Waltham, MA) was utilised for running the eluted library DNA. Products between 300 and 350 bp were used without further purification in the PCR mixture of the NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent kit (New England Biolabs). Library amplification was accomplished in 12 cycles. The products were purified by the Gel/PCR DNA fragments extraction kit (Geneaid). Library DNA was eluted in 50 µl of nuclease-free water then quantified fluorometrically on Qubit 2.0 equipment using Qubit dsDNA BR assay kit (Invitrogen). Finally, the library DNA was diluted to 10 to 14 pM and then clonally amplified by emulsion PCR, according to the manufacturer's protocol using the Ion PGM template kit on a OneTouch v2 instrument. Enrichment of the templated beads (on an Ion One Touch ES machine) and further steps for presequencing setup were made according to the 200-bp protocol of the manufacturer. The sequencing protocol recommended for the Ion PGM sequencing kit on a 316 chip was strictly followed.

Sequence analysis of the MLST genes (*efp*, *metG*, *pgiB*, *recA*, *adk*, *rpoB*, *tpiA*) was performed using Geneious software 10.2.3 ® (Biomatters Ltd., New Zealand). Sequences of the isolates were mapped to the type strain (Genbank Accesion Number: NC_007295), thereafter the genes of interests were sorted and aligned. Reading errors were corrected manually and the sequences were trimmed. Regions of certain sizes (*efp* - 459 bp, *metG* - 735 bp, *pgiB* - 754 bp, *recA* - 721 bp, *adk* - 581 bp, *rpoB* - 602 bp, *tpiA* - 641 bp) (Mayor *et al.*, 2008) were collected, analysed and concatenated. For each gene fragment, allele numbers are assigned to the nucleotide sequences, and the sequence type (ST) of the isolates is defined by the corresponding allele numbers of each locus. Isolates with a common ST are assumed to have a recent common ancestor (Spratt, 1999). Analysis of the concatenated MLST sequences was accomplished with Maximum Likelihood method using 1000 bootstraps in MEGA 7.0 (Tamura *et al.*, 2011). Nucleotide sequences of the studied isolates were compared with publicly available database at https://pubmlst.org/bigsdb?db=pubmlst_mhyopneumoniae_seqdef (Jolley and Maiden, 2010).

4.2.2. Multi-locus variable number tandem repeat analysis

MLVA method is based on the differences in the number of repeat units of tandem repeat regions in the bacterial genome. The high genetic variability of *M. hyopneumoniae* strains and therefore the different number of repeat units makes this method a useful tool for epizootiologic investigations (Vranckx *et al.*, 2011; Charlebois *et al.*, 2014).

MLVA based on four tandem repeat regions (Locus1, Locus2, P97-RR1, P97-RR2) was performed with the amplification primers described by Charlebois *et al.* (2014). Furthermore, the assay was expanded with the analysis of the serine repeat numbers of gene *p146* (Mayor *et al.*, 2007); and used for resolution of the identical STs of MLST based on seven genes. Different names of the MLVA loci from previous publications are listed in Table 4. Primer sequences used for the MLVA assay are presented in Table 5. Forty-four *M. hyopneumoniae* field isolates and the type strain (ATCC 25934) were included in the analysis; the latter one was used as a quality control of the process. PCRs were performed in 25 µl final volume containing 1 µl of target DNA (10 to 100 ng) diluted in nuclease-free water. The PCR mixture for loci P97-RR1/-RR2 and Locus-1/-2 are summarised in Table 6.

Table 4. Different names of the MLVA loci used in previous publications

MLVA loci	Locus1	Locus2	p146 (VNTR)	P97-RR1	P97-RR2
VNTR[a]	H4[b]/h1[c]	H1[b]	P146R3[b]/p146 repeat 3[c]	P97R1[b]/P97 repeat 1[c]	P97R2a[b]
Protein name	H4, MHJ0032	H1, MHJ0441	p146, MHJ0663	P97, MHJ0194	MHJ0194

[a] VNTR's name in previous publications; [b] de Castro *et al.*, 2006; [c] Vranckx *et al.*, 2011

An aliquot (4 µl) of each PCR product was subjected to electrophoresis (8 V/cm) in 3% high resolution agarose gel (MetaPhor Agarose, Lonza Inc., Rockland, ME). Amplified DNA products were visualised with ECO Safe nucleic acid staining solution (Pacific Image Electronics Co, Ltd., New Taipei City, Taiwan). Molecular weight markers were used after every sixth sample: 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific Inc.) was utilised for PCR products below 200 bps and 100-bp DNA ladder (GeneRuler 100 bp Plus, Thermo Fisher Scientific Inc.) over the length of 200 bps. Stained gels were visualised by UV light and band sizes were estimated with the help of Kodak MI SE software package (Kodak Inc., Rochester, NY). Band size results of MLVA were converted to number of repeat units (Charlebois *et al.*, 2014). Clustering analysis was accomplished with the Neighbor-joining method with 1000 bootstraps in MEGA 7.0 (Tamura *et al.*, 2011).

4.2.3. Analysis of gene *p146*

Genotyping based on the sequence analyses of the gene *p146* was performed using the amplification primers and PCR conditions described by Mayor *et al.* (2007) (Tables 5-6). The reaction mixture and PCR conditions are described in Table 6. PCR products were sequenced on ABI 3130XL genetic analyser (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Geneious software 10.2.3 ® (Biomatters Ltd.). Nucleotide sequence data of *p146* gene, including both the flanking region and the serine repeat region, were analysed with Neighbor-joining method with FastGap (Borchsenius, 2009) and PAUP 4.0 software (Swofford, 2003). All PCRs were performed in a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Inc.).

4.2.4. Comparison of methods

The discriminatory power of the different typing methods was determined using Simpson's index of diversity with 95% confidence intervals (CI) (Hunter and Gaston, 1988). The quantitative level of congruence and the interchangeability with the respective confidence intervals between typing methods were calculated based on the data of the isolates analysed with all methods using the adjusted Rand and Wallace coefficients with the help of the Comparing Partitions website (*Comparing Partitions Website In http://darwin.phyloviz.net/ ComparingPartitions/index.php?link=Home.*) (Carriço *et al.*, 2006; Severiano *et al.*, 2011).

Table 5. Primers used for MLVA assays of the examined *M. hyopneumoniae* isolates

Primer	Primer sequence (5'-3')	Repeat size[a]	Reference
Locus1	AAACTCCAAAAAAATCGATAAAAA CTTTTTTTGCGGTAATAAGGTTAT	18	(Marois-Créhan *et al.*, 2012)
Locus2	TTTGTGCTTGGTAAAGTTAAATT AGAGATTACAACCAAGAAGCAA	12	(Marois-Créhan *et al.*, 2012)
p146	TCCAAGACGAAGATCTTGACTATC TTAGAACTTGCAAGATAAAGCTTG	3	(Mayor *et al.*, 2007)
P97-RR1	GAAGCTATCAAAAAAGGGGAAACTA GGTTTATTTGTAAGTGAAAAGCCAG	15	(Stakenborg *et al.*, 2006)
P97-RR2	AGCGAGTATGAAGAACAAGAA TTTTTACCTAAGTCAGGAAGG	30	(Stakenborg *et al.*, 2006)

[a] Data of *M. hyopneumoniae* J strain counted in bp

Table 6. Reaction mixtures and conditions used for genotyping of *M. hyopneumoniae* isolates

Reaction mixture (volumes/µ/1 sample)	P97-RR1/RR2[a]	Locus-1/-2[b]	*p146*[c]
Target DNA (10 to 100 ng)	1	1	1
5×Green GoTaq Flexi Buffer (Promega, Inc., Madison, WI)	5	5	5
$MgCl_2$ (25 mM; Promega, Inc.)	2	3	2.5
dNTP (10 mM; Qiagen Inc.)	0.8	0.8	0.5
Primers (10 pmol/µl)	0.5	0.5	1
GoTaq Flexi DNA Polymerase (5 U/µl; Promega Inc.)	0.2	0.2	0.25
PCR program			
Initial denaturation	94 °C - 5 min	94 °C - 5 min	95 °C - 15 min
Number of cycles	30	40	35
Denaturation	94 °C - 30 s	94 °C - 30 s	94 °C - 30 s
Annealing	50 °C - 30 s	52 °C - 30 s	50 °C - 30 s
Extension	72 °C - 60 s	72 °C - 30 s	72 °C - 30 s
Final extension	72 °C - 5 min	72 °C - 5 min	72 °C - 7 min

Abbreviations: [a] - Stakenborg *et al.*, 2006; [b] - Marois-Créhan *et al*., 2012; [c] - Mayor *et al.*, 2007

4.3. Antibiotic susceptibility testing

4.3.1. Antimicrobial agents

Antibiotic susceptibility profiles of 44 *M. hyopneumoniae* strains were defined by microbroth dilution method for 15 antimicrobial agents. The number of colour changing units (CCU) was determined by microbroth dilution method after four weeks of incubation (Hannan, 2000).

The following antimicrobial classes and individual agents were examined during the microbroth dilution tests: fluoroquinolones (enrofloxacin, marbofloxacin), tetracyclines (oxytetracycline, doxycycline), aminoglycosides (gentamicin), aminocyclitols (spectinomycin), macrolides (tylosin, tilmicosin, tylvalosin, tulathromycin, gamithromycin) pleuromutilins (tiamulin, valnemulin), lincosamides (lincomycin) and phenicols (florfenicol).

Table 7. Antibiotics and concentration ranges (µg/ml) used in susceptibility tests of the examined *M. hyopneumoniae* strains

Antibiotic group	Antibiotic	Concentration range (µg/ml)
Fluoroquinolones	Enrofloxacin	0.039-10
	Marbofloxacin	0.039-10
Tetracyclines	Oxytetracycline	0.25-64
	Doxycycline	0.039-10
Aminoglycoside	Gentamicin	0.25-64
Aminocyclitol	Spectinomycin	0.25-64
Macrolides	Tylosin	0.25-64
	Tilmicosin	0.25-64
	Tylvalosin	0.25-64
	Tulathromycin	0.25-64
	Gamithromycin	0.25-64
Pleuromutilines	Tiamulin	0.039-10
	Valnemulin	0.039-10
Lincosamides	Lincomycin	0.25-64
Phenicols	Florfenicol	0.125-32

Tylvalosin originated from ECO Animal Health Ltd., UK (Aivlosin), tulathromycin originated from Pfizer Inc., USA, and the rest of the products originated from VETRANAL, Sigma-Aldrich, Germany. The antibiotics were diluted and stored according to the recommendation of Hannan (2000). Stock solutions of 1 mg/ml were prepared in sterile distilled water, except the fluoroquinolones, tulathromycin, gamithromycin and florfenicol. Stock solutions of 1 mg/ml enrofloxacin and marbofloxacin were prepared in 0.1 M NaOH and stock solutions of 1 mg/ml tulathromycin, gamithromycin and florfenicol were prepared in 96% ethanol and sterile distilled water. Aliquots were stored at -70 °C until required, precipitation on thawing was checked before usage and dilutions for each test were freshly prepared. Twofold dilutions were made according to the concentration ranges listed in Table 7.

4.3.2. Microbroth dilution method

Microbroth dilution test was accomplished using a 96-well microtiter plate (Figure 5). The plate contained dilutions of the examined antibiotics in columns 1-9 (100 µl broth media containing certain concentration of antibiotic and 100 µl of bacterial suspension), sterility control in column 10 (200 µl broth media without bacterium culture), endpoint control in column 11 (200 µl sterile broth media adjusted to pH 6.8) and growth control in column 12 (100 µl

bacterium culture in 100 µl broth media). By reason of the more pronounced colour change of the media, Mycoplasma Experience broth medium (Mycoplasma Experience Ltd., Bletchingley, United Kingdom) was applied for determining the number of CCU of strains and the susceptibility tests. The antibiotic susceptibility test was accomplished on 10^4-10^5 CCU/ml of the strains as recommended by Hannan (Hannan, 2000). All strains were tested in duplicates and all plates contained a duplicate of the reference strain (NCTC 10110) as quality control.

MIC was established as the lowest antibiotic concentration where no colour change of the broth was observed as a consequence of the absence of bacterial metabolism. Initial MIC values were recorded when colour change of the broth media of the growth control was visible (4-14 days after inoculation), and final MIC values were registered when no further colour change was observed. MIC_{50} and MIC_{90} values were determined as the lowest concentrations that inhibited the growth of 50% or 90% of the strains (Hannan, 2000).

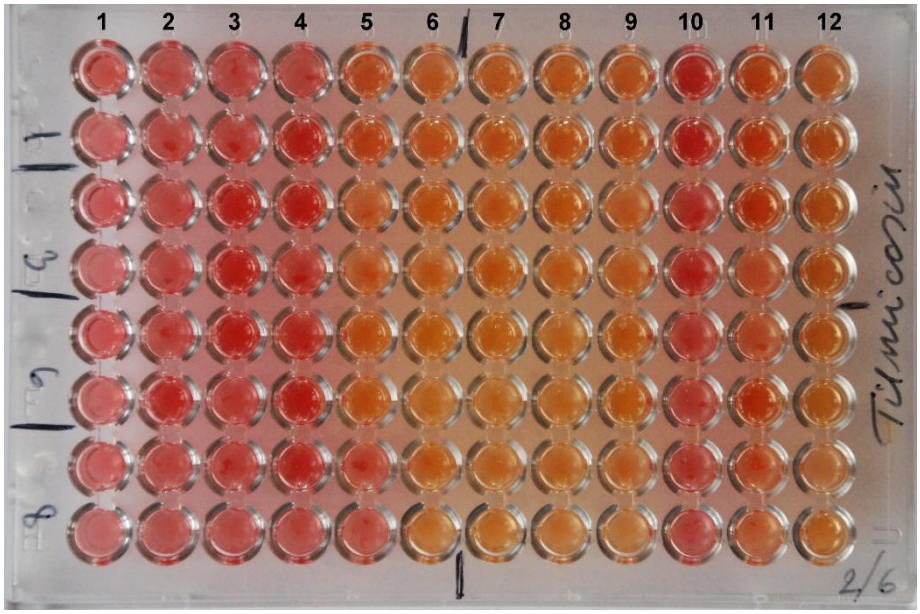

Figure 5. Minimum inhibitory concentration (MIC) determination of tilmicosin with microbroth dilution method against the type strain (NCTC 10110) (1) and three *M. hyopneumoniae* field isolates (3; 6; 8). The columns are 1-9: descending concentrations of tilmicosin (64-0.25 µg/ml); 10: sterility control; 11: endpoint control; 12: growth control. MIC values of tilmicosin were 8 µg/ml against the isolates 1; 3 and 6 and 4 µg/ml against the isolate 8.

4.4. Development of molecular assays for the rapid detection of antibiotic susceptibility of *M. hyopenumoniae*

4.4.1. Identification of mutations responsible for increased minimum inhibitory concentration values of fluoroquinolones, macrolides and lincosamides

Genetic markers correlating with fluoroquinolone, macrolide and lincosamide susceptibility in *M. hyopneumoniae* were examined in genes *gyrA*, *gyrB*, *parC*, *parE* and 23S rRNA (Stakenborg *et al.*, 2005a; Le Carrou *et al.*, 2006; Vicca *et al.*, 2007; Gautier-Bouchardon, 2018). Nucleotide sequences of the listed genes were studied using the whole genome sequences (described in chapter 4.2.1.) as a preliminary step, and then nucleotide alterations were confirmed with Sanger sequencing. The primers utilised for Sanger sequencing are summarised in Table 8, and reaction mixtures with PCR conditions are listed in Table 9. PCR products were sequenced on ABI 3130XL genetic analyser (Applied Biosystems, Foster City, CA) and sequence analysis was performed by using Geneious software 10.2.3 ® (Biomatters Ltd.) (Kearse *et al.*, 2012).

The validity of SNPs was confirmed by manual examination of the assembled sequences. The numbering of nucleotide and amino acid positions is based on genes and proteins of *Escherichia coli* strain K-12 substrain MG1655 (GenBank accession number CP014225). Susceptibility profiles and correlating genetic markers were evaluated in relation with previously determined genotypes of the examined strains also.

Although there is no official breakpoint for the antibiotic susceptibility testing of *M. hyopneumoniae*, each strain showing higher final MIC value of enrofloxacin of 2 µg/ml was marked resistant according to Hannan *et al.* (1997a). The strains showing lower MIC ranges were called sensitive. Amino acid alterations of interest were chosen according to the literature, thus the changes Ser80Phe/Tyr, Asp84Asn and Ala116Glu in the *parC* gene, and Gly81Ala, Ala83Val and Glu87Gly in the *gyrA* gene were examined (Le Carrou *et al.*, 2006; Vicca *et al.*, 2007; Sulyok *et al.*, 2017; Gautier-Bouchardon, 2018).

According to the literature, SNPs correlating with macrolide and lincosamide resistance in *M. hyopneumoniae* are in the 2057-2059 region (Stakenborg *et al.*, 2005a; Sulyok *et al.*, 2017; Gautier-Bouchardon, 2018).

Table 8. Amplification primers specific for the genes correlating with increased MIC values of *M. hyopneumoniae* isolates

Primer	Primer sequence (5'-3')	Reference
gyrA	CTKCCRGATGTCCGWGATGG GTSGGRAARTCYGGCYCCGG	(Vicca *et al.*, 2007)
gyrB	ACATTCATAACCCTGAAGGC GTCTCTCAAAGTTGTTCCGG	(Vicca *et al.*, 2007)
parC	TGATTCAGTAATTAATTCCCGG TCACTATCATCAAAATTAGGGC	(Le Carrou *et al.*, 2006)
parE	TCAATTCTTGAATTTGTTGGGC TCAATAAATTTGTCTTCAAGGG	(Le Carrou *et al.*, 2006)
23S rRNA	GATGAGTATTCTAAGGTGAGCGAG CAGTCAAACTACCCACCACG	

Table 9. Reaction mixtures and PCR conditions for the assays targeting genes correlating with increased MIC values of *M. hyopneumoniae* isolates

Reaction mixture (volumes/µ/1 sample)	*gyrA*[a]	*gyrB*[a]	*parC, parE*[b]	23S rRNA[c]
Target DNA (10 to 100 ng)	2	1	1	1
5×Green GoTaq Flexi Buffer (Promega, Inc., Madison, WI)	5	5	5	5
$MgCl_2$ (25 mM; Promega, Inc.)	1.5	2.5	1	2.5
dNTP (10 mM; Qiagen Inc.)	0.5	0.5	0.5	0.5
Primers (10 pmol/µl)	1	1	1.25	1
GoTaq Flexi DNA Polymerase (5 U/µl; Promega Inc.)	0.25	0.25	0.5	0.25
PCR program				
Initial denaturation	94 °C - 5 min	94 °C - 5 min	95 °C - 2 min	95 °C - 2 min
Number of cycles	35	35	40	34
Denaturation	94 °C - 60 s	94 °C - 60 s	94 °C - 30 s	95 °C - 30 s
Annealing	55 °C - 60 s	55 °C - 60 s	56 °C - 30 s	56 °C - 30 s
Extension	72 °C - 60 s	72 °C - 60 s	72 °C - 30 s	72 °C - 60 s
Final extension	72 °C - 5 min	72 °C - 5 min	72 °C - 5 min	72 °C - 5 min

Abbreviations: a - Vicca *et al.*, 2007; b - Le Carrou *et al*., 2006; c - Stakenborg et al., 2005a

4.4.2. Mismatch amplification mutation and high resolution melt design

Mismatch amplification mutation assay (MAMA) is a PCR-based molecular biological method used for SNP determination in many bacteria (Birdsell *et al.*, 2012). In brief, MAMAs are based on allele-specific primers that are SNP specific at the 3' end. A single destabilizing mismatch at the 3' end of each allele-specific primer enhances the discrimination capacity of the assay. One of the allele-specific primers is marked with an additional 15-20 bp long GC-clamp that increases the melting temperature and the size of the amplicon as well. The temperature shift can be easily detected in the presence of intercalating fluorescent dye on a real-time PCR platform (melt-MAMA) and the difference in the sizes of the amplicons can be observed in 2% agarose gel electrophoresis (agarose-MAMA). In the present study, MAMAs were designed and tested for the detection of SNPs related to fluoroquinolone, macrolide and lincosamide resistance (Tables 10-12).

Table 10. PCR mixtures for melt-MAMA and HRM assays used for identification of SNPs in genes correlating with decreased antibiotic susceptibility of *M. hyopneumoniae* isolates

Reaction mixture (volumes/µ/1 sample)			
	Melt-MAMA		HRM
	parC (248;259)	23S rRNA	*parC*
Target DNA (10 to 100 ng)	1	1	1
5×Colorless GoTaq Flexi Buffer (Promega, Inc., Madison, WI)	2	2	2
$MgCl_2$ (25 mM; Promega, Inc.)	1	2	1.5
dNTP (10 mM; Qiagen Inc.)	0.3	0.3	0.3
EvaGreen (20X, Biotum Inc., Hayward, CA)	0.5	0.5	0.5
GoTaq Flexi DNA Polymerase (5 U/µl; Promega Inc.)	0.08	0.08	0.08
PCR program			
Initial denaturation	95 °C - 10 min	95 °C - 10 min	94 °C - 10 min
Number of cycles	34	30	34
Denaturation	95 °C - 15 s	95 °C - 15 s	95 °C - 15 s
Annealing	55 °C - 60 s	60 °C - 60 s	55 °C - 60 s
Melt analysis			
Dissociation	95 °C - 15 s	95 °C - 15 s	95 °C - 15 s
Melting (0.3 °C)	60 - 95 °C	60 - 95 °C	60 - 95 °C

Numbers: 248 and 259 indicate the base positions in the *parC* gene targeted by the MAMA and HRM assays.

Besides individual SNPs, a “hot-spot” region with two possible mutations in the *parC* gene was identified in strains with declined fluoroquinolone susceptibility (Le Carrou *et al*., 2006). This region of the *parC* gene (nucleotide region 194-287 according to *M. hyopneumoniae* numbering) was targeted by high resolution melt (HRM) assay (Tables 10 and 12). HRM is based on thermodynamic differences between small amplicons, therefore it is a suitable technique for the analysis of different SNPs (Palais *et al*., 2005). Melt-MAMA and HRM assays were optimised for Applied Biosystems Step-One Plus real-time PCR system with StepOne Software version 2.3 (Thermo Fisher Scientific). EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature. Agarose-MAMAs were performed in C1000^{TM} Touch Thermal Cycler (Bio-Rad Laboratories Inc.), and the electrophoresis was carried out in 2% agarose gel (SeaKem LE Agarose, Lonza Inc.) using a 20-bp DNA ladder (O’RangeRuler 20 bp, Thermo Fisher Scientific Inc.) as molecular weight marker. All MAMA and HRM primers were designed and tested on the *M. hyopneumoniae* type strain (J, NCTC 10110) and on field strains (Table 1). Primer melting temperature and general suitability were calculated using NetPrimer software (Premier Biosoft International, Palo Alto, CA). Sequences and amounts of the primers as well as the utilised thermocycler parameters for the assays can be found in Tables 10-12.

Table 11. PCR mixture of agarose-MAMA assays for the identification of the SNPs in genes correlating with decreased antibiotic susceptibility of the examined *M. hyopneumoniae* isolates

Reaction mixture (volumes/µ/1 sample)		
	*par*C	23S rRNA
Target DNA (10 to 100 ng)	1	2
5×Green GoTaq Flexi Buffer (Promega, Inc., Madison, WI)	5	5
$MgCl_2$ (25 mM; Promega, Inc.)	2.5	2.5
dNTP (10 mM; Qiagen Inc.)	0.5	0.5
GoTaq Flexi DNA Polymerase (5 U/µl; Promega Inc.)	0.25	0.25
PCR program		
Initial denaturation	95 °C - 2 min	95 °C - 2 min
Number of cycles	34	34
Denaturation	95 °C - 30 s	95 °C - 30 s
Annealing	55 °C - 30 s	57 °C - 30 s
Extension	72 °C - 60 s	72 °C - 60 s
Final extension	72 °C - 5 min	72 °C - 5 min

4.4.3. Mismatch amplification mutation and high resolution melt validation

In order to test the sensitivity of the assays, tenfold dilutions of the sensitive and resistant phenotypes were used in the range of 10^6-10^1 copy number/µl. Template copy number was calculated with the help of an online tool (Staroscik, 2004) based on the length of the whole genome sequence and concentration of DNA of pure *M. hyopneumoniae* cultures measured by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.). The lowest DNA concentrations (template copy number) yielding melting temperature (T_m) specific to the genotype were considered the detection limits of the assays. All members of the *M. hyopneumoniae* strain collection (*n*=44) was examined with all MAMA and HRM assays to compare the antibiotic susceptibility results made by molecular biological assays and microbroth dilution method. The specificity of the assays was tested by including the following porcine *Mycoplasma* species in the analysis: *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare*.

Table 12. Primers and PCR conditions used for MAMA and HRM assays

Assay type	Target gene	SNP[a]	SNP[b]	Antibiotic group	Primer name	Primer sequence (5'-3')	Primer[c]
MAMA	*parC*	248	239	Fluoroquinolones	parC_248_cons	AATCTGCTAGAGTTGTCGGTG	0.15
					parC_248_sen	CAAGAGCATC/TATAGATTGgAG	0.15
					parC_248_res	ggggcggggcggggcCAAGAGCATC/TATAGATTGcAA/T	0.15
MAMA	*parC*	259	250	Fluoroquinolones	parC_259_cons	AATCTGCTAGAGTTGTCGGTG	0.15
					parC_259_sen	GCAAGTCTGACAAGAGCgTC	0.15
					parC_259_res	ggggcggggcggggcGCAAGTCTGACAAGAGCcTt	0.15
MAMA	23S rRNA	2072	2059	Macrolides, Lincosamides	23S_2072_cons	CCACCTATCCTACACATAATAAACC	0.15
					23S_2072_sen	GTTA/TCCCGCATCAAGACaAA	0.15
					23S_2072_res	ggggcggggcggggcGTTA/TCCCGCATCAAGACtAg	0.6
HRM	*parC*	248	239	Fluoroquinolones	parC_248-259_ResR	CATTCCTGGGCAAGTCTG	0.25
		259	250		parC_248-259_ResF	AATCTGCTAGAGTTGTCGGTG	0.25

Abbreviations: [a] - nucleotide position according to *M. hyopneumoniae* numbering, [b] – nucleotide position according to *E. coli* numbering, [c] – primer amounts in the PCR mixture (µl)

5. Results

5.1. Genotyping

5.1.1. Multi-locus sequence typing

Sequence data of the studied isolates are deposited in Genbank with the accession numbers: MG387660-387946; MG574873-574893, while allele types and STs of the examined strains are summarised in Table 13, and sequence data are available from PubMLST database under the IDs 186-229.

The examined 44 *M. hyopneumoniae* isolates clustered into 27 STs by MLST using either all seven housekeeping genes of the conventional (Figure 6), or only three housekeeping genes of the minimal MLST (Figure S1). The majority of the isolates originating from the same herds had identical STs and were placed in the same subclades by the MLSTs (e.g. MycSu5-6; MycSu80-82). However, there were some exceptions, and in certain cases isolates clustered on different branches, despite their common origin of herds (MycSu3-4; MycSu7-8 and 41). The most frequent sequence type was ST82, which included 11 Hungarian isolates (MycSu1-2; MycSu8; MycSu10-11; MycSu13; MycSu42; MycSu53 and MycSu80-82).

All of the studied isolates represented novel STs, but matches on allele levels were observed with allele type 5 (MycSu34), 6 (MycSu20; 43-44; 52; 83), 19 (MycSu46; 50) and 23 (MycSu9; 12; 45) of the *adk* gene, allele type 15 (MycSu39) and 18 (MycSu79) of the *rpoB* gene and allele type 1 (MycSu3; 52), 7 (MycSu15-16; MycSu18; MycSu41) and 26 (MycSu46) of the *tpiA* gene based on the comparison with data published at pubMLST (https://pubmlst.org/bigsdb?db=pubmlst_mhyopneumoniae_seqdef). Among the seven MLST genes *rpoB* showed the highest resolution power categorising the field isolates into 22 different allele types, while genes *efp* and *pgiB* had the lowest resolution power with 11 allele types (Table 13).

Table 13. MLST allele and sequence types of the examined *M. hyopneumoniae* field isolates and the type strain

Sample ID	STs*	*efp*	*metG*	*pgiB*	*recA*	*adk**	*rpoB**	*tpiA**	*p146**
MycSu1	82	1	4	11	14	26	34	37	87
MycSu2	82	1	4	11	14	26	34	37	87
MycSu3	97	8	8	11	15	28	35	1*	88
MycSu4	98	7	12	11	9	29	36	38	89
MycSu5	96	1	12	11	6	27	37	38	89
MycSu6	96	1	12	11	6	27	37	38	90
MycSu7	83	11	5	11	5	26	38	39	91
MycSu8	82	1	4	11	14	26	34	37	92
MycSu9	95	1	5	7	12	23*	39	40	93
MycSu10	82	1	4	11	14	26	34	37	92
MycSu11	82	1	4	11	14	26	34	37	92
MycSu12	80	1	5	1	10	23*	40	41	94
MycSu13	82	1	4	11	14	26	34	37	87
MycSu14	86	1	6	3	8	27	37	42	95
MycSu15	89	2	1	2	4	30	41	7*	96
MycSu16	89	2	1	2	4	30	41	7*	96
MycSu17	83	11	5	11	5	26	38	39	97
MycSu18	89	2	1	2	4	30	41	7*	96
MycSu19	90	4	3	10	1	31	42	43	99
MycSu20	74	10	9	11	7	6*	43	44	98
MycSu33	90	4	3	10	1	31	42	43	99
MycSu34	72	9	1	11	9	5*	37	42	100
MycSu37	91	5	2	10	1	32	44	38	101
MycSu39	85	1	4	11	14	27	15*	38	102
MycSu40	93	9	1	1	1	33	45	45	97
MycSu41	89	2	1	2	4	30	41	7*	96
MycSu42	82	1	4	11	14	26	34	37	103
MycSu43	73	7	1	11	9	6*	37	38	104
MycSu44	75	3	7	5	5	6*	46	46	105
MycSu45	81	1	5	8	11	23*	47	41	106
MycSu46	79	6	1	11	13	19*	38	26*	107
MycSu47	87	7	10	9	16	29	48	47	108
MycSu49	93	9	1	1	1	33	45	45	97
MycSu50	78	11	1	11	3	19*	37	42	109
MycSu52	76	9	1	4	2	6*	49	1*	110
MycSu53	82	1	4	11	14	26	34	37	111
MycSu70	88	6	11	10	13	29	50	38	112
MycSu79	92	6	1	9	16	33	18*	45	117
MycSu80	82	1	4	11	14	26	34	37	113
MycSu81	82	1	4	11	14	26	34	37	113
MycSu82	82	1	4	11	14	26	34	37	113
MycSu83	77	6	1	9	9	6*	51	48	114
MycSu84	84	9	11	6	7	26	52	49	115
MycSu85	94	6	1	1	2	34	53	45	116
J strain	28*	12	13	12	17	5*	16*	16*	26*
		0.745 (CI: 0.609-0.881)	0.773 (CI: 0.686-0.861)	0.667 (CI: 0.503-0.830)	0.880 (CI: 0.796-0.963)	0.860 (CI: 0.784-0.937)	0.884 (CI: 0.810-0.959)	0.887 (CI: 0.816-0.958)	0.976 (CI: 0.956-0.996)
Simpson's index						0.907 (CI: 0.828-0.985)			
								0.977 (CI: 0.957-0.998)	
		0.907(CI: 0.828-0.985)							

CI= confidential interval; * previously defined allele and sequence types from pubMLST database

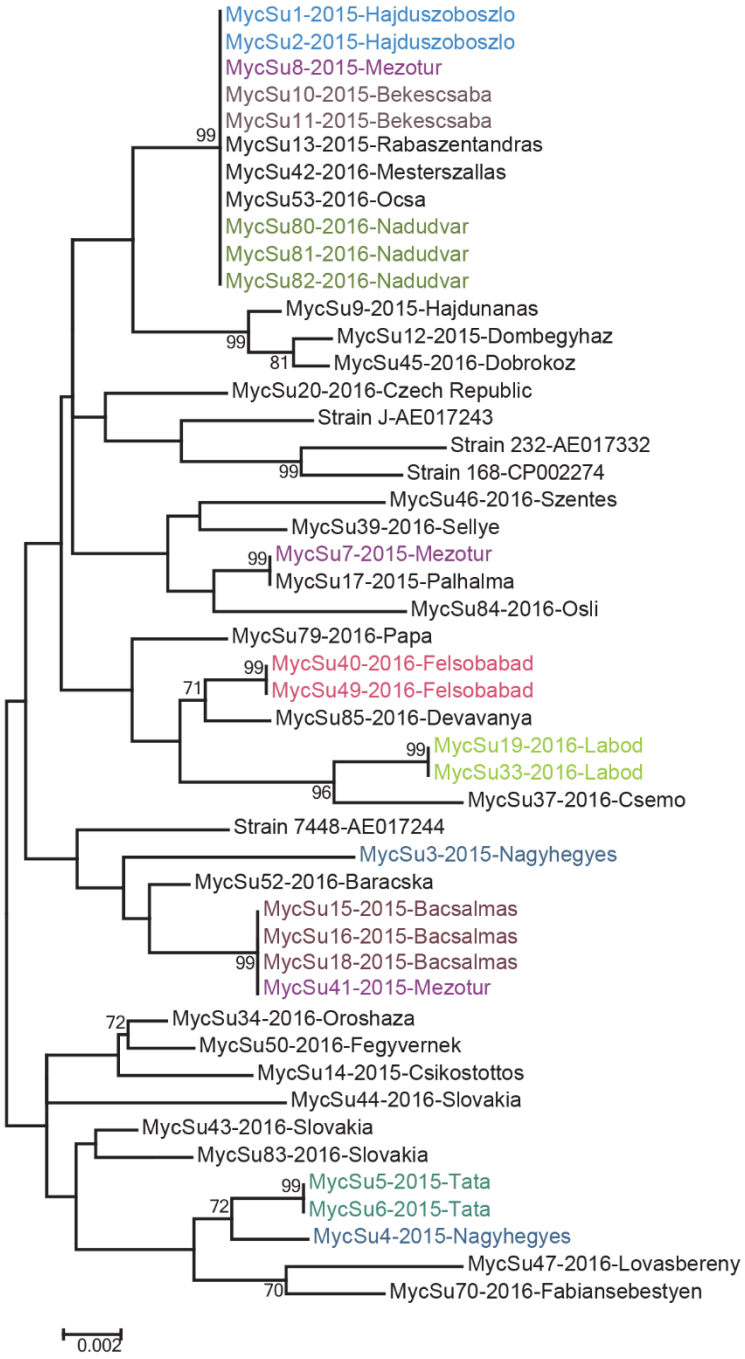

Figure 6. Molecular typing of 44 *M. hyopneumoniae* isolates by conventional MLST (multi locus sequence typing).

The MLST phylogenetic tree was constructed by Maximum-Likelihood method using *efp*, *metS*, *pgiB*, *recA*, *adk*, *rpoB* and *tpiA* genes, with 1000 bootstraps; only bootstrap values > 70 are presented. The scale bar represents the average number of substitutions per site. Colours indicate the common origin of the isolates.

5.1.2. Multi-locus variable number tandem repeat analysis

In this study, the original MLVA assays using the alleles of Charlebois et al. (2014) (Locus1 and 2, P97-RR1 and -RR2) (Figure 7), the common loci of Charlebois et al. (2014) and Vranckx et al. (2011) (Locus1 and P97-RR1) (Figure S2) and the listed schemes extended with the VNTR of *p146* gene were compared (Figures S3-4).

The repeat number of the poly-serine region in gene *p146* ranged from 8 to 42 among the examined field isolates (Table 14). The MLVA profile of the type strain J was the same at all measurement, corresponding to literary data except one locus: one more repeat unit was detected on Locus1 compared to that in the literature (Table 14). The 44 *M. hyopneumoniae* field isolates constituted 38 genotypes (GT) by the MLVA assay described by Charlebois et al. (2014), including 35 individual GTs (Figure 7). Correlation with geographic origin and GT was observed only in case of the strains originating from Nádudvar (MycSu80-82). These isolates originated from one single sampling. Certain strains showed multiple alleles on one or two loci by the MLVA (Table 14), which were omitted from the statistical analyses.

5.1.3. Analysis of gene *p146*

Analysis based on the point mutations of the flanking region and the serine repeat region of gene *p146* using Neighbor-joining method resulted in 31 different types (Figure 8). The strains clustered into two major subclades, but no correlations were found between the origin of the samples and their sequences. As the extension of the minimal MLST, the analysis of the partial nucleotide sequence of gene *p146* seems to be able to refine the phylogenetic examinations, because this method successfully differentiated common MLST STs of strains with a different origin (MycSu1-2 and MycSu10-11) (Figure S5).

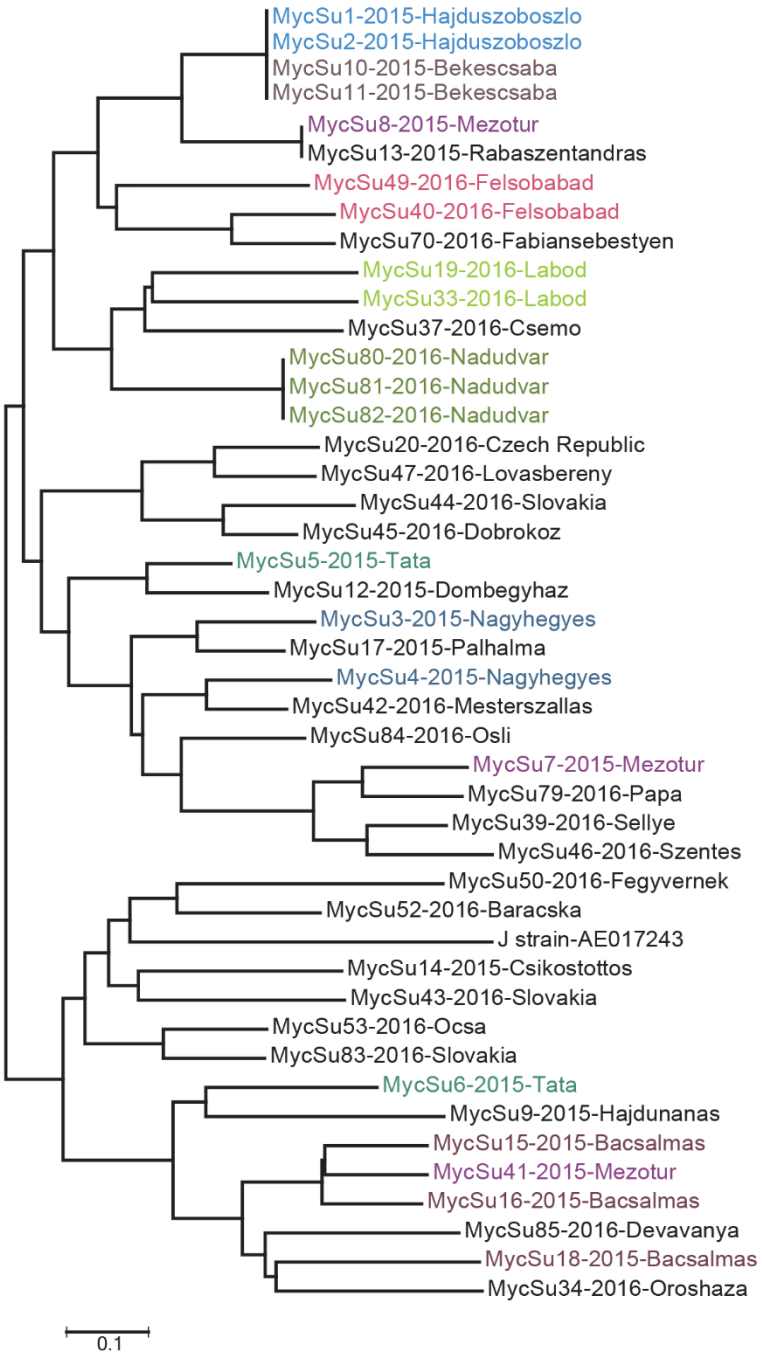

Figure 7. MLVA (multiple-locus variable-number tandem repeat analysis) of 44 *M. hyopneumoniae* isolates.

The MLVA tree was constructed by Neighbor-Joining method, with 1000 bootstraps using Locus1, Locus2, P97-RR1 and P97-RR2 loci. Colours indicate the common origin of the isolates.

Table 14. Repeat unit numbers of the MLVA loci of the studied *M. hyopneumoniae* isolates

Sample ID	P97-RR2	Locus2	P97-RR1	Locus1	*p146* (VNTR)
MycSu1	4	2	8	2	29
MycSu2	4	2	8	2	29
MycSu3	3	2	11	3	18
MycSu4	3	2	7	6; 7; 9	18
MycSu5	3	2	8	1	18
MycSu6	3	3	8	1	17
MycSu7	3	3	6; 11; 19	2	10
MycSu8	4	2	5	2	32
MycSu9	4	3	8	10	13
MycSu10	4	2	8	2	32
MycSu11	4	2	8	2	32
MycSu12	3	2	8	8	17
MycSu13	4	2	5	2	29
MycSu14	5	2	13	8	35
MycSu15	5	3	8	14	17
MycSu16	5	3	8	13	17
MycSu17	3	2	11	2	17
MycSu18	5	3	9	11	17
MycSu19	3; 4; 8	2	8; 10; 17	1	36
MycSu20	4	2	6	5	15
MycSu33	2; 3; 7	2	7; 8; 15	1	36
MycSu34	5	3	12	7	33
MycSu37	2	2	11	1	28
MycSu39	3	3	9	2	36
MycSu40	4	2	11; 12; 19	4	17
MycSu41	5	3	8	18	17
MycSu42	3	2	7	2	31
MycSu43	5	2	9	15	42
MycSu44	2	2	6	9	17
MycSu45	3	2	6	9	27
MycSu46	3	1	9	2	26
MycSu47	3	2	6	5	12
MycSu49	4	2	13	3	17
MycSu50	5	1	10	2	36
MycSu52	5	2	10	7	26
MycSu53	5	2	8	17	31
MycSu70	4	2	4; 9; 21	4	27
MycSu79	3	3	12	2	8
MycSu80	4	2	10	1	34
MycSu81	4	2	10	1	34
MycSu82	4	2	10	1	34
MycSu83	5	2	8	13	27
MycSu84	3	2	7; 8; 15	2	19
MycSu85	5	3	11	5	35
J strain	5	4	7	9	18
Simpson's index	0.718 (CI: 0.678-0.757)	0.480 (CI: 0.341-0.620)	0.836 (CI: 0.755-0.918)	0.866 (CI: 0.785-0.947)	0.923 (CI: 0.869-0.978)
	0.985 (CI: 0.967-1.000)				
			0.977 (CI: 0.960-0.995)		
				0.992 (CI: 0.982-1.000)	
	0.992 (CI: 0.982-1.000)				

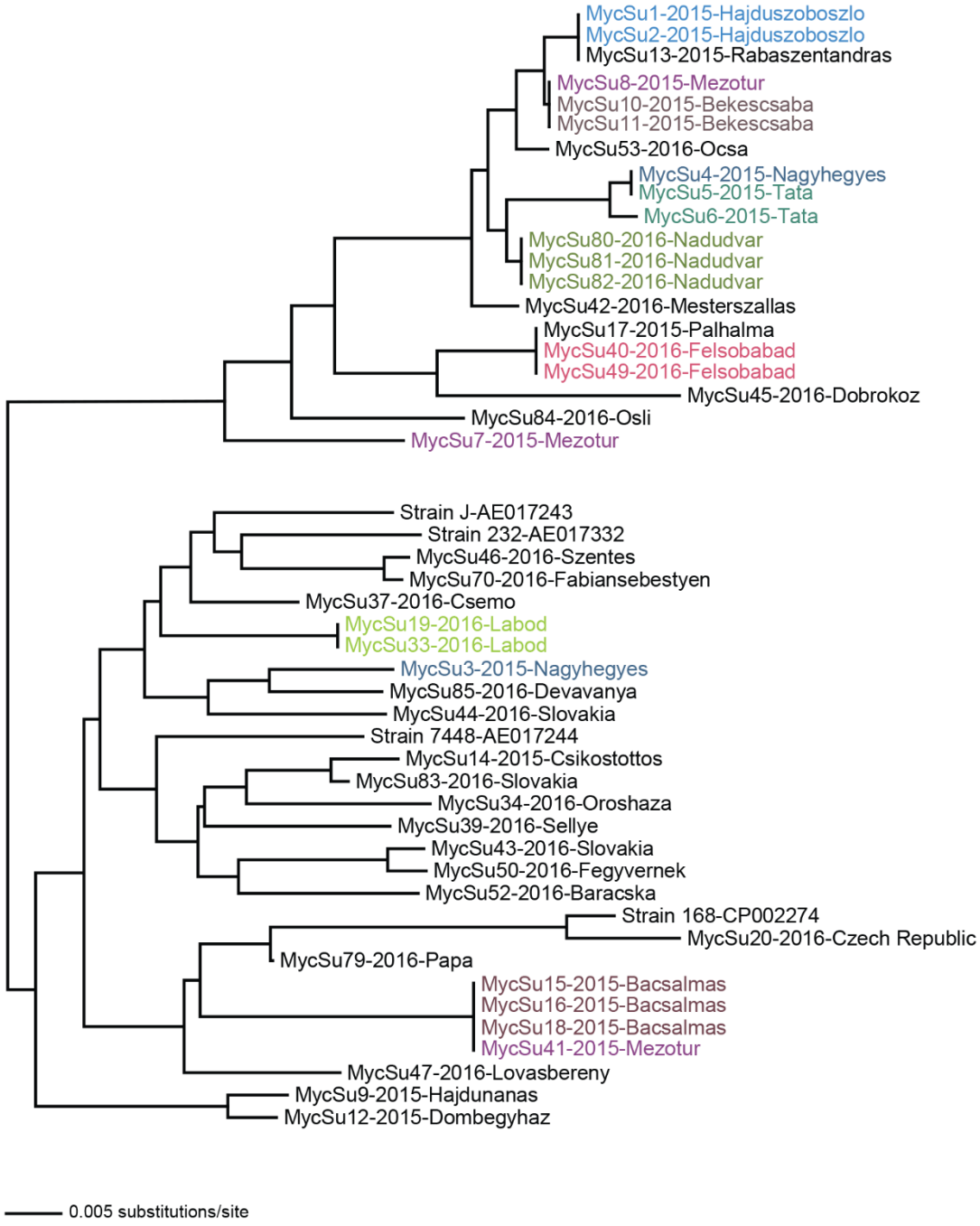

Figure 8. Phylogenetic tree based on the sequence data of gene *p146* of the *M. hyopneumoniae* isolates

The tree based on the sequence data of *p146* gene was constructed by Neighbor-Joining method with pairwise deletion with FastGap (Borchsenius, 2009) and PAUP 4.0 (Swofford, 2003) software. The scale bar represents the average number of substitutions per site. Colours indicate the common origin of the isolates.

5.1.4. Evaluation of the studied methods

The minimal MLST and the conventional scheme showed congruency (Tables S1 and S2) and identical discriminatory power (0.907) (Table 13). In the comparison of MLVA loci combinations, the use of loci P97-RR1 and Locus1 resulted slightly lower resolution of the isolates as the use of all four loci (Locus1 and 2, P97-RR1 and -RR2) (Charlebois et al., 2014) (Table 14). MLVA (Charlebois *et al.*, 2014) extended with the serine repeat numbers of gene *p146* as a fifth locus, had the highest discriminatory power (0.992) among all the studied methods, either using all five loci or only loci P97-RR1, Locus1 and gene *p146* (Table 14). Analysing the sequence data of gene *p146* independently (0.976) or as an extension of the minimal MLST (0.977) showed similarly high discriminatory power (Table 13).

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 (Table S1). The sequence analysis of gene *p146* showed higher congruency with the MLVA assays than with the MLST methods (Table S1). Extended MLVA assays showed the highest interchangeability with the other studied methods providing more refined data (Table S2). The MLVA assay based on the common loci (P97-RR1 and Locus1) of Charlebois *et al.* (2014) and Vranckx *et al.* (2011) extended with the serine repeat numbers of the gene *p146* was able to discriminate strains of different origin with the same MLST sequence type (MycSu1-2 and MycSu10-11) (Figure 9).

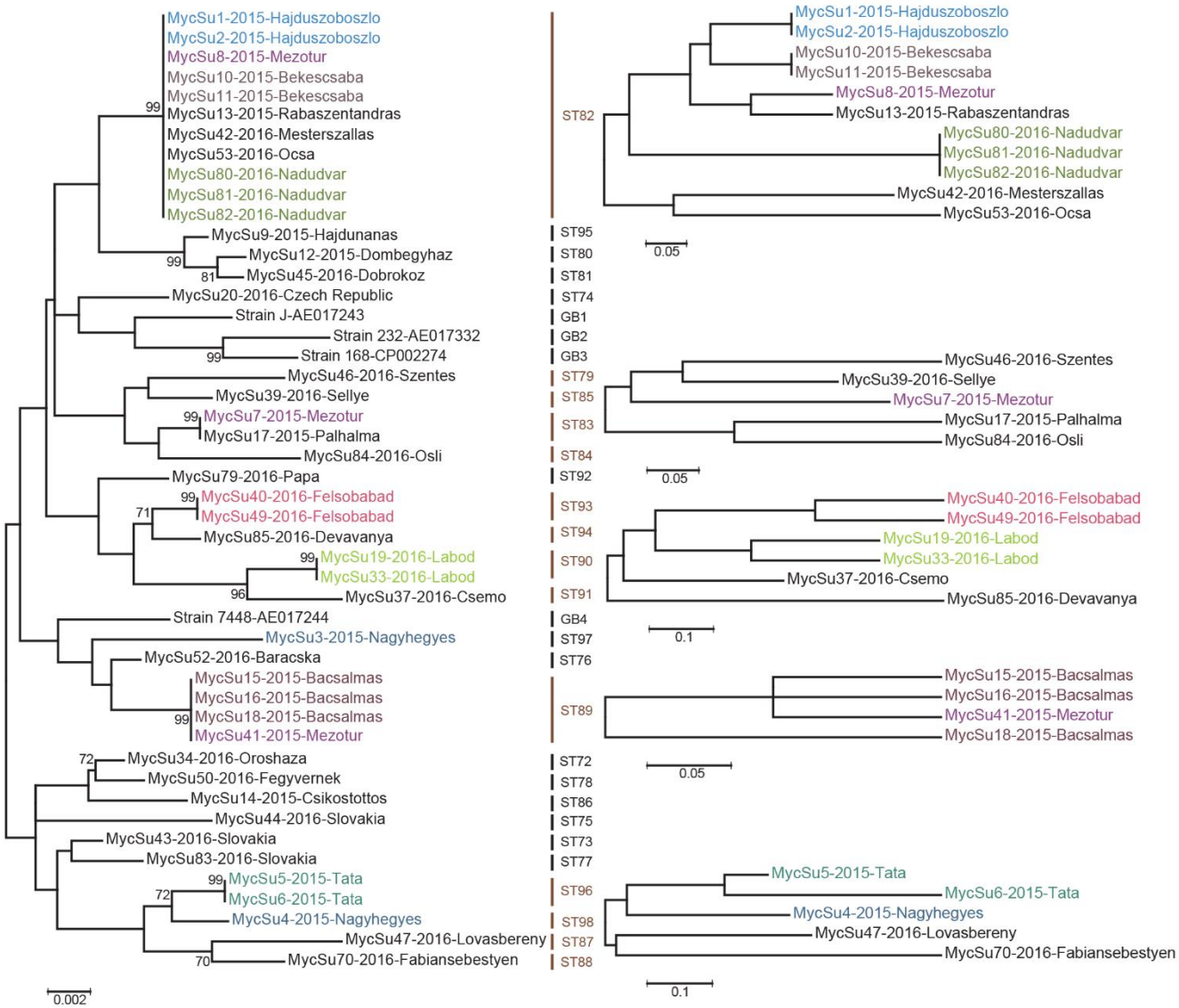

Figure 9. Resolution of identical sequence types of conventional MLST (multi-locus sequence typing) of 44 *M. hyopneumoniae* isolates by MLVA (multiple-locus variable-number tandem repeat analysis).

The MLST phylogenetic tree was constructed by Maximum-Likelihood method using *efp*, *metS*, *pgiB*, *recA*, *adk*, *rpoB* and *tpiA* genes, with 1000 bootstraps; only bootstrap values > 70 are presented. MLST sequence types further resolved by MLVA (using Locus1, -2, P97-RR1, -2 and *p146* loci) are marked brown. Resolution of the MLST STs by MLVA was accomplished by Neighbor-Joining method with 1000 bootstraps. The scale bar represents the average number of substitutions per site. Abbreviations: ST- sequence type, GB- sequence type of strains originating from public database. Colours indicate the common origin of the isolates.

5.2. Antibiotic susceptibility testing

The initial MIC values are evaluated and discussed throughout the study (Hannan, 2000), however, differences were registered between initial and final MIC values in certain cases. As official breakpoints of antibiotics against *M. hyopneumoniae* are not standardized, MIC values were compared to previously published, unofficial breakpoints (Hannan *et al.*, 1997a) in the present study. MIC values of the studied antimicrobial agents against the type strain (NCTC 10110) were consistent throughout the study (Table 15), and these results were mostly in accordance with previously defined values gained by microbroth dilution method (enrofloxacin 0.015-0.2 µg/ml, marbofloxacin 0.031 µg/ml, oxytetracycline 0.12-1 µg/ml, gentamicin 0.25-5 µg/ml, tylosin ≤0.015-0.06 µg/ml, tylvalosin 0.06 µg/ml, lincomycin 0.05-0.125 µg/ml, tiamulin 0.008-0.125 µg/ml, valnemulin ≤0.001-0.008 µg/ml) (Hannan *et al.*, 1997a,b; Vicca *et al.*, 2004; Thongkamkoon *et al.*, 2013; Tavío *et al.*, 2014; Klein *et al.*, 2017). However, minor differences (two-fold increase or decrease) were observed in the MIC values against the type strain compared to earlier data in case of doxycycline (0.06-0.5 µg/ml), spectinomycin (0.5 µg/ml), tilmicosin (0.25-1 µg/ml) and florfenicol (0.25-0.5 µg/ml) (Vicca *et al*., 2004; Thongkamkoon *et al*., 2013; Tavío *et al*., 2014; Klein *et al*., 2017). Moreover, the MIC value of tulathromycin was noticeably higher (10^3 difference between MIC values) than that reported in the literature (≤0.001-0.002 µg/ml) (Klein *et al.*, 2017). Previously published MIC values for gamithromycin were not available at the time of the present study. The MIC ranges, the MIC_{50} and MIC_{90} values of each antibiotic against the type strain and examined strains are recorded in Table 15 and illustrated in Figure 10. Initial and final MIC values of the individual strains are listed in Table S3-4.

All of the studied antimicrobial agents were effective against the examined *M. hyopneumoniae* isolates. However, increased MIC values of fluoroquinolones were registered, and an outlier strain (MycSu18) was inhibited by extremely high concentrations of macrolides and lincosamides. The distribution of the MIC values of fluoroquinolones (enrofloxacin and marbofloxacin) showed one main peak coinciding with MIC_{50} value at the examined lowest antibiotic concentration (≤0.039 µg/ml), while the other values represented equipartition with the highest MIC values (2.5 µg/ml and 5 µg/ml, respectively) (Figure 10A and B). One strain (MycSu17) exceeded the unofficial breakpoint (Hannan *et al.*, 1997a), with the initial MIC value of 2.5 µg/ml of enrofloxacin, however more strains showed increased final MIC values exceeding the breakpoint (Table 16). All of the examined tetracyclines had low MIC values with MIC_{50} and MIC_{90} values of ≤0.25 µg/ml and 2 µg/ml of oxytetracycline; and 0.078 µg/ml and 0.312 µg/ml of doxycycline (Figure 10C and D). The lowest examined concentration of gentamicin (≤0.25 µg/ml) was effective against most of the studied strains (Figure 10E). MIC_{50} and MIC_{90} values of spectinomycin were 2 µg/ml, with MIC 4 µg/ml being the highest detected

value (Figure 10F). Five macrolides were tested (Figure 10G-K), out of which tilmicosin showed a Gaussian distribution with 2 µg/ml and 4 µg/ml MIC_{50} and MIC_{90} values, respectively. One main peak at the examined lowest antibiotic concentration (≤0.25 µg/ml) was observed in the MIC values of tylosin and tylvalosin against the strains. MIC_{50} values of gamithromycin and tulathromycin were 0.5 µg/ml, while MIC_{90} values were 2 µg/ml and 1 µg/ml, respectively. Both MIC_{50} and MIC_{90} values of lincomycin coincided with the lowest examined antibiotic concentration (≤0.25 µg/ml) (Figure 10L). For all macrolides and for lincomycin high MIC values (>64 µg/ml of tilmicosin and tulathromycin; 64 µg/ml of gamithromycin; 32 µg/ml of tylosin; 2 µg/ml of tylvalosin; and >64 µg/ml of lincomycin) were detected against an outlier strain (MycSu18). Both studied pleuromutilins had low MIC values (Figure 10M and N). The MIC_{50} and MIC_{90} values of valnemulin were ≤0.039 µg/ml, while that of tiamulin ≤0.039 µg/ml and 0.078 µg/ml. MIC_{50} and MIC_{90} values of florfenicol were 1 µg/ml and 2 µg/ml (Figure 10O). No correlation was found between antibiotic susceptibility profiles and earlier assigned genotypes of the examined strains.

Table 15. MIC values against the type strain and summary of MIC ranges, MIC_{50} and MIC_{90} values (µg/ml) against the *M. hyopneumoniae* strains involved in this study

	NCTC 10110 initial	NCTC 10110 final	Range initial	Range final	MIC_{50} initial	MIC_{50} final	MIC_{90} initial	MIC_{90} final
Fluoroquinolones								
Enrofloxacin	≤0.039	0.078	≤0.039-2.5	≤0.039-5	≤0.039	0.312	1.25	2.5
Marbofloxacin	≤0.039	0.156	≤0.039-5	≤0.039-10	≤0.039	1.25	2.5	5
Tetracyclines								
Oxytetracycline	≤0.25	4	≤0.25-4	0.5-32	≤0.25	4	2	16
Doxycycline	≤0.039	0.625	≤0.039-0.625	0.078-2.5	0.078	0.625	0.312	2.5
Aminoglycoside								
Gentamicin	≤0.25	1	≤0.25-0.5	0.5-2	≤0.25	1	0.5	2
Aminocyclitol								
Spectinomycin	1	4	≤0.25-4	1-8	2	4	2	4
Macrolides								
Tylosin	≤0.25	0.5	≤0.25-32	≤0.25-64	0.25	0.5	≤0.25	0.5
Tilmicosin	2	8	≤0.25-≥64	2->64	2	8	4	16
Tylvalosin	≤0.25	≤0.25	≤0.25-2	≤0.25-8	≤0.25	≤0.25	≤0.25	≤0.25
Gamithromycin	1	4	≤0.25-64	1->64	0.5	4	2	8
Tulathromycin	1	4	≤0.25-≥64	0.5->64	0.5	2	1	4
Lincosamide								
Lincomycin	≤0.25	1	≤0.25-≥64	≤0.25->64	≤0.25	0.5	≤0.25	1
Pleuromutilins								
Tiamulin	≤0.039	0.156	≤0.039-0.156	0.078-0.312	≤0.039	0.156	0.078	0.156
Valnemulin	≤0.039	≤0.039	≤0.039	≤0.039	≤0.039	≤0.039	≤0.039	≤0.039
Phenicol								
Florfenicol	1	2	≤0.125-2	1-4	1	2	2	4

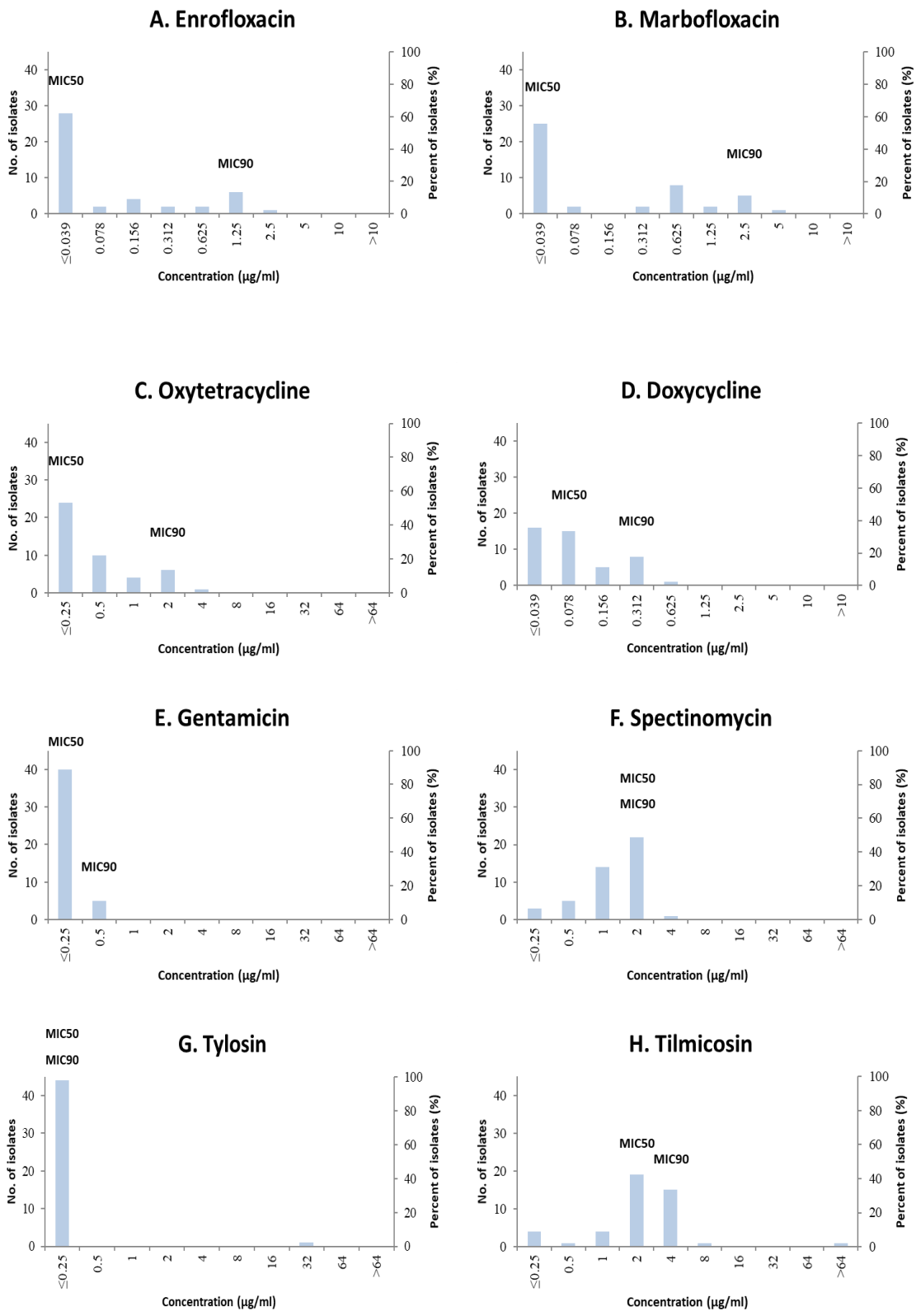

Figure 10. Distribution of the minimal inhibitory concentrations of each tested antibiotic against the studied *M. hyopneumoniae* isolates. MIC_{50} and MIC_{90} values are marked, respectively.

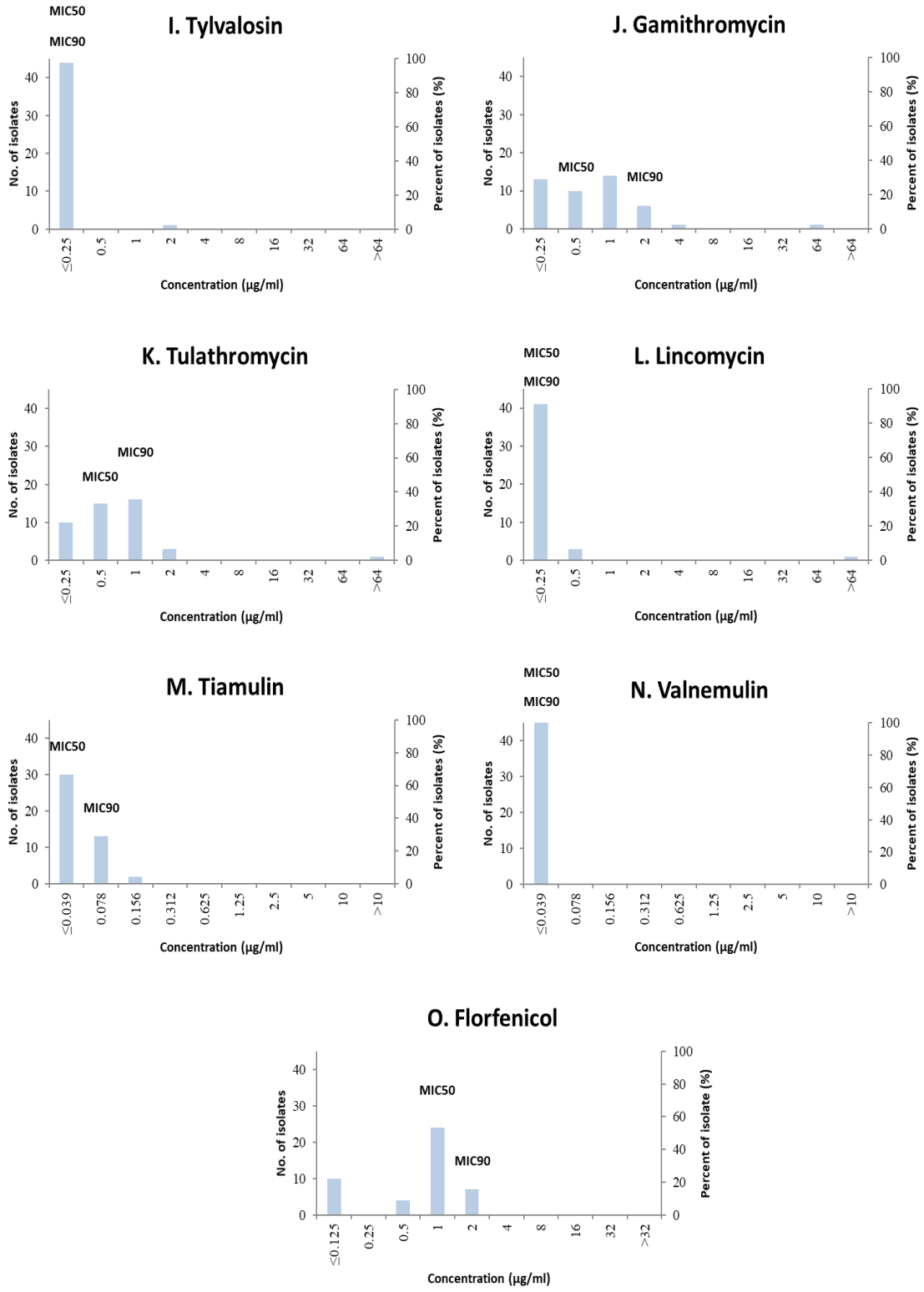

Figure 10 (continued). Distribution of the minimal inhibitory concentrations of each tested antibiotic against the studied *M. hyopneumoniae* isolates. MIC_{50} and MIC_{90} values are marked, respectively.

5.3. Identification of mutations responsible for increased minimum inhibitory concentration values of fluoroquinolones, macrolides and lincosamides

5.3.1. Fluoroquinolones

Both synonymous and non-synonymous substitutions were observed in genes associated with susceptibility to fluoroquinolones (*gyrA*, *gyrB*, *parC* and *parE*); however, only SNPs resulting in amino acid alterations were further examined in the present study. None of the amino acid changes in the genes *gyrB* and *parE* showed correlation with the defined MIC values. On the other hand, amino acid changes in the *gyrA* gene (Gly81Ala, Ala83Val and Glu87Gly) and in the *parC* gene (Ser80Phe, Ser80Tyr or Asp84Asn) correlated with decreased susceptibility of fluoroquinolones (Table 16). Single alterations in the *parC* gene seem to have no crucial effect on fluoroquinolone susceptibility when initial MIC values are examined. On the other hand, at least 12-fold concentration difference is observed in the final MIC values against strains, which contain a single alteration in the *parC* gene. As opposed to the observed slight increase of MIC values of fluoroquinolones in association with the single substitution event in gene *parC,* double substitutions in genes *parC* and *gyrA* correlated with final MIC values higher than 2 µg/ml (MycSu12; 15-18; 20; 41; 44-45; 50). Only one exception (MycSu44) was observed in case of marbofloxacin. The double substitutions in strain MycSu44 consisted of Ala83Val in gene *gyrA* and Asp84Asn in *parC*, while the rest of the strains showed various amino acid substitution types in gene *gyrA* but only the change of serine at amino acid position 80 in gene *parC* (Ser80Phe or Ser80Tyr). The observed SNPs in the strains originating from the same herds were consistent with one exception: the strains originating from Mezőtúr (MycSu7; 8 and 41), which also clustered into completely different sequence types according to the genotyping analysis showed distinct susceptibility profiles and genetic alterations correlating with antibiotic susceptibility.

5.3.2. Macrolides and lincosamides

Correlation was described between increased MIC values of macrolides and lincosamides against *Mycoplasma* species/*M. hyopneumoniae* and SNPs in the 23S rRNA sequence (Stakenborg *et al.*, 2005a; Gautier-Bouchardon, 2018). A nucleotide substitution at the position A2059G in the 23S rRNA sequence was found in the outlier strain (MycSu18) showing extremely decreased susceptibility to macrolides and lincosamides (Table 16 and Table S4).

Table 16. Initial and final minimum inhibitory concentration (MIC) ranges (µg/ml) of fluoroquinolones, macrolides and lincomycin against the examined *M. hyopneumoniae* isolates with the amino acid substitutions in the *gyrA* and *parC* genes and nucleotide substitutions in the 23S rRNA sequence.

Sample ID	Range of MIC values (µg/ml)								Substitutions		
	Fluoroquinolones		16-membered macrolides		15-membered macrolides		Lincomycin		*gyrA*	*parC*	23S rRNA
	initial	final	initial	final	initial	final	initial	final			
MycSu4	≤0.039	≤0.039	≤0.25-4	≤0.25-8	≤0.25-0.5	2-4	≤0.25	0.5			
MycSu9	≤0.039	≤0.039	≤0.25-2	≤0.25-8	≤0.25-0.5	1-2	≤0.25	0.5			
MycSu34	≤0.039	≤0.039	≤0.25-4	≤0.25-8	≤0.25-0.5	2-4	≤0.25	0.5			
MycSu43	≤0.039	≤0.039	≤0.25-8	≤0.25-16	1	2-8	≤0.25	1			
MycSu5	≤0.039	≤0.039-0.078	≤0.25-2	≤0.25-4	≤0.25-0.5	1-4	≤0.25	0.5			
MycSu40	≤0.039	≤0.039-0.078	≤0.25-4	≤0.25-16	1-2	2-16	≤0.25	0.5			
MycSu42	≤0.039	≤0.039-0.078	≤0.25-4	≤0.25-16	2-4	4-8	0.5	1			
MycSu49	≤0.039	≤0.039-0.078	≤0.25-4	≤0.25-8	0.5-2	2-4	≤0.25	0.5			
MycSu70	≤0.039	≤0.039-0.078	≤0.25-1	≤0.25-2	≤0.25	0.5-2	≤0.25	1			
MycSu83	≤0.039	≤0.039-0.078	≤0.25-4	≤0.25-8	1-2	1-8	≤0.25	0.5			
MycSu84	≤0.039	≤0.039-0.078	≤0.25-2	≤0.25-4	0.5	2	≤0.25	0.5			
MycSu47	≤0.039	≤0.039-0.156	≤0.25-2	≤0.25-32	2	4-8	≤0.25	1			
MycSu6	≤0.039	0.078	≤0.25-2	≤0.25-8	0.5-1	2-4	≤0.25	1			
MycSu7	≤0.039	0.078	≤0.25-0.5	≤0.25-4	≤0.25	2	≤0.25	0.5			
MycSu14	≤0.039	0.078	≤0.25-2	≤0.25-8	0.5-1	2-8	≤0.25	1			
MycSu52	≤0.039	0.078	≤0.25-2	≤0.25-8	1	2-4	≤0.25	0.5			
MycSu85	≤0.039	0.078	≤0.25-2	≤0.25-8	0.5	1-4	≤0.25	1			
MycSu3	≤0.039	0.078-0.156	≤0.25-2	≤0.25-4	1	2-4	≤0.25	0.5			
MycSu2	≤0.039	0.312-1.25	≤0.25-4	≤0.25-4	2	4	0.5	1		Ser80Phe	
MycSu10	≤0.039	0.312-1.25	≤0.25-2	≤0.25-4	0.5-1	1-4	≤0.25	0.5		Ser80Phe	
MycSu11	≤0.039	0.312-1.25	≤0.25-2	≤0.25-8	0.5-1	2-4	≤0.25	1		Ser80Phe	
MycSu39	≤0.039	0.312-1.25	≤0.25-2	≤0.25-4	≤0.25	0.5-2	≤0.25	1		Asp84Asn	
MycSu33	≤0.039-0.078	0.312-1.25	≤0.25-2	≤0.25-8	≤0.25-0.5	2-4	≤0.25	1		Ser80Phe	
MycSu79	≤0.039-0.078	0.312-1.25	≤0.25-4	≤0.25-16	1	2-4	≤0.25	0.5		Ser80Tyr	
MycSu1	≤0.039-0.312	0.312-1.25	≤0.25-4	≤0.25-8	1	2-4	≤0.25	1		Ser80Phe	
MycSu13	≤0.039-0.312	0.312-1.25	≤0.25-2	≤0.25-4	1	1-2	≤0.25	1		Ser80Phe	
MycSu8	0.078-0.625	0.312-1.25	≤0.25-2	≤0.25-4	0.5	1-2	≤0.25	0.5		Ser80Phe	
MycSu46	0.078-0.625	0.312-1.25	≤0.25-4	≤0.25-16	1-2	2-8	≤0.25	1		Ser80Phe	
MycSu19	0.156-0.625	0.312-1.25	≤0.25-1	≤0.25-4	0.5	1-2	≤0.25	1		Ser80Phe	

Table 16 (continued). Initial and final minimum inhibitory concentration (MIC) ranges (μg/ml) of fluoroquinolones, macrolides and lincomycin against the examined *M. hyopneumoniae* isolates with the amino acid substitutions in the *gyrA* and *parC* genes and nucleotide substitutions in the 23S rRNA sequence.

Sample ID	Range of MIC values (μg/ml)								Substitutions		
	Fluoroquinolones		16-membered macrolides		15-membered macrolides		Lincomycin		*gyrA*	*parC*	23S rRNA
	initial	final	initial	final	initial	final	initial	final			
MycSu53	≤0.039	0.625-1.25	≤0.25	≤0.25-4	≤0.25-0.5	1-4	≤0.25	≤0.25		Ser80Phe	
MycSu81	0.156-0.625	0.625-1.25	≤0.25-2	≤0.25-8	1	1-4	≤0.25	0.5		Ser80Phe	
MycSu82	0.156-0.625	0.625-1.25	≤0.25-4	≤0.25-8	0.5	2-4	≤0.25	0.5		Ser80Phe	
MycSu37	≤0.039-0.312	0.625-2.5	≤0.25	≤0.25-4	≤0.25-1	2-4	≤0.25	1		Ser80Phe	
MycSu80	0.312-0.625	0.625-2.5	≤0.25-2	≤0.25-4	0.5	2-4	≤0.25	1		Ser80Phe	
MycSu44	0.625	1.25-2.5	≤0.25-4	≤0.25-16	≤0.25-0.5	2-4	≤0.25	0.5	Ala83Val	Asp84Asn	
MycSu20	0.156-0.625	2.5-5	≤0.25-4	≤0.25-8	1	1-4	≤0.25	0.5	Ala83Val	Ser80Tyr	
MycSu12	0.625-1.25	2.5-5	≤0.25-1	≤0.25-8	1	2-4	≤0.25	0.5	Ala83Val	Ser80Tyr	
MycSu15	1.25-2.5	2.5-5	≤0.25-4	≤0.25-8	≤0.25-1	1-2	≤0.25	≤0.25	Gly81Ala	Ser80Tyr	
MycSu16	1.25-2.5	2.5-5	≤0.25-2	≤0.25-8	≤0.25	1	≤0.25	0.5	Gly81Ala	Ser80Tyr	
MycSu18	1.25-2.5	2.5-5	2>64	8->64	64->64	>64	>64	>64	Gly81Ala	Ser80Tyr	A2059G
MycSu45	1.25-2.5	2.5-5	≤0.25	≤0.25-8	≤0.25	2-4	≤0.25	1	Glu87Gly	Ser80Phe	
MycSu41	1.25	5	≤0.25-1	≤0.25-4	≤0.25	1-2	≤0.25	1	Gly81Ala	Ser80Tyr	
MycSu50	1.25-2.5	5	≤0.25	≤0.25-8	≤0.25	2-8	≤0.25	0.5	Glu87Gly	Ser80Tyr	
MycSu17	2.5-5	5-10	≤0.25-4	≤0.25-8	0.5-1	2-8	0.5	1	Ala83Val	Ser80Phe	

5.4. Development of molecular assays for the rapid detection of antibiotic susceptibility of *M. hyopneumoniae*

5.4.1. Validation of mismatch amplification mutation and high resolution melt assays

All melt- and agarose-MAMAs and the HRM assay clearly differentiated the sensitive and resistant genotypes (Figures 11-14), correlating with the results of the microbroth dilution method of the 44 *M. hyopneumoniae* field isolates and the type strain (*M. hyopneumoniae* J, NCTC 10110) (Tables S3-4 and Figure 10). Melting temperatures and sizes of amplicons are listed in Table 17.

Two MAMAs (both agarose and melt assays) were developed for the detection of decreased fluoroquinolone susceptibility. The assays target the nucleotide substitutions C239T/A or G250A in the *parC* gene (Figures 11-12), which showed correlation with the final MIC range 0.312-10 µg/ml (Table 16). The sensitivity of the melt- and agarose-MAMA assays targeting the nucleotide substitution C239T/A were 10^4 copies for the SNPs correlating with decreased MIC values and 10^3 copies for the fluoroquinolone sensitive genotype. The sensitivity of the melt- and agarose-MAMA assays targeting the nucleotide substitution C250A were 10^2-10^3 copies for the SNPs correlating with decreased MIC values and 10^3 copies for the fluoroquinolone sensitive genotype. No cross-reaction was noted, however, non-specific product of *M. flocculare* (Tm=78.7 °C) might be present (Figure 12). Melt curve analysis for PCR amplicons showed slight shifts in the melting temperatures between independent runs, however, the melt curve shapes and temperature differences remained unchanged. All samples reached a plateau before the end of the real-time PCR amplification stage.

As the SNPs (C235T/A; G250A) in the *parC* gene are close to each other, an HRM assay was also designed for the rapid discrimination of sensitive and resistant strains (Figure 13). Normalization interval of 74.0-74.5 °C and 81.0-81.5 °C were used in the analysis of the amplicons. No cross-reaction was noted with other porcine Mycoplasmas. The sensitivity of the assay was 10^5 copies for the fluoroquinolone sensitive type strain and for the strains containing Ser80Tyr or Asp84Asn substitutions in the *parC* gene. The sensitivity of the system was 10^6 copies per reaction in case of Ser80Phe alteration. All of the 44 examined *M. hyopneumoniae* isolates were successfully differentiated sensitive or resistant.

Table 17. Results of the MAMA and HRM assays

Assay type	Target SNP	Antibiotic group	Type	T_m (°C)	Amplicon (bp)	Sensitivity	Cross-reactions
MAMA	*parC* C239T/A	Fluoroquinolone	S R	75.6 79.9	74 86	10^3 10^4	-
	parC G250A	Fluoroquinolone	S R	77.7 80.9	84 96	10^3 10^{2-3}	*M. flocculare*
	23S rRNA A2059G	Macrolide, Lincosamide	S R	77.1 79.2	85 97	10^{3-4} 10^{3-4}	*M. hyorhinis* *M. flocculare*
HRM	*parC* C239T/A, G250A	Fluoroquinolone	S R	78.6-78.7 78.1-78.5	93	10^5 10^{5-6}	-

Abbreviations: S- sensitive, R- resistant

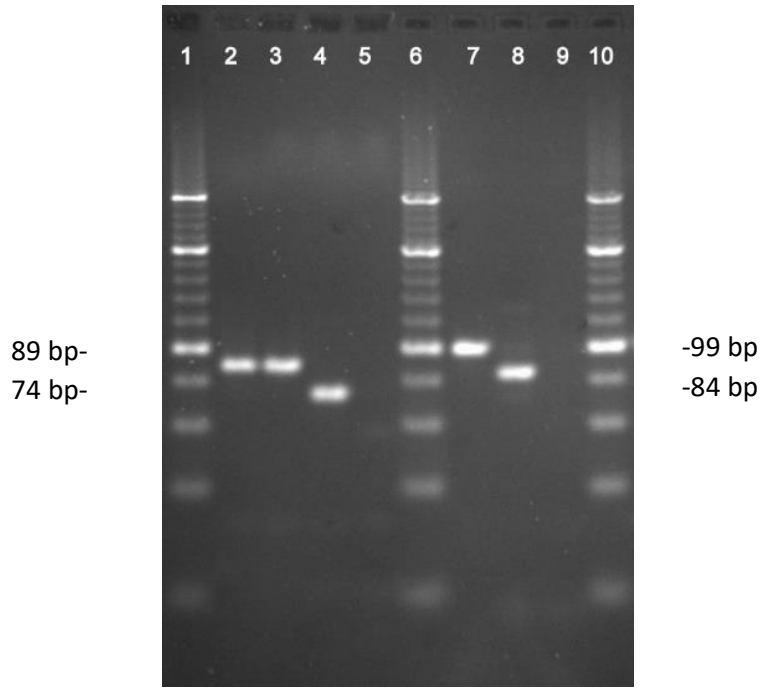

Figure 11. Agarose-MAMAs for the detection of C239T/A and G250A of the *parC* gene. Electrophoresis was performed in 2% agarose gel (SeaKem LE Agarose Lonza Inc.). Lanes 1, 6 and 10: 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific Inc.); Lane 2: fluoroquinolone resistant MycSu1 (C239T substitution; 89 bp); Lanes 3: fluoroquinolone resistant MycSu18 (C239A substitution; 89 bp); Lanes 4 and 8: fluoroquinolone sensitive *M. hyopneumoniae* type strain (J strain, NCTC10110; 74 bp and 84 bp); Lanes 5 and 9: negative control; Lane 7: fluoroquinolone resistant MycSu39 (G250A substitution; 99 bp).

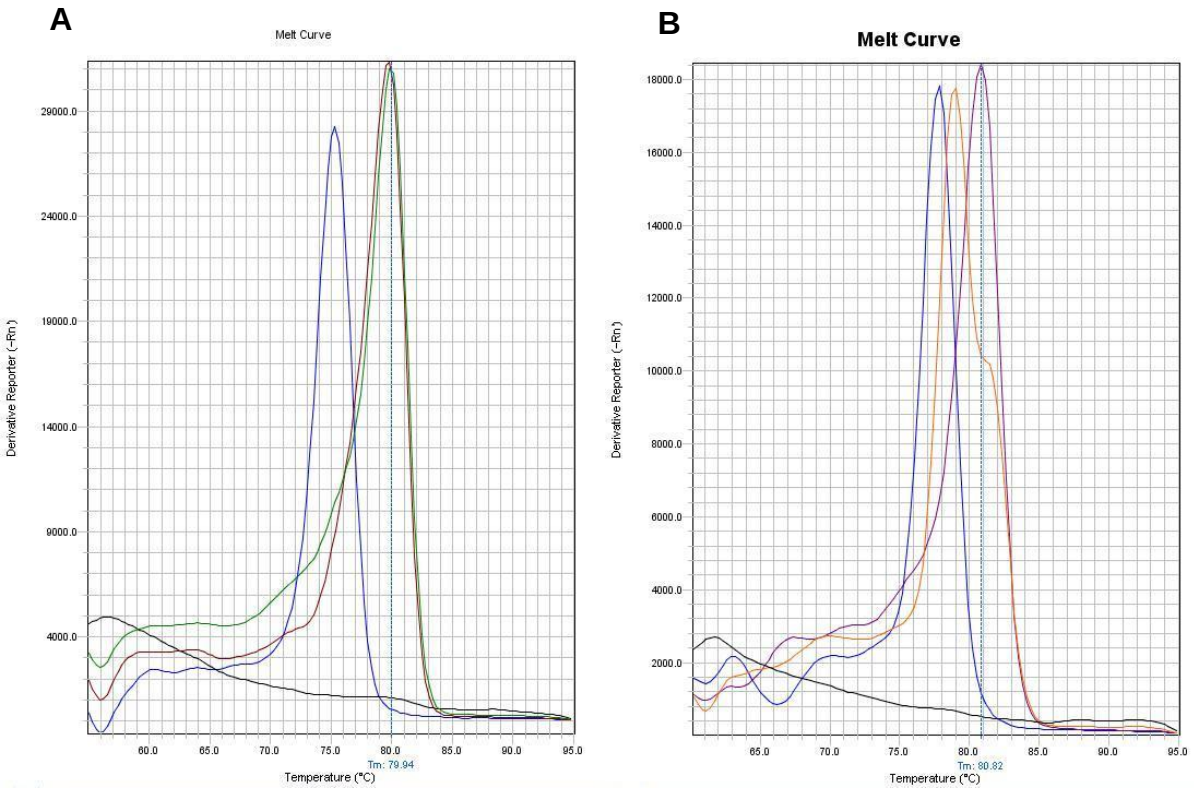

Figure 12. Melting curves of melt-MAMAs designed for *parC* gene of *M. hyopenumoniae*
A. Melt-MAMA designed for C239T/A substitution according to *E. coli* numbering.
The melting curves show melting temperatures of the fluoroquinolone sensitive *M. hyopneumoniae* type strain (J strain, NCTC10110) (blue line) (T_m=75.6 °C) and fluoroquinolone resistant strains MycSu1 (dark red), MycSu18 (green line) (T_m=79.9 °C). The negative control (black line) did not show a specific melting curve.

B. Melt-MAMA designed for G250A substitution according to *E. coli* numbering.
The melting curves show melting temperatures of the fluoroquinolone sensitive *M. hyopneumoniae* type strain (J strain, NCTC10110) (blue line) (T_m=77.7 °C), fluoroquinolone resistant strain MycSu39 (violet line) (T_m=80.9 °C) and the aspecific *M. flocculare* (orange line) (T_m=78.8 °C). The negative control (black line) did not show a specific melting curve.

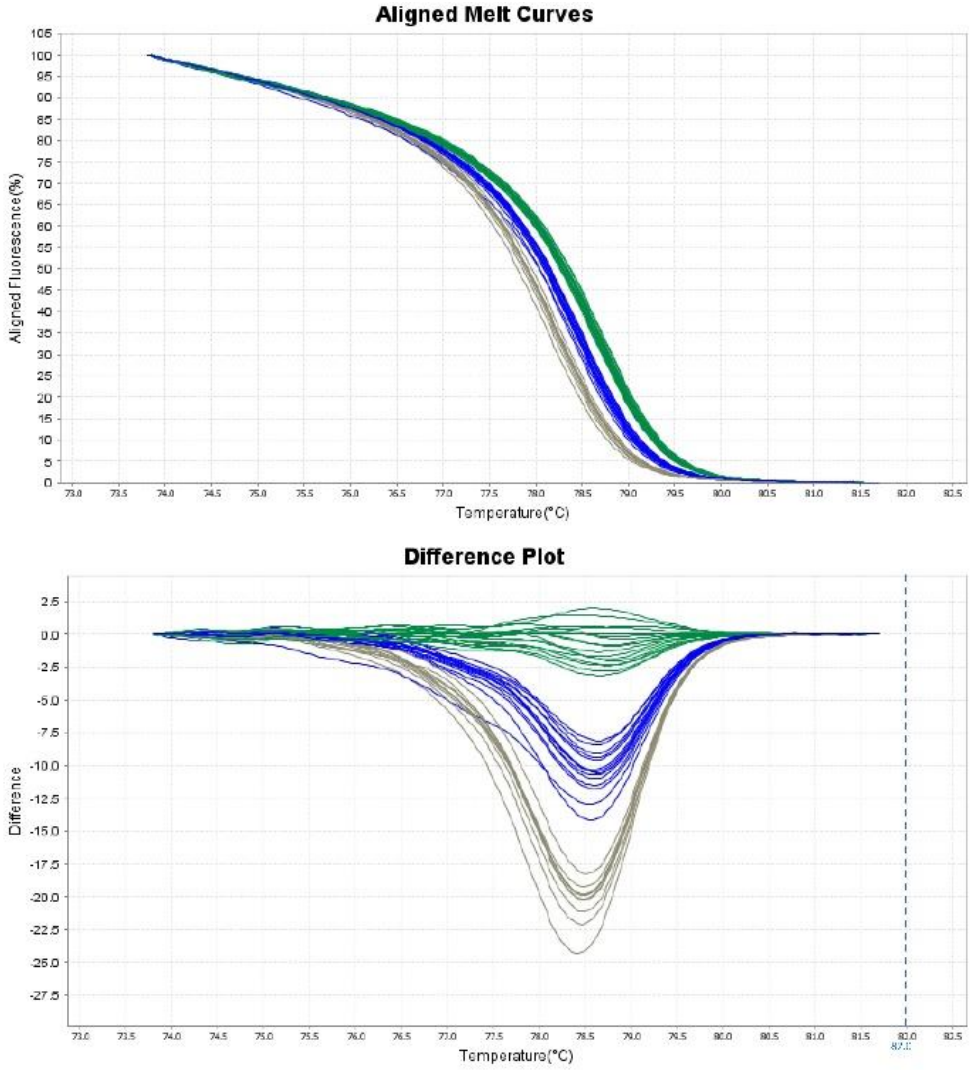

Figure 13. HRM analyses of 44 *M. hyopneumoniae* isolates for the detection of fluoroquinolone resistance.
Aligned melt curves and difference plots of the assay *parC* (hot spot region 239-250 of *parC*). Fluoroquinolone sensitive type (green lines) (T_m= 78.6-78.7 °C) and fluoroquinolone resistant type C239T (blue lines) (Tm=78.4-78.5 °C), C239A or G250A (grey lines) (Tm=78.1-78.2 °C).

One MAMA (both agarose- and melt assays) was designed for the detection of macrolide and lincosamide resistant strains containing an A2059G substitution in the nucleotide sequence of the 23S rRNA (Figure 14). The resistant and sensitive genotypes are clearly differentiated in both the agarose- and the melt assay. The sensitivity of the agarose -MAMA assay targeting the nucleotide substitution A2059G was 10^2-10^3 copies for the SNPs correlating with decreased MIC values and 10^3 copies for the sensitive genotype. The sensitivity of the melt-MAMA assay targeting the same substitution was 10^4 copies for both genotypes. However, the resistant genotype shows bimodal peak in the melting analysis (with one specific peak at 80 °C and a non-specific peak at 83 °C) and cross-reaction can be observed with *M. hyorhinis* (Tm=76.8 °C) and *M. flocculare* (Tm=77.2 °C) (Figure 14).

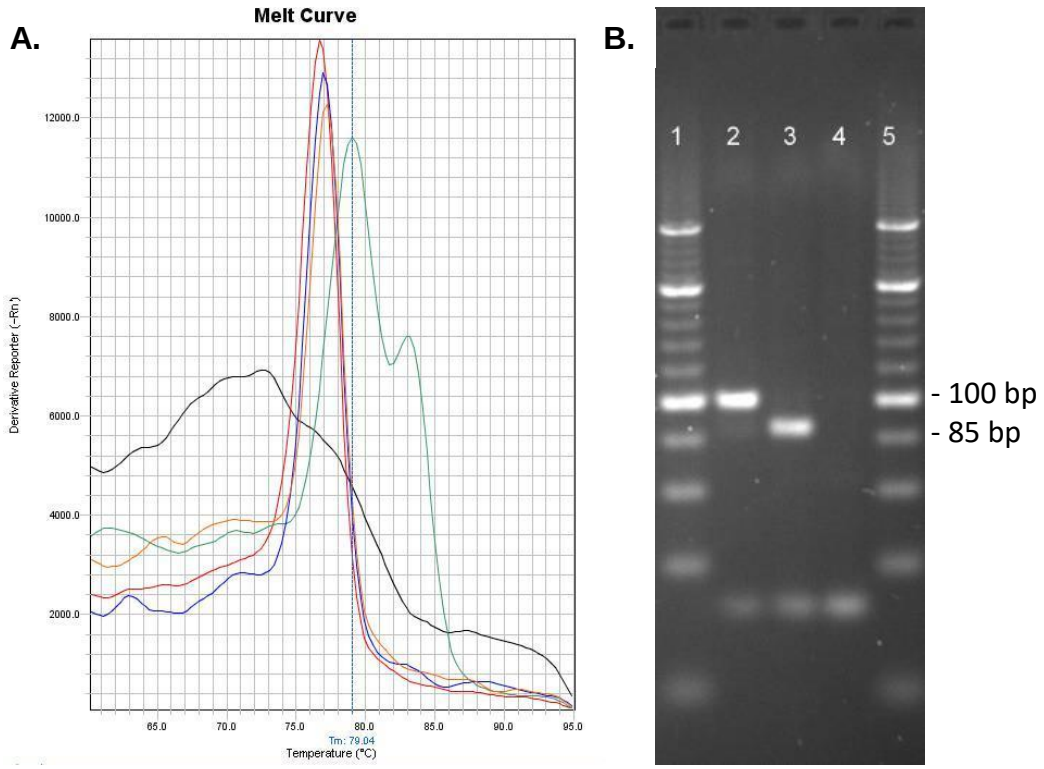

Figure 14. Mismatch amplification mutation assays for the detection of macrolide resistance

A. Melt-MAMA designed for 23S rRNA of *M. hyopenumoniae* (A2059G according to *E. coli* numbering).

The melting curves show melting temperatures of the fluoroquinolone sensitive *M. hyopneumoniae* type strain (J strain, NCTC10110) (blue line) (T_m=77.1 °C), the resistant field isolate MycSu18 (green line) (T_m=79.2 °C) and cross-reactions of *M. hyorhinis* (red line) (T_m=76.8 °C) and *M. flocculare* (orange line) (T_m=77.2 °C). The negative control (black line) showed non-specific melting temperature.

B. Agarose-MAMA for the detection of A2059G substitution in the 23S rRNA sequence.

Electrophoresis was performed in 2% agarose gel (SeaKem LE Agarose Lonza Inc.). Lane 1 and 5: 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific Inc.); Lane 2: macrolide and lincosamide resistant MycSu18 (A2059G substitution; 100 bp); Lane 3: macrolide and lincosamide sensitive *M. hyopneumoniae* type strain (J strain, NCTC10110; 85 bp); Lane 4: negative control.

6. Discussion

6.1. Genotyping

Transmission routes of *M. hyopneumoniae* involve direct contact of infected animals or airborne transmission, and intensive animal transport may facilitate the spread of the pathogen, thus PCR-based typing assays able to discriminate the bacteria on strain level, are useful tools in epizootiological investigations (Mayor *et al.*, 2007; Mayor *et al.*, 2008; Vranckx *et al.*, 2011; Charlebois *et al.*, 2014). In addition, molecular typing studies allowed better understanding of the variability of *M. hyopneumoniae* strains circulating worldwide (Michiels *et al.*, 2017). However, the comparison of the different typing methods and the determination of their most appropriate application based on their discriminatory power and robustness have not been performed yet.

In the present study, genotyping of Hungarian (n=40), Czech (n=1) and Slovakian (n=3) *M. hyopneumoniae* isolates was accomplished by different schemes of MLST and MLVA assays with and without the extension of the assays by the gene *p146* analysis. While certain isolates with common herd origin shared the same ST or MLVA profile (e.g. MycSu80-82), divergences were also found (e.g. MycSu7-8; 41 or MycSu3-4). High variability of the strains was defined by all molecular typing methods used, in consistence with previous observations (de Castro *et al.*, 2006; Stakenborg *et al.*, 2006; Calus *et al.*, 2007; Pulgarón *et al.*, 2015; Pantoja *et al.*, 2016). Strains which had identical MLST STs, were further differentiated by the MLVA assays, representing multiple variants in certain herds, which also confirms earlier findings (Michiels *et al.*, 2017). Although only pure cultures were intended to be examined in the present study, the MLVA assay detected multiple allele-variants in certain cases (MycSu4, MySu7, MycSu19, MycSu33, MycSu40, MycSu70 and MycSu84) either indicating the presence of more than one strain in the isolates (Vranckx *et al*., 2011; Charlebois *et al.*, 2014) or the presence of alternate alleles revealing potential mutations (Gyuranecz *et al.*, 2013). Neither the MLST assays nor the analyses of gene *p146* revealed multiple allele variants, which assumes that these methods are less sensitive to slight mutations detected by MLVA.

MLST is a robust and reproducible method, used for intermediate scale typing of *M. hyopneumoniae* strains in epizootiologic and phylogenetic examinations. Although certain matches on allele levels were defined, all of the studied isolates represented new MLST STs, which also indicates high variability of the *M. hyopneumoniae* strains. The minimal MLST and the conventional scheme showed congruency and shared identical discriminatory power (0.907) in the present study, supporting the use of the minimal MLST for phylogenetic investigations (Mayor *et al.*, 2008; Kuhnert *et al.,* 2011). Moreover, the combination of the minimal MLST with gene *p146* (Overesch and Kuhnert, 2017) differentiated common MLST

STs of strains with different herd of origin (MycSu1-2 and MycSu10-11), providing more detailed data for phylogenetic studies. Therefore, the minimal MLST combined with gene *p146* proved to be a cost-effective solution for the high resolution of *M. hyopneumoniae* strains.

Extended MLVA assays, applicable in laboratories with basic equipment, showed the highest interchangeability with the other studied methods, and they provided the most refined data, therefore their utilisation offers cost-effective and rapid tools for epizootiologic purposes (Charlebois *et al*., 2014). Since the extended MLVA is suitable for the resolution of common MLST STs, the combination of the assays could support deep phylogenetic studies in the case of this highly variable pathogen.

Analysing the nucleotide sequence of the gene *p146* is feasible for the majority of the laboratories using clinical material (Savic *et al.* 2010), and it showed higher congruency with the MLVA assays than with the MLST methods. However, the use of gene *p146* analysis in epizootiologic studies is not recommended, as the highly variable nature of this gene may determine the same STs for independent isolates incorrectly. Accordingly, the comparison of the current results with previously published data revealed that the sequence of gene *p146* of the Belgian *M. hyopneumoniae* strain B2V1W20 (GenBank Accession Number: JF461513) and four Hungarian isolates (ST96, MycSu15-16; 18 and 41) were identical, however, no further agreement was defined. Therefore, the method is proposed to be used either as an extension of MLST analysis by nucleotide sequence comparison or of an MLVA assay by determining the poly-serine region of gene *p146*.

In the present study molecular typing of *M. hyopneumoniae* strains originating from a relatively restricted geographic origin and short time interval was accomplished by different combinations of MLST and MLVA assays and the sequence analysis of gene *p146*. Our results revealed high diversity of the pathogen circulating in the Central European region. MLVA is a cost-effective and highly discriminative method for epizootiologic investigations, while for the reliable exploration of genetic relationships of *M. hyopneumoniae* isolates, with increasing refinement of the data, the MLST alone, expanded with *p146* gene or as a combination with the extended MLVA analyses is recommended, respectively.

6.2. Antibiotic susceptibility testing

Directed antibiotic therapy can decrease the emergence of resistant bacteria. However, antibiotic susceptibility testing of porcine mycoplasmas is not performed routinely, because it is fastidious, time-consuming and requires special techniques and media (Hannan, 2000). Furthermore, the lack of official standards makes the interpretation of the results difficult. The Clinical and Laboratory Standards Institute (CLSI) has provided official breakpoints for certain antibiotics but only for human pathogen mycoplasmas (Wayne, 2011) and the procedures and media vary according to each of the examined species (Waites *et al.*, 2012). Although all of the examined antimicrobial agents were effective against the majority of the *M. hyopneumoniae* field isolates, declined susceptibility was also observed in case of fluoroquinolones (e.g. MycSu17), macrolides and lincosamides (MycSu18).

Fluoroquinolones are potentially active antimicrobial agents against *M. hyopneumoniae* through inhibition of the bacterial DNA gyrase and topoisomerase IV enzymes (Hooper, 2000; Gautier-Bouchardon, 2018). In the present study, a broad range of MIC values was recorded with low MIC_{50} value of enrofloxacin, similarly to previous results in other European publications in the last 20 years (Hannan *et al.*, 1997a,b; Vicca *et al.*, 2004; Klein *et al.*, 2017). Some of the examined strains (e.g. MycSu17) were inhibited by higher enrofloxacin concentration, the final MIC value against these strains exceeded the unofficial breakpoint determined by Hannan *et al.* (Hannan *et al.*, 1997a). Similar observations have already been recorded with high MIC values in Thailand (≥2 µg/ml) and in Belgium (>1 µg/ml) (Vicca *et al.*, 2004; Thongkamkoon *et al.*, 2013), which forewarns the importance of susceptibility testing before choosing antibiotics for treatment. Although MIC_{50} value of marbofloxacin against the studied strains was mostly in accordance with recent data (Klein et al., 2017), MIC_{90} value against the Hungarian isolates was higher than those against Belgian, Spanish and British strains (0.5-1 µg/ml). The increasing susceptibility against fluoroquinolones is a notable problem, because these agents are important antibiotics for human therapy (Collignon *et al.*, 2009). To maximize efficacy and reduce mutant selection in case of fluoroquinolones, the ratio of maximum serum concentration to the MIC (C_{max}/MIC ratio) of equal or higher than 10 was proposed (Rodvold *et al.*, 2001). Marbofloxacin administered at 4 or 8 mg/kg intramuscularly resulted in 6.3 and 3.38 µg/ml C_{max} in pigs (Schneider *et al.*, 2014) respectively, resulting in maximum activity against strains with MICs of 0.625 and 0.3125 µg/ml or lower in case of the two dosages, respectively.

Tetracyclines are frequently used to control *M. hyopneumoniae* infections, and they act by binding to the decoding centre of the small bacterial ribosomal subunit (Maes *et al.*, 2008; Nguyen *et al.*, 2014). Most of the previous publications from Europe defined similar MIC_{50} and MIC_{90} values of oxytetracycline (Hannan *et al.*, 1997a; Vicca *et al.*, 2004; Klein *et al.*, 2017);

but higher MIC_{50} and MIC_{90} values of doxycycline were described against strains originating from Spain (1 µg/ml both) and Thailand (3.12 µg/ml and 6.25 µg/ml) than against the Hungarian isolates. According to other publications also supported by our results, tetracyclines are still active against *M. hyopneumoniae* despite their long-standing usage in human and veterinary medicine (Thongkamkoon *et al.*, 2013; Tavío *et al.*, 2014).

The aminoglycoside gentamicin seems to be an effective antimicrobial agent against *M. hyopneumoniae*, as low MIC_{50} and MIC_{90} values were observed in the present study, similar to earlier data (Vicca *et al.*, 2004; Tavío *et al.*, 2014). Although MIC range of the aminocyclitol antibiotic spectinomycin was broadly similar to the findings of a previous Spanish study, the MIC_{50} and MIC_{90} values were higher in the present study compared to Spanish and Belgian MIC values (Vicca *et al.*, 2004; Tavío *et al.*, 2014).

Macrolides are among the most frequently used antibiotics in the swine industry to treat *M. hyopneumoniae* infections (Maes *et al.*, 2008). Both 16-membered (tylosin, tilmicosin and tylvalosin) and 15-membered (tulathromycin and gamithromycin) macrolides were effective against the studied strains. However, the MIC value of tulathromycin against the type strain (J strain, NCTC 10110) was three orders of magnitude higher, than in the literature (Klein *et al.*, 2017). The reason for the discrepancy might be a different passage number or the different medium/antibiotic solution used during the test. However, the MIC value of tulathromycin against the type strain did not exceed 16 µg/ml (a possible unofficial breakpoint according to other porcine respiratory pathogens [Godinho, 2008]), in either case. In the current study, a slight increase of MIC_{50} and MIC_{90} values of macrolides was described compared to the literature (Klein *et al.*, 2017), and extremely high MIC values against an outlier strain (MycSu18) was noted. According to the habituation study of Hannan *et al*. (1997b) and the high MIC values presented in this study, emergence of macrolide-resistance could be a considerable problem, which was confirmed by earlier reported results from Belgium (Vicca *et al.*, 2004), Thailand (Thongkamkoon *et al.*, 2013) and Spain (Tavío *et al.*, 2014).

Lincomycin is also active against *M. hyopneumoniae*, but extremely high MIC values appear every now and then (Vicca *et al.*, 2004; Thongkamkoon *et al.*, 2013; Tavío *et al.*, 2014), like the outlier strain (MycSu18) in the present study. The reason of the decreased susceptibility can be the cross-resistance with macrolides, as reported in an earlier publication, which described decreasing susceptibility against tylosin and lincomycin in strains originating from a lincomycin-treated herd (Vicca *et al.*, 2004). The simultaneously appearing change in susceptibility may lead back to the same mode of action of macrolides and lincosamides, inhibiting bacterial protein synthesis on the 50S ribosomal subunit (Weisblum, 1995).

Pleuromutilins are important antibiotics to control *M. hyopneumoniae* infections through inhibiting bacterial protein synthesis (Poulsen *et al.*, 2001). According to our results and other publications, tiamulin seems to be one of the most effective antimicrobial agents against

M. hyopneumoniae with low *in vitro* inhibitory concentrations (Hannan *et al.*, 1997a,b; Vicca *et al.*, 2004; Thongkamkoon *et al.*, 2013; Tavío *et al.*, 2014; Klein *et al.*, 2017). Valnemulin is the most effective antibiotic against all of the studied strains, which supported the earlier published observations (Hannan *et al.,* 1997b; Tavío *et al.*, 2014; Klein *et al.*, 2017).

The chloramphenicol derivative florfenicol is an inhibitor of bacterial protein synthesis, used exclusively for veterinary purposes (Priebe and Schwarz, 2003). The moderate distribution of the MIC range and the relatively low MIC_{50} and MIC_{90} values of florfenicol, were similar to earlier observations from different parts of Europe and Thailand (Vicca *et al.*, 2004; Thongkamkoon *et al.*, 2013; Klein *et al.*, 2017), and they may indicate that this antibiotic is an effective agent against *M. hyopneumoniae*.

In vitro MIC values do not necessarily correlate with the effectiveness of the antimicrobials *in vivo* and interpretation of the MIC distributions is difficult as *Mycoplasma* species with veterinary relevance do not have official clinical breakpoints (Klein *et al.*, 2017). Furthermore, strains with different antibiotic susceptibility can coexist within a herd (Thongkamkoon *et al.*, 2013). Pharmacokinetic-pharmacodynamic analysis is an important tool to maximize *in vivo* antimicrobial activity (Ahmad *et al.*, 2016; Somogyi *et al*., 2018). Most of our results were in accordance with other results of the European region, this involves, that all the tested agents are most probably still suitable to control enzootic pneumonia. Nonetheless, the results of this study may help veterinarians to choose the proper antimicrobial agent against *M. hyopneumoniae.* Although the isolation of *M. hyopneumoniae* strains is a time-consuming and fastidious process, the regularly accomplished antibiotic susceptibility testing of the swine herds should enable appropriate antibiotic use during treatment.

6.3 Identification of mutations responsible for increased minimum inhibitory concentration values of fluoroquinolones, macrolides and lincosamides

Although antimicrobials cannot eliminate *M. hyopneumoniae*, they play an important role in reducing clinical signs, furthermore antimicrobials or combinations of antibiotic agents that are also active against secondary bacteria complicating mycoplasma-pneumonia are indicated to be used. Long term antibiotic therapy enables the accumulation of drug residues and the emergence of resistant bacteria (Maes *et al.*, 1996, 2008). Fluoroquinolones and macrolides are among the most frequently utilised antibiotic agents to control mycoplasma pneumonia in Hungary (EMA, 2015). Resistance-related SNPs and regions have been investigated and identified in many *Mycoplasma* species including porcine *Mycoplasma* species, and accordingly, the quinolone resistance-determining regions (QRDR) in genes encoding subunits of the topoisomerase enzymes (*gyrA*, *gyrB*, *parC*, *parE*) (Hooper, 2000; Gautier-Bouchardon, 2018), and certain regions in the 23S rRNA (Kobayashi *et* al., 2005; Stakenborg *et al.*, 2005a; Gautier-Bouchardon, 2018) have been associated with resistance against these agents. No amino acid substitutions, correlating with increased MIC values, were observed in the genes *gyrB* and *parE*, corroborating earlier findings (Vicca *et al.*, 2007). Although single amino acid substitutions in the *parC* gene (Ser80Phe, Ser80Tyr or Asp84Asn) showed correlation with increased MIC values of fluoroquinolones in earlier publications (Le Carrou *et al.*, 2006; Vicca *et al.*, 2007; Gautier-Bouchardon, 2018), the degree of increase seems to be negligible according to the initial MIC values detected in the present study. However, a definite increase of MIC values was detected when double substitutions in *parC* and *gyrA* genes were described in the examined strains, especially in the case of final MIC values. The observed effect of the double substitutions is in accordance with previous findings (Vicca *et al.*, 2007). Various combinations of amino acid changes were detected in the examined strains containing double substitutions in the genes *gyrA* and *parC*. Moreover, new amino acid alterations (Glu87Gly and Gly81Ala) have been described in *gyrA* gene of *M. hyopenumoniae* in the present study, which had been observed only in *M. bovis* and *M. gallisepticum* before (Reinhardt, 2002; Sulyok *et al.*, 2017). Factors influencing the degree of the decrease of susceptibility to fluoroquinolones, such as the type of amino acid changes or mechanisms are yet to be discovered. Although initial MIC values are advised to be taken into account in the interpretation of the results of antibiotic susceptibility tests (Hannan, 2000), correlations between the amino acid substitutions and increased final MIC values were more defined in the current examinations. Some of the strains containing a single mutation in the *parC* gene were inhibited with the lowest antibiotic concentration according to the initial MIC values (≤0.039 µg/ml), however the final MIC values of these strains were always higher, than that of the strains without the SNP. Results of the

present study highlight the usefulness of determining initial and final MIC values in case of the slow growing *M. hyopneumoniae* isolates.

In the current study, a slight increase of MIC_{50} and MIC_{90} values of macrolides was described compared to the literature (Klein *et al.*, 2017), and extremely high MIC values against an outlier strain (MycSu18) was noted. According to the literature, nucleotide substitutions at the bases 2057-2059 of the 23S rRNA sequence play an important role in acquired resistance to macrolides (Hansen *et al.*, 2002; Stakenborg *et al.*, 2005a; Gautier-Bouchardon, 2018). Analysis of the 23S rRNA sequence of the strain MycSu18 revealed the nucleotide substitution A2059G (*E. coli* numbering), which was also described in macrolide and lincosamide resistant *M. hyorhinis* and *M. bovis* strains before (Kobayashi *et* al., 2005; Sulyok *et al.*, 2017). The resistance-associated SNPs identified in the present study support the understanding of genetic background of antibiotic resistance in *M. hyopneumoniae* and also may serve as targets for PCR-based assays for the discrimination of strains with increased resistance to quinolones, macrolides and lincosamides.

6.4. Development of molecular assays for the rapid detection of antibiotic susceptibility of *M. hyopneumoniae*

Since vaccination does not provide complete protection against *M. hyopneumoniae* (Meyns *et al.*, 2004, 2006; Stakenborg *et al.*, 2006; Villarreal *et al.*, 2009), antimicrobial therapy is important for the pig industry, because it is able to reduce clinical symptoms, moderate the mortality rate and increase weight-gain of the animals (Maes *et al.*, 1996; Pallarés *et al.*, 2015). However, the conventional method of antibiotic susceptibility testing is usually not accomplished before the therapy, because it requires the isolation of the bacteria, special media and the method is time-consuming (Hannan, 2000). Therefore, the choice of the agent is usually based on earlier experiences. The development of PCR-based assays for the rapid detection of antibiotic susceptibility of *M. hyopneumoniae* would improve the treatment of mycoplasma pneumonia.

Although certain mutations in the target regions of the antibiotics seem to be correlating with increased MIC values (Stakenborg *et al.*, 2005a; Le Carrou *et al.*, 2006; Vicca *et al.*, 2007; Gautier-Bouchardon, 2018), PCR-based susceptibility testing of *M. hyopneumoniae* has not been published yet. Therefore, in the present study, three MAMAs (both agarose- and melt-MAMAs) and one HRM assay were designed for the detection of fluoroquinolone, macrolide and lincosamide resistance of *M. hyopneumoniae*. All of the assays were challenged with DNA of filter-cloned members of the strain collection. Cross-reaction was checked with DNA of other porcine *Mycoplasmas*, and sensitivity was estimated with ten-fold dilution series of the strains.

Furthermore, the results of the MAMA and HRM assays were compared with that of the conventional microbroth dilution test.

Two MAMAs and an HRM assay were developed for the detection of C239A/T and G250A substitutions in the *parC* gene. HRM assay is able to detect the presence of any of the mentioned SNPs and differentiate fluoroquinolone sensitive and resistant strains. However, it requires special equipment and this method is less sensitive than the agarose- and melt-MAMA assays. Therefore, MAMA assays are suggested to be used on clinical material, and HRM can rather be used on pure cultures. A disadvantage of the MAMA assay targeting the G250A substitution in the *parC* gene is, that the presence of *M. flocculare* must be excluded with species-specific PCR.

Agarose- and melt-MAMA were designed for the detection of the A2059G nucleotide change, correlating with extremely high final MIC values to macrolides and lincosamides (8->64 µg/ml of macrolides and >64 µg/ml of lincomycin) (Table 11). Although the MAMA assay targeting the A2059G substitution can successfully differentiate the resistant and sensitive genotypes, the presence of contaminating Mycoplasmas (*M. flocculare*, *M. hyorhinis*) must be excluded when the assays are applied directly on clinical samples.

PCR-based detection of the mentioned SNPs correlating with increased MIC values of fluoroquinolones, macrolides and lincosamides, either with HRM or MAMA assays from clinical material would diminish the time of susceptibility testing from several months to just a couple of days. The use of genetic methods in the determination of antibiotic susceptibility provides rapid and reliable guidance for antibiotic therapy, however, the presence of factors complicating the genetic detection of certain mechanisms should be considered as well (e.g. variable mutation events on single nucleotides, undetectable efflux systems, high mutation rate of mycoplasmas [Citti and Blanchard, 2013]). A considerable advantage of the developed assays next to their rapidness and cost-effectiveness is, that agarose-MAMA can be performed on basic PCR equipment, therefore the genetic-based susceptibility testing can be accomplished in any laboratory. According to our knowledge, this is the first report describing molecular-based susceptibility testing of *M. hyopneumoniae* against fluoroquinolones, macrolides and lincosamides.

7. Overview of the new scientific results

Ad 1. Comparative genetic analysis of the Hungarian isolates was performed. MLVA extended with the serine repeat numbers of 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 fifteen 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. Single nucleotide polymorphisms described in the literature of the genes *parC* and *gyrA* were marked in the fluoroquinolone resistant isolates, while a SNP in the 23S rRNA region, previously associated with antibiotic resistance in other *Mycoplasma* species, was first detected in a macrolide and lincosamide resistant *M. hyopneumoniae* isolates.

Ad 4. MAMA and HRM assays were designed for the rapid detection of SNPs correlating with a decreased fluoroquinolone, macrolide and lincosamide susceptibility in *M. hyopneumoniae* isolates.

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9. Scientific publications

In peer-reviewed journals

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Conference oral presentation

Felde O., Kreizinger Z., Sulyok K.M., Hrivnák V., Kiss K., Biksi I., Gyuranecz M.: **Development of mismatch amplification mutation assay (MAMA) and high resolution melt (HRM) analyses of Mycoplasma hyopneumoniae strains for the fast detection of antibiotic resistance**, European Mycoplasma Conference, London, United Kingdom, 2019.

Felde O., Kreizinger Z., Sulyok K.M., Marton S., Bányai K., Korbuly K., Kiss K., Biksi I., Gyuranecz M.: **Genotyping of *Mycoplasma hyopneumoniae* strains from Central Europe**, Annual conference of the Hungarian Society for Microbiology, Eger, Hungary, 2018.

Conference poster presentation

Felde O., Bekő K., Hrivnák V., Kiss K., Biksi I., Kreizinger Z., Sulyok K.M., Gyuranecz M.: **Antibiotic susceptibility profiles of *Mycoplasma hyopneumoniae* isolates from Hungary**, Annual conference of the Hungarian Society for Micribiology, Keszthely, Hungary, 2017.

Other publications in peer-reviewed journals

Bekő K., Felde O., Sulyok K.M., Kreizinger Z., Hrivnák V., Kiss K., Biksi I., Jerzsele Á., Gyuranecz M.: **Antibiotic susceptibility profiles of *Mycoplasma hyorhinis* strains isolated from swine in Hungary,** Vet. Mic. 228. 196-201, 2019.

Marosi A., Dufkova L., Forró B., Felde O., Erdélyi K., Sirmarova J., Palus M., Honig V., Salat J., Tikos R., Gyuranecz M., Ruzek D., Martina B., Koraka P., Osterhaus A.D.M.E., Bakonyi T.: **Combination therapy of rabies-infected mice with inhibitors of pro-inflammatory host response, antiviral compounds and human rabies immunoglobulin.** Vaccine 37. 4724-4735, 2019.

Kreizinger Z., Sulyok K.M., Bekő K., Kovács Á.B., Grózner D., Felde O., Marton S., Bányai K., Salvatore C., Dusan B., Gyuranecz M.: **Genotyping *Mycoplasma synoviae*: Development of a multi-locus variable number of tandem-repeats analysis and comparison with current molecular typing methods.** Vet. Mic. 226. 41-49, 2018

Kreizinger Z., Erdélyi K., Felde O., Fabbi M., Sulyok K.M., Magyar T., Gyuranecz M.: **Comparison of virulence of *Francisella tularensis ssp. holartica* genotypes B.12 and B.FTNF002-00** BMC Vet. Res. 13. 46. 2017.

Wernery U., Gyuranecz M., Kinne J., Raghavan R., Syriac G., Johanson B., Kreizinger Z., Dénes B., Felde O., Magyar T., Jose Sh., Raja S., John J., Wernery R.: **Laboratory investigations after eye drop immunisation of dromedaries with live attenuated *Brucella melitensis* Rev 1 vaccine,** J. Camel Pract. Res. 24. 9-14, 2017.

Gyuranecz M., Wernery U., Kreizinger Z., Juhász J., Felde O., Nagy P.: **Genotyping of *Brucella melitensis* strains from dromedary camels (*Camelus dromedarius*) from the United Arab Emirates with multiple-locus variable-number tandem repeat analysis** Vet. Mic. 186. 8-12, 2016.

Kreizinger Z., Sulyok K.M., Pásztor A., Erdély K., Felde O., Povazsán J., Kőrösi L., Gyuranecz M.: **Rapid, simple and cost-efective molecular method to differentiate the temperature sensitive (ts+) MS-H vaccine strain and the wild-type *Mycoplasma synoviae* isolates.** Plos One 10. e0133554. 8, 2015.

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

Other conference poster presentation

Ludmerszki E., Felde O., Oláh C., Rácz I., Rudnóy S.: **Quenching thirst: an alternative compound to combat the harmful effects of drught and salt stress in maize plants.** International Conference Plant Abiotic Stress Tolerance III, Vienna, Austria, 2015

10. Supplements

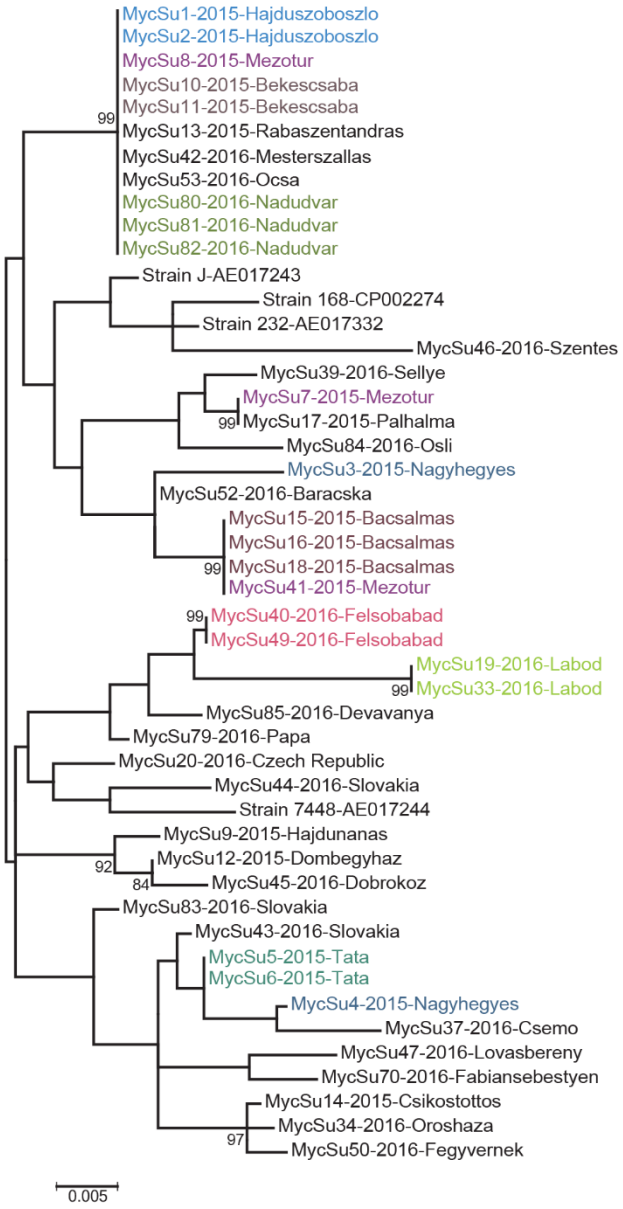

Figure S1. Minimal MLST (multi locus sequence typing) assay of the 44 *M. hyopneumoniae* isolates.

Phylogenetic tree was constructed by Maximum-likelihood method using *adk*, *rpoB* and *tpiA* genes, with 1000 bootstraps; only bootstrap values >70 are presented. The scale bar represents the average number of substitutions per site. Colours indicate the common origin of the isolates.

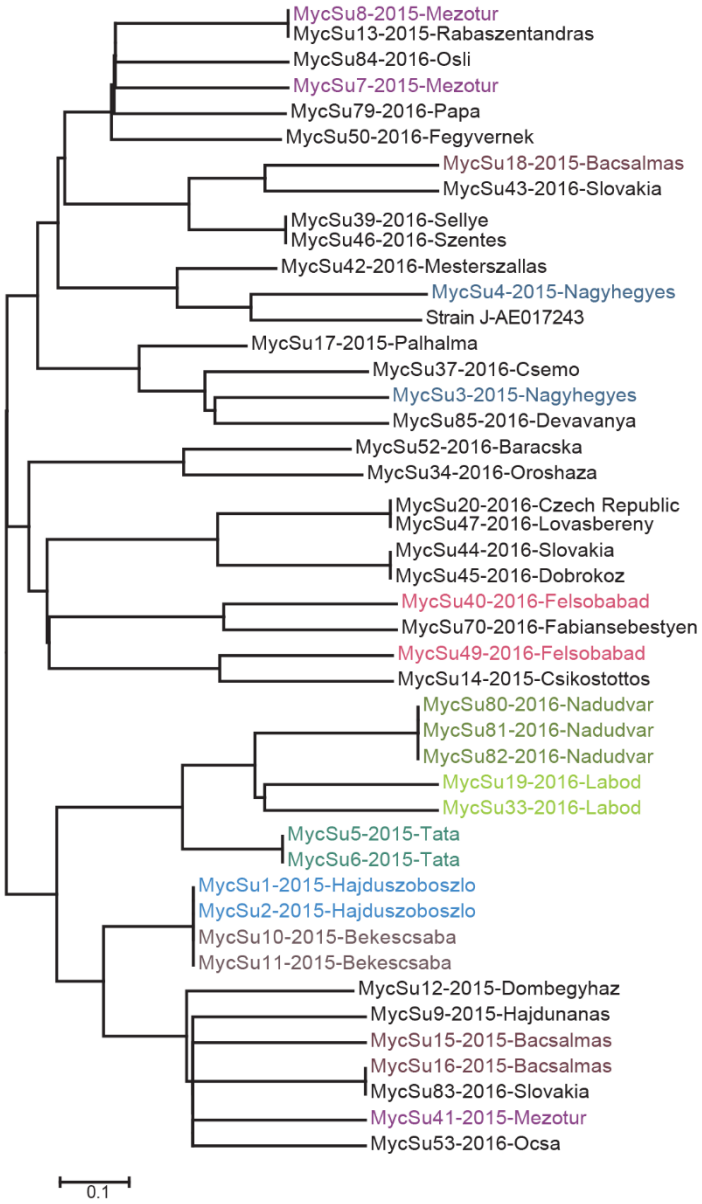

Figure S2. MLVA (multiple-locus variable-number tandem repeat analysis) of the 44 *M. hyopneumoniae* isolates according to the common loci of Charlebois *et al.* (2014) and Vranckx *et al.* (2011).

The MLVA tree was constructed by Neighbor-Joining method, with 1000 bootstraps using Locus1 and P97-RR1 loci. The scale bar represents the average number of substitutions per site. Colours indicate the common origin of the isolates.

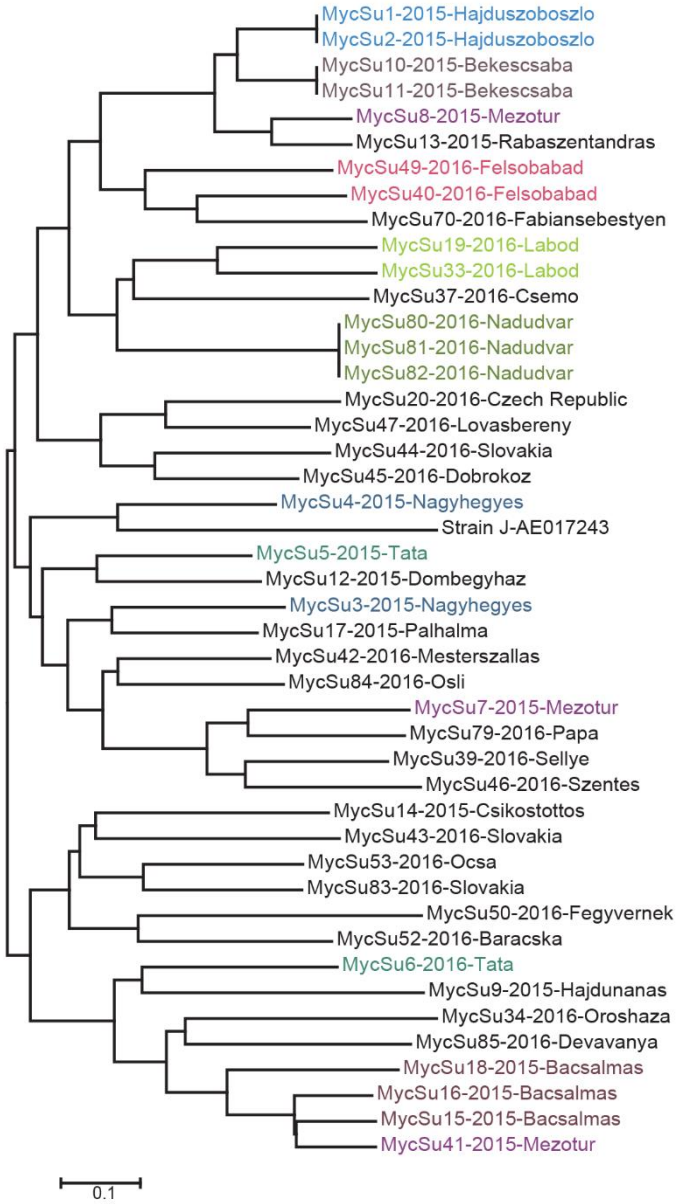

Figure S3. MLVA (multiple-locus variable-number tandem repeat analysis) according to the loci of Charlebois *et al.* (2014), extended with the serine repeat numbers of the gene *p146* of the 44 *M. hyopneumoniae* isolates.

The MLVA tree was constructed by Neighbor-Joining method, with 1000 bootstraps. The scale bar represents the average number of substitutions per site. Colours indicate the common origin of the isolates.

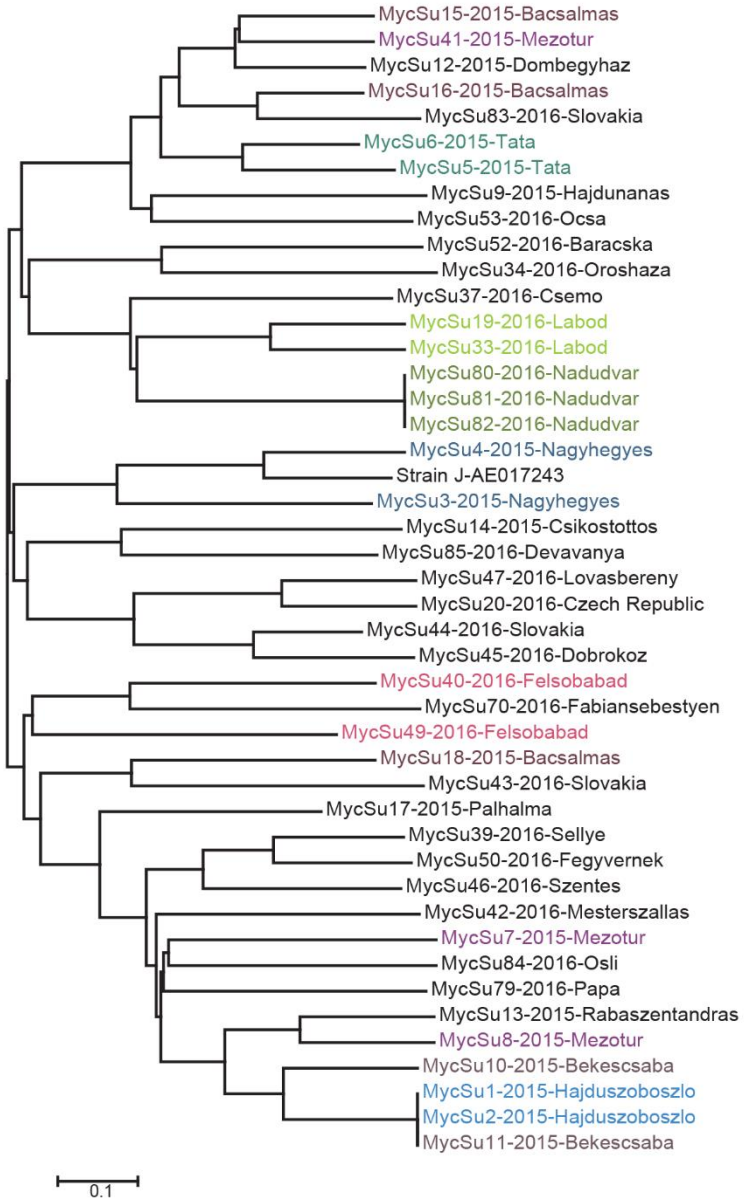

Figure S4. MLVA (multiple-locus variable-number tandem repeat analysis) of the 44 *M. hyopneumoniae* isolates according to the common loci of Charlebois *et al.* (2014) and Vranckx *et al.* (2011) extended with the serine repeat numbers of the gene *p146*.
The MLVA tree was constructed by Neighbor-Joining method, with 1000 bootstraps. The scale bar represents the average number of substitutions per site. Colours indicate the common origin of the isolates.

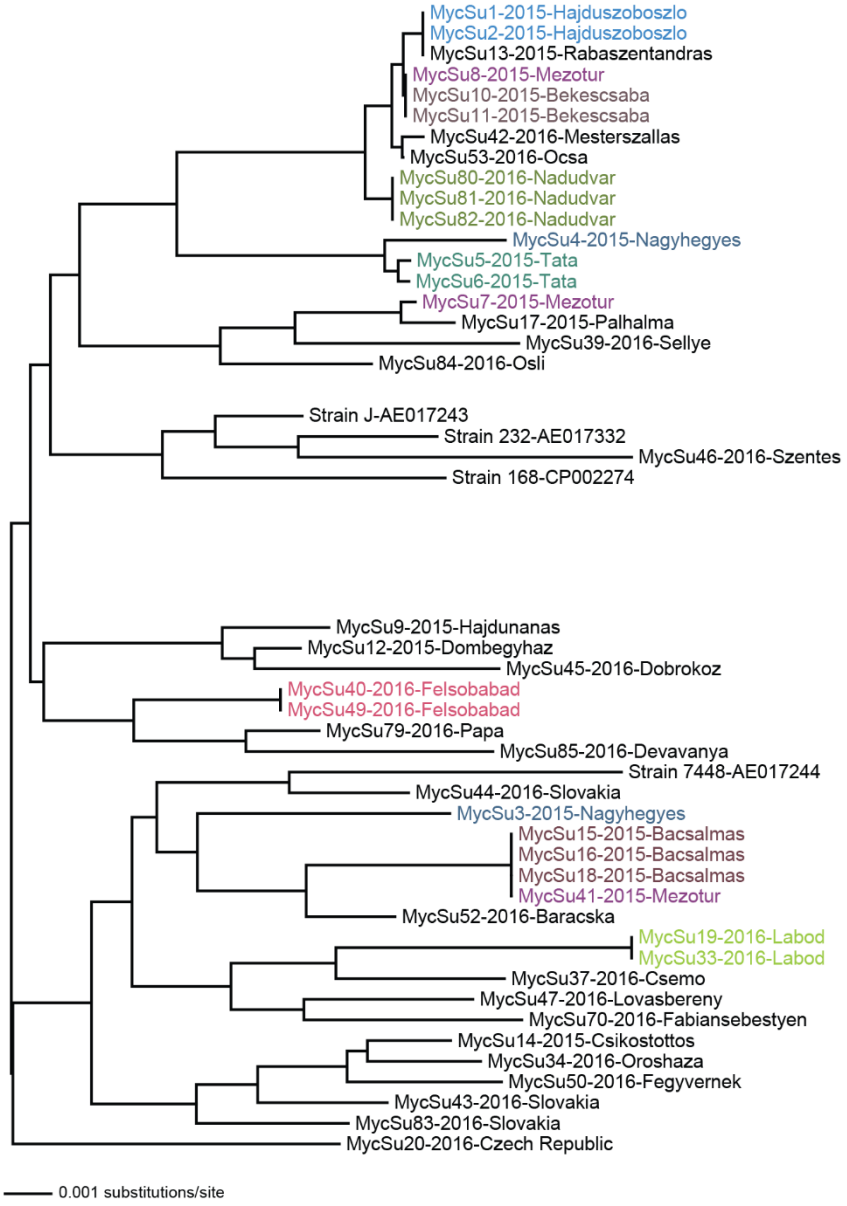

Figure S5. Minimal MLST (multi locus sequence typing) assay extended with the nucleotide sequence of the gene *p146* of the 44 *M. hyopneumoniae* isolates.

Tree based on the minimal MLST assay using *adk*, *rpoB* and *tpiA* genes extended with the sequence data of *p146* gene were analysed with Neighbor-joining method with pairwise deletion with FastGap (Borchsenius, 2009) and PAUP 4.0 (Swofford, 2003) software. The scale bar represents the average number of substitutions per site. Colours indicate the common origin of the isolates.

Table S1: Adjusted Rand coefficient values of the genotyping methods used on 44 *M. hyopneumoniae* isolates

	conventional MLST	minimal MLST	*p146* sequence analysis	minimal MLST + *p146* sequence	MLVA4	MLVA4 + *p146* VNTR	MLVA2 + p146 VNTR	MLVA2
conventional MLST								
minimal MLST	1.000							
p146 sequence analysis	0.360	0.360						
minimal MLST + *p146* sequence	0.367	0.367	0.967					
MLVA4	0.259	0.259	0.373	0.389				
MLVA4 + *p146* VNTR	0.137	0.137	0.470	0.494	0.663			
MLVA2 + *p146* VNTR	0.137	0.137	0.470	0.494	0.663	1.000		
MLVA2	0.259	0.259	0.306	0.318	0.796	0.494	0.494	

Congruencies between the different genotyping methods were determined with the help of the adjusted Rand coefficients. Conventional MLST using seven genes (*efp*, *metG*, *pgiB*, *recA*, *adk*, *rpoB*, *tpiA*), minimal MLST using three genes (*adk*, *rpoB*, *tpiA*), sequence analysis of gene *p146*, minimal MLST extended with the sequence data of gene *p146* and MLVA (multiple-locus variable-number tandem repeat analysis) assay utilising different locus combinations were compared. Abbreviations for MLVA types are: MLVA4 - loci described by Charlebois *et a*l. (2014); MLVA2 -common MLVA loci suggested by Charlebois *et al*. (2014) and Vranckx *et al.* (2011).

Table S2: Adjusted Wallace coefficient values for the genotyping methods used on 44 *M. hyopneumoniae* isolates

	conventional MLST	minimal MLST	*p146* sequence analysis	minimal MLST + *p146* sequence	MLVA4	MLVA4 + *p146* VNTR	MLVA2 + *p146* VNTR	MLVA2
conventional MLST		1.000 (CI:1.000-1.000)	0.223 (CI:0.095-0.352)	0.224 (CI:0.096-0.353)	0.149 (CI:0.000-0.302)	0.074 (CI:0.000-0.197)	0.074 (CI:0.000-0.197)	0.158 (CI:0.005-0.312)
minimal MLST	1.000 (CI:1.000-1.000)		0.223 (CI:0.095-0.352)	0.224 (CI:0.096-0.353)	0.149 (CI:0.000-0.302)	0.074 (CI:0.000-0.197)	0.074 (CI:0.000-0.197)	0.158 (CI:0.005-0.312)
p146 sequence analysis	0.931 (CI:0.862-1.000)	0.931 (CI:0.862-1.000)		0.936 (CI:0.872-1.000)	0.302 (CI:0.077-0.527)	0.307 (CI:0.084-0.531)	0.307 (CI:0.084-0.531)	0.297 (CI:0.070-0.524)
minimal MLST + *p146* sequence	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)		0.323 (CI:0.093-0.554)	0.328 (CI:0.099-0.557)	0.328 (CI:0.099-0.557)	0.318 (CI:0.086-0.550)
MLVA4	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	0.488 (CI:0.217-0.759)	0.488 (CI:0.218-0.759)		0.496 (CI:0.230-0.763)	0.496 (CI:0.230-0.763)	1.000 (CI:1.000-1.000)
MLVA4 + *p146* VNTR	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)		1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)
MLVA2 + *p146* VNTR	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)	1.000 (CI:1.000-1.000)		1.000 (CI:1.000-1.000)
MLVA2	0.706 (CI:0.559-0.853)	0.706 (CI:0.559-0.853)	0.317 (CI:0.080-0.554)	0.318 (CI:0.082-0.554)	0.662 (CI:0.510-0.813)	0.328 (CI:0.096-0.561)	0.328 (CI:0.096-0.561)	

Interchangeability between the different genotyping methods were described with help of the adjusted Wallace coefficients including values of the confidence intervals (CI). Conventional MLST using seven genes (*efp*, *metG*, *pgiB*, *recA*, *adk*, *rpoB*, *tpiA*), minimal MLST using three genes (*adk*, *rpoB*, *tpiA*), sequence analysis of gene *p146*, minimal MLST extended with the sequence data of gene *p146* and MLVA (multiple-locus variable-number tandem repeat analysis) assay utilising different locus combinations were involved. Abbreviations for MLVA types are: MLVA4 - loci described by Charlebois *et al.* (2014); MLVA2 - common MLVA loci suggested by Charlebois *et al.* (2014) and Vranckx *et al.* (2011).

Table S3. Initial and final minimum inhibitory concentration (MIC) values (μg/ml) of fluoroquinolones, tetracyclines, aminoglycosides, aminocyclitoles and pleuromutilines against the studied *M. hyopneumoniae* strains

Sample ID	MIC values (μg/ml)															
	EFX		MFX		OTC		DX		GTC		SPC		TIA		VAL	
	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final
MycSu1	≤0.039	0.312	0.312	1.25	≤0.25	4	0.078	0.312	0.5	2	1	4	0.078	0.156	≤0.039	≤0.039
MycSu2	≤0.039	0.312	≤0.039	1.25	≤0.25	4	0.078	0.312	≤0.25	1	1	8	0.078	0.156	≤0.039	≤0.039
MycSu3	≤0.039	0.078	≤0.039	0.156	≤0.25	4	0.078	0.625	≤0.25	1	1	2	≤0.039	0.156	≤0.039	≤0.039
MycSu4	≤0.039	≤0.039	≤0.039	≤0.039	1	8	0.312	1.25	≤0.25	0.5	2	4	0.078	0.156	≤0.039	≤0.039
MycSu5	≤0.039	≤0.039	≤0.039	0.078	2	32	0.312	1.25	≤0.25	1	2	4	≤0.039	0.156	≤0.039	≤0.039
MycSu6	≤0.039	0.078	≤0.039	0.078	0.5	8	0.156	0.625	≤0.25	1	0.5	2	≤0.039	0.156	≤0.039	≤0.039
MycSu7	≤0.039	0.078	≤0.039	0.078	≤0.25	1	≤0.039	0.156	≤0.25	2	1	4	≤0.039	0.156	≤0.039	≤0.039
MycSu8	0.078	0.312	0.625	1.25	≤0.25	4	0.078	0.312	≤0.25	0.5	2	4	≤0.039	0.156	≤0.039	≤0.039
MycSu9	≤0.039	≤0.039	≤0.039	≤0.039	≤0.25	0.5	≤0.039	0.156	≤0.25	1	2	4	≤0.039	0.078	≤0.039	≤0.039
MycSu10	≤0.039	0.312	≤0.039	1.25	0.5	4	0.078	0.312	≤0.25	1	2	2	≤0.039	0.156	≤0.039	≤0.039
MycSu11	≤0.039	0.312	≤0.039	1.25	≤0.25	8	≤0.039	0.625	≤0.25	1	2	4	0.078	0.156	≤0.039	≤0.039
MycSu12	0.625	2.5	1.25	5	≤0.25	2	≤0.039	0.312	≤0.25	2	2	4	≤0.039	0.156	≤0.039	≤0.039
MycSu13	≤0.039	0.312	0.312	1.25	0.5	4	0.078	0.312	≤0.25	1	2	4	0.078	0.156	≤0.039	≤0.039
MycSu14	≤0.039	0.078	≤0.039	0.078	≤0.25	0.5	≤0.039	0.078	0.5	2	1	8	≤0.039	0.156	≤0.039	≤0.039
MycSu15	1.25	2.5	2.5	5	4	16	0.625	2.5	≤0.25	0.5	2	2	0.078	0.078	≤0.039	≤0.039
MycSu16	1.25	2.5	2.5	5	2	16	0.312	2.5	≤0.25	0.5	2	4	≤0.039	0.156	≤0.039	≤0.039
MycSu17	2.5	5	5	10	0.5	4	0.078	0.312	≤0.25	0.5	1	2	≤0.039	0.156	≤0.039	≤0.039
MycSu18	1.25	2.5	2.5	5	2	8	0.312	1.25	≤0.25	0.5	2	2	0.156	0.312	≤0.039	≤0.039
MycSu19	0.156	0.312	0.625	1.25	0.5	16	0.156	1.25	≤0.25	1	2	4	0.078	0.156	≤0.039	≤0.039
MycSu20	0.156	2.5	0.625	5	1	32	0.156	1.25	≤0.25	0.5	2	4	≤0.039	0.156	≤0.039	≤0.039
MycSu33	≤0.039	0.312	0.078	1.25	≤0.25	4	0.078	0.625	≤0.25	0.5	1	4	0.078	0.156	≤0.039	≤0.039
MycSu34	≤0.039	≤0.039	≤0.039	≤0.039	0.5	8	0.078	0.625	≤0.25	0.5	2	2	≤0.039	0.156	≤0.039	≤0.039
MycSu37	0.312	0.625	≤0.039	2.5	1	16	0.312	1.25	≤0.25	0.5	≤0.25	2	≤0.039	0.156	≤0.039	≤0.039
MycSu39	≤0.039	0.312	≤0.039	1.25	0.5	2	≤0.039	0.312	≤0.25	0.5	4	4	≤0.039	0.078	≤0.039	≤0.039
MycSu40	≤0.039	≤0.039	≤0.039	0.078	0.5	8	0.156	1.25	≤0.25	0.5	2	4	0.078	0.156	≤0.039	≤0.039
MycSu41	1.25	5	1.25	5	0.5	8	0.078	1.25	≤0.25	0.5	0.5	1	≤0.039	0.078	≤0.039	≤0.039
MycSu42	≤0.039	≤0.039	≤0.039	0.078	0.5	8	0.078	0.625	≤0.25	0.5	2	2	0.156	0.156	≤0.039	≤0.039
MycSu43	≤0.039	≤0.039	≤0.039	≤0.039	2	32	0.312	2.5	0.5	1	2	4	≤0.039	0.156	≤0.039	≤0.039
MycSu44	0.625	2.5	0.625	1.25	≤0.25	1	≤0.039	0.156	≤0.25	1	0.5	4	≤0.039	0.156	≤0.039	≤0.039
MycSu45	1.25	2.5	2.5	5	≤0.25	4	≤0.039	0.625	≤0.25	2	≤0.25	8	≤0.039	0.156	≤0.039	≤0.039
MycSu46	0.078	0.312	0.625	1.25	≤0.25	4	0.078	0.625	≤0.25	0.5	1	2	0.078	0.156	≤0.039	≤0.039
MycSu47	≤0.039	≤0.039	≤0.039	0.156	≤0.25	0.5	≤0.039	0.078	≤0.25	1	0.5	2	0.078	0.156	≤0.039	≤0.039
MycSu49	≤0.039	≤0.039	≤0.039	0.078	≤0.25	8	0.078	0.625	≤0.25	0.5	2	4	≤0.039	0.156	≤0.039	≤0.039

Table S3 (continued). Initial and final minimum inhibitory concentration (MIC) values (μg/ml) of fluoroquinolones, tetracyclines, aminoglycosides, aminocyclitoles and pleuromutilines against the studied *M. hyopneumoniae* strains

Sample ID	MIC values (μg/ml)															
	EFX		MFX		OTC		DX		GTC		SPC		TIA		VAL	
	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final
MycSu50	1.25	5	2.5	5	≤0.25	4	0.078	0.625	≤0.25	0.5	0.5	4	≤0.039	0.156	≤0.039	≤0.039
MycSu52	≤0.039	0.078	≤0.039	0.078	≤0.25	4	≤0.039	0.625	≤0.25	0.5	1	2	≤0.039	0.156	≤0.039	≤0.039
MycSu53	≤0.039	0.625	≤0.039	1.25	≤0.25	4	≤0.039	0.312	≤0.25	1	≤0.25	4	≤0.039	0.156	≤0.039	≤0.039
MycSu70	≤0.039	≤0.039	≤0.039	0.078	2	32	0.156	2.5	≤0.25	2	2	4	≤0.039	0.078	≤0.039	≤0.039
MycSu79	≤0.039	0.312	0.078	1.25	≤0.25	4	≤0.039	0.625	≤0.25	0.5	1	2	≤0.039	0.156	≤0.039	≤0.039
MycSu80	0.312	0.625	0.625	2.5	≤0.25	4	≤0.039	0.625	≤0.25	1	1	2	≤0.039	0.156	≤0.039	≤0.039
MycSu81	0.156	0.625	0.625	1.25	≤0.25	4	≤0.039	0.312	≤0.25	1	1	2	≤0.039	0.156	≤0.039	≤0.039
MycSu82	0.156	0.625	0.625	1.25	≤0.25	4	0.078	0.625	0.5	1	2	2	≤0.039	0.156	≤0.039	≤0.039
MycSu83	≤0.039	≤0.039	≤0.039	0.078	≤0.25	4	≤0.039	0.312	≤0.25	1	2	4	0.078	0.156	≤0.039	≤0.039
MycSu84	≤0.039	≤0.039	≤0.039	0.078	2	16	0.312	1.25	≤0.25	1	2	2	0.078	0.156	≤0.039	≤0.039
MycSu85	≤0.039	0.078	≤0.039	0.078	1	16	0.312	2.5	0.5	1	1	2	≤0.039	0.156	≤0.039	≤0.039

MIC values of enrofloxacin (EFX), marbofloxacin (MFX), oxytetracycline (OTC), doxycycline (DX), gentamicin (GTC), spectinomycin (SPC), tiamulin (TIA), valnemulin (VAL), are presented.

Table S4. Initial and final minimum inhibitory concentration (MIC) values (µg/ml) of macrolides, lincosamides and phenicols against the studied *M. hyopneumoniae* strains

Sample ID	MIC values (µg/ml)													
	TYL		TIL		TVN		GTM		TTM		LCM		FFC	
	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final
MycSu1	≤0.25	0.5	4	8	≤0.25	≤0.25	1	4	1	2	≤0.25	1	1	2
MycSu2	≤0.25	0.5	4	4	≤0.25	≤0.25	2	4	2	4	0.5	1	≤0.125	2
MycSu3	≤0.25	0.5	2	4	≤0.25	≤0.25	1	4	1	2	≤0.25	0.5	1	2
MycSu4	≤0.25	0.5	4	8	≤0.25	≤0.25	≤0.25	4	0.5	2	≤0.25	0.5	1	2
MycSu5	≤0.25	≤0.25	2	4	≤0.25	≤0.25	≤0.25	4	0.5	1	≤0.25	0.5	2	2
MycSu6	≤0.25	0.5	2	8	≤0.25	≤0.25	0.5	4	1	2	≤0.25	1	≤0.125	2
MycSu7	≤0.25	0.5	0.5	4	≤0.25	≤0.25	≤0.25	2	≤0.25	2	≤0.25	0.5	1	4
MycSu8	≤0.25	0.5	2	4	≤0.25	≤0.25	0.5	2	0.5	1	≤0.25	0.5	0.5	2
MycSu9	≤0.25	≤0.25	2	8	≤0.25	≤0.25	≤0.25	2	0.5	1	≤0.25	0.5	1	2
MycSu10	≤0.25	≤0.25	2	4	≤0.25	≤0.25	1	4	0.5	1	≤0.25	0.5	1	2
MycSu11	≤0.25	0.5	2	8	≤0.25	≤0.25	1	4	0.5	2	≤0.25	1	1	4
MycSu12	≤0.25	0.5	1	8	≤0.25	≤0.25	1	4	1	2	≤0.25	0.5	≤0.125	2
MycSu13	≤0.25	0.5	2	4	≤0.25	≤0.25	1	2	1	1	≤0.25	1	1	2
MycSu14	≤0.25	0.5	2	8	≤0.25	≤0.25	0.5	8	1	2	≤0.25	1	≤0.125	2
MycSu15	≤0.25	≤0.25	4	8	≤0.25	≤0.25	1	2	≤0.25	1	≤0.25	≤0.25	1	2
MycSu16	≤0.25	≤0.25	2	8	≤0.25	≤0.25	≤0.25	1	≤0.25	1	≤0.25	0.5	1	2
MycSu17	≤0.25	0.5	4	8	≤0.25	≤0.25	1	8	0.5	2	0.5	1	1	2
MycSu18	32	64	>64	>64	2	8	64	>64	>64	>64	>64	>64	≤0.125	2
MycSu19	≤0.25	≤0.25	1	4	≤0.25	≤0.25	0.5	2	0.5	1	≤0.25	1	1	2
MycSu20	≤0.25	0.5	4	8	≤0.25	≤0.25	1	4	1	1	≤0.25	0.5	1	2
MycSu33	≤0.25	≤0.25	2	8	≤0.25	≤0.25	≤0.25	4	0.5	2	≤0.25	1	≤0.125	2
MycSu34	≤0.25	0.5	4	8	≤0.25	≤0.25	0.5	4	≤0.25	2	≤0.25	0.5	1	2
MycSu37	≤0.25	0.5	≤0.25	4	≤0.25	≤0.25	≤0.25	4	1	2	≤0.25	1	≤0.125	1
MycSu39	≤0.25	≤0.25	2	4	≤0.25	≤0.25	≤0.25	2	≤0.25	0.5	≤0.25	1	2	2
MycSu40	≤0.25	1	4	16	≤0.25	≤0.25	2	16	1	2	≤0.25	0.5	2	4
MycSu41	≤0.25	≤0.25	1	4	≤0.25	≤0.25	≤0.25	2	≤0.25	1	≤0.25	1	≤0.125	1
MycSu42	≤0.25	0.5	4	16	≤0.25	≤0.25	4	8	2	4	0.5	1	2	4
MycSu43	≤0.25	≤0.25	8	16	≤0.25	≤0.25	1	8	1	2	≤0.25	1	0.5	2
MycSu44	≤0.25	0.5	4	16	≤0.25	≤0.25	0.5	4	≤0.25	2	≤0.25	0.5	1	2
MycSu45	≤0.25	>0.25	≤0.25	8	≤0.25	≤0.25	≤0.25	4	≤0.25	2	≤0.25	1	≤0.125	2
MycSu46	≤0.25	0.5	4	16	≤0.25	≤0.25	2	8	1	2	≤0.25	1	1	2
MycSu47	≤0.25	0.5	2	32	≤0.25	≤0.25	2	8	2	4	≤0.25	1	1	4
MycSu49	≤0.25	0.5	4	8	≤0.25	≤0.25	2	4	0.5	2	≤0.25	0.5	1	2

Table S4 (continued). Initial and final minimum inhibitory concentration (MIC) values (μg/ml) of macrolides, lincosamides and phenicols against the studied *M. hyopneumoniae* strains

Sample ID	MIC values (μg/ml)													
	TYL		TIL		TVN		GTM		TTM		LCM		FFC	
	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final
MycSu50	≤0.25	0.5	≤0.25	8	≤0.25	≤0.25	≤0.25	8	≤0.25	2	≤0.25	0.5	2	4
MycSu52	≤0.25	0.5	2	8	≤0.25	≤0.25	1	4	1	2	≤0.25	0.5	0.5	2
MycSu53	≤0.25	≤0.25	≤0.25	4	≤0.25	≤0.25	≤0.25	4	0.5	1	≤0.25	≤0.25	1	2
MycSu70	≤0.25	≤0.25	1	2	≤0.25	≤0.25	≤0.25	2	≤0.25	0.5	≤0.25	1	2	4
MycSu79	≤0.25	≤0.25	4	16	≤0.25	≤0.25	1	4	1	2	≤0.25	0.5	1	4
MycSu80	≤0.25	0.5	2	4	≤0.25	≤0.25	0.5	4	0.5	2	≤0.25	1	0.5	2
MycSu81	≤0.25	0.5	2	8	≤0.25	≤0.25	1	4	1	1	≤0.25	0.5	1	4
MycSu82	≤0.25	0.5	4	8	≤0.25	≤0.25	0.5	4	0.5	2	≤0.25	0.5	1	4
MycSu83	≤0.25	0.5	4	8	≤0.25	≤0.25	2	8	1	1	≤0.25	0.5	1	2
MycSu84	≤0.25	≤0.25	2	4	≤0.25	≤0.25	0.5	2	0.5	2	≤0.25	0.5	2	2
MycSu85	≤0.25	0.5	2	8	≤0.25	≤0.25	0.5	4	0.5	1	≤0.25	1	≤0.125	2

MIC values of tylosin (TYL), tilmicosin (TIL), tylvalosin (TVN), gamithromycin (GTM), tulathromycin (TTM), lincomycin (LCM) and florfenicol (FFC) are presented.

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