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**Core genome multi-locus sequence typing of *Mycoplasma
hyorhinis***

Thesis

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Abbreviation

adk	adenylate kinase
BAL	Bronchoalveolar lavage
cgMLST	Core genome multi locus sequence typing
DNA	Deoxyribonucleic acid
dnaA	Internal fragments of the chromosomal replication initiation protein
gltX	glutamyl-tRNA synthetase
gmK	guanylate kinase
gyrB	DNA gyrase subunit B
lepA	leader peptidase A
<i>M. hyorhinis</i>	<i>Mycoplasma hyorhinis</i>
MLST	Multi locus sequence typing
PCR	Polymerase chain reaction
PCV2	Porcine Circovirus 2
PRDC	Porcine Respiratory Disease Complex
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RNA	Ribonucleic acid
rpoB	RNA polymerase subunit
rpoC	DNA-directed RNA polymerase subunit beta
SNP	Single nucleotide polymorphism
uvrA	UvrABC system protein A
valS	valine-tRNA ligase
VNTR	Variable number tandem repeat
WGS	Whole genome sequencing

Table of Contents

1. Introduction	4
1.1. <i>Mycoplasma hyorhinis</i>	4
1.2. Clinical Signs	5
1.3. Sample Collection for Laboratory Diagnosis	6
1.4. Diagnostics and Molecular Typing	7
1.5. Therapy and Prevention	8
1.6. Core Genome Multi-Locus Sequence Typing	9
2. Original Publication	11
3. Discussion and Conclusion	19
4. Summary	23
5. Zusammenfassung	24
6. References	25

1. Introduction

Mycoplasma hyorhinis (*M. hyorhinis*) is an important pathogen of pigs. Infection with *M. hyorhinis* can lead to various clinical manifestations, which may occur in isolation or concurrently. While the natural habitat for *M. hyorhinis* proliferation is the upper respiratory tract of pigs, various pathogenic mechanisms allow for dissemination and infection of other organs. For instance, if serous membranes are affected by *M. hyorhinis*, severe lesions and inflammatory processes can develop. This can result in significant medical and economic damages for both the pigs and the farmers. Core genome multi locus sequence typing (cgMLST) is a valuable tool to investigate outbreaks and for long-term surveillance of diverse strains. For the multi locus sequence typing (MLST) of the core genomes, the *M. hyorhinis* strains isolated and stored at the University of Veterinary Medicine Vienna between 2009 and 2020 were genotyped and discussed in this publication and the accompanying thesis.

1.1. *Mycoplasma hyorhinis*

Mycoplasma hyorhinis is a small pleomorphic bacterium, belonging to the class *Mollicutes* and the family *Mycoplasmataceae* (Petzoldt and Kirchoff, 1986). *Mollicutes* represent the simplest form of self-replicating cells and are highly adapted to their hosts (Kornspan et al., 2015). *Mollicutes* are distinguished from other prokaryotes primarily by their lack of a cell wall. They are most closely related to gram-positive bacteria from which they have evolved by significant genome reduction (Rottem, 2003).

M. hyorhinis ferments glucose and other sugars but does not hydrolyze arginine or urea, which is important for cultivation. It forms colonies with a diameter of approximately 0.5-1 mm, which exhibit a typical fried-egg morphology under the stereomicroscope (Kobisch and Friis, 1996). As other *Mollicutes*, the bacterium lacks a cell wall and is surrounded only by a cytoplasmic membrane, which accounts for its pleomorphism (Bisping, 1979), indicating a lack of fixed cell structure morphology. The genomes of the *M. hyorhinis* strains sequenced to date are approximately 820-840 kb in size, meaning that the species possesses smaller genomes even within the *Mycoplasma* genus (Calcutt et al., 2012).

M. hyorhinis is a ubiquitous microorganism that is widely distributed worldwide (Schreiber and Thacker, 2012). Furthermore, *M. hyorhinis* has often been identified as a contaminant in cell cultures (Razin et al., 1998). Initially, *M. hyorhinis* was isolated from the respiratory tract of young pigs (Switzer, 1955). Its habitat resides in the mucosa of the upper respiratory tract and

tonsils (Kobisch and Friis, 1996). Infected pigs can be found in all age groups, with post-weaning piglets being the most affected age class (Luehrs et al., 2017). A study by Clavijo et al. (Clavijo et al., 2019) has shown prevalence of fewer than 10 % in sows and weaning piglets, while the prevalence reached up to 100 % in post-weaning piglets. It is assumed that a few sows infect their offspring, which then spread throughout the post-weaning piglet herd. These remain hypotheses, as the exact epidemiology remains unclear, although direct contact has been proven as a main source of transmission (Clavijo et al., 2019).

M. hyorhinis has been isolated and identified in various diseases and clinical conditions, subsequently recognized as a primary pathogen (Kobisch and Friis, 1996). The main clinical manifestations involve polyserositis and arthritis, affecting the majority of post-weaning piglets (Luehrs et al., 2017).

1.2. Clinical Signs

An infection with *M. hyorhinis* can manifest in various clinical signs, which may appear individually or in combination. Most infections progress sub-clinically, and infected pigs show no clinical signs (Luehrs et al., 2017). The microorganism resides as commensal in the upper respiratory tract and tonsils of asymptotically infected pigs (Friis and Feenstra, 1994). Generally, depending on the study and population, *M. hyorhinis* can be isolated from approximately 10 % of sows and 30-40 % of weaning piglets from nasal swabs (Kobisch and Friis, 1996). The prevalence increases from the age of post-weaning piglets at three weeks, with evidence of the pathogen in up to 100 % of these piglets (Clavijo et al., 2019).

An infection occurs when *M. hyorhinis* spreads or ascends. This may occur as an ascending infection into the lungs or through hematogenous dissemination, attaching to serous body cavities such as the pericardium, pleura, peritoneum, meninges and joints (Bünger et al., 2020; Friis and Feenstra, 1994).

Hematogenous dissemination into the joints results in arthritis across different age groups. Lameness due to *M. hyorhinis* has been observed predominantly in pigs aged 12-15 weeks. However, piglets can show symptomatic signs as early as three weeks old. This is usually noticed by the farmer when one or more pigs show an abnormal gait. It is possible that one or multiple limbs are affected (Leuwerke, 2009). *M. hyorhinis* causes painful lesions on the joint surfaces (Kobisch and Friis, 1996). Noticeably post-weaning piglets exhibit signs of slow rising movements. Due to the discomfort when standing, affected pigs tend to lie down more often or shift their weight repeatedly to relieve the affected limbs. The clinically affected animal tends

to minimize movement of the joints, resulting in a stiff and jerky gait (Leuwerke, 2009). The affected joints exhibit swelling and localized hyperthermia. While the disease may heal spontaneously, the lesions caused by *M. hyorhinis* can lead to lasting damage. Affected piglets may experience lifelong discomfort while moving (Kobisch and Friis, 1996).

Symptoms of arthritis can occur independently or in combination with other clinical conditions caused by *M. hyorhinis*. Additional indications of a *M. hyorhinis* infection include polyserositis. It can affect every serous membrane in the body. It is an inflammation of the pleura, pericardium, or peritoneum. This condition may lead to fever, decreased feed intake, lethargy, and dyspnea (Schreiber and Thacker, 2012). Often triggered by poor overall health, affected piglets are often runts and fail to gain the desired weight.

Depending on which serous membranes are affected, certain clinical signs may be more prevalent. Pleuritis often coincides with dyspnea.

M. hyorhinis has also occasionally been isolated from cases of conjunctivitis (Friis, 1976), otitis media, and eustachitis, and has been implicated in the Porcine Respiratory Disease Complex (PRDC) as a contributing pathogen (Lee et al., 2016). Furthermore, *M. hyorhinis* is often found in conjunction with other pathogens and coinfections frequently occur, for instance, with Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Porcine Circovirus 2 (PCV2) (Lee et al., 2016).

Depending on the severity of the disease and affected organ systems, pigs may recover within one to two weeks but might have chronic impairment. Some, however, may succumb to the infection or need to be culled (Schreiber and Thacker, 2012).

1.3. Sample Collection for Laboratory Diagnosis

As the clinical presentations are nonspecific, an unequivocal diagnosis of a *M. hyorhinis* infection cannot be determined based solely on clinical examination. Diagnosis always necessitates the detection of the bacterium. Typically, swab samples are taken for bacteriological examination or molecular detection by PCR. Various approaches are available for this purpose. Samples can be taken from both live and deceased or culled animals. In the case of deceased pigs, samples are collected during necropsy from the affected tissue sites. This can be done through swabbing of joints and serous surfaces. It is advisable to take multiple samples from affected pigs to increase the likelihood of an accurate diagnosis (Leuwerke, 2009).

In live pigs, various methods can be employed to obtain informative samples. In cases of respiratory diseases, swabs from the upper respiratory tract and/or bronchoalveolar lavage (BAL) fluid sampling are recommended for the detection of *M. hyorhinis*. Generally, and this applies to the detection of many other pathogens as well, samples should be collected before any antibiotic treatment (Schreiber and Thacker, 2012).

In cases of lameness or swollen joints, samples for pathogen detection are obtained under sterile conditions. Synovial fluid is collected from the joint (Leuwerke, 2009).

Depending on the location of the lesion, different pathogens must be considered as differential diagnoses. For lameness or arthritis, besides *M. hyorhinis*, five other pathogens are to consider. These include *Streptococcus suis*, *Glaesserella parasuis*, *Actinobacillus suis*, *Erysipelothrix rhusiopathiae*, and *Mycoplasma hyosynoviae* (Leuwerke, 2009).

In cases of polyserositis, *Glaesserella parasuis*, *Actinobacillus pleuropneumoniae* and *Streptococcus suis* can cause similar lesions and clinical signs in affected pigs (Schreiber and Thacker, 2012).

1.4. Diagnostics and Molecular Typing

Laboratory diagnosis of *M. hyorhinis* is usually performed by cultivation and/or PCR. *M. hyorhinis* grows well in and on Friis medium (Kobisch and Friis, 1996). Its growth is indicated by a color change of the broth medium due to acidification based on glucose fermentation. Colonies of *M. hyorhinis* usually develop within 3-5 days on Friis agar, exhibit a typical fried egg morphology, and show variability in colony size and opacity (Palzer et al., 2020). After cultivation, *M. hyorhinis* is identified at the species-level using matrix-assisted laser desorption/ionization – time of flight (MALDI-ToF) mass spectrometry (Spergser et al., 2019). For the molecular detection of *M. hyorhinis* in clinical specimens, end-point and real-time PCR systems (Caron et al., 2000; Clavijo et al., 2014) have been developed, allowing a more rapid detection and quantification (real-time PCR) of the pathogen in samples examined. Multi-locus sequence typing (MLST) of *M. hyorhinis* strains was first described by Tocqueville et al. targeting the six housekeeping genes of the chromosomal replication initiation protein (*dnaA*), RNA polymerase subunit (*rpoB*), DNA gyrase subunit B (*gyrB*), glutamyl-tRNA synthetase (*gltX*), adenylate kinase (*adk*), and guanylate kinase (*gmk*) (Tocqueville et al., 2014).

In 2020, a new MLST scheme was described employing a different combination of six housekeeping genes including leader peptidase A (*lepA*), DNA-directed RNA polymerase

subunit beta (*rpoB*), DNA-directed RNA polymerase subunit beta (*rpoC*), glutamyl-tRNA synthetase (*gltX*), UvrABC system protein A (*uvrA*) and valine-tRNA ligase (*valS*) (Földi et al., 2020).

MLST is used for typing various *Mycoplasma* species including *M. hyorhinis* (Brown et al., 2015; El-Gazzar et al., 2016; Mayor et al., 2008).

1.5. Therapy and Prevention

Since they do not have cell walls, mycoplasmas are naturally resistant to antibiotics that interfere with cell wall synthesis, such as beta-lactams. In this regard, typical antibiotics effective against diseases caused by *Streptococcus suis*, *Glaesserella parasuis*, *Actinobacillus suis*, and *Erysipelothrix rhusiopathiae* show little to no success in treating *M. hyorhinis*-related illnesses. Particularly promising is the therapy involving macrolides and lincosamide antibiotics such as tylosin, tilmicosin, and lincomycin (Leuwerke, 2009). Success has also been observed with pleuromutilines (tiamulin), phenicoles (florfenicol), and tetracyclines (chlortetracycline) (Schreiber and Thacker, 2012). Both the antibiotic selection and the route of administration influence therapeutic success. Higher therapeutic efficacy has been reported with intramuscular administration rather than administration of the medications through food or water. However, as a general principle, the sooner the affected animals are treated, the better the chances of recovery and the lesser the likelihood of lasting damage (Leuwerke, 2009).

Prevention mainly involves in reducing stressors, which includes ensuring appropriate stocking density and maintaining stable environmental conditions. This means avoiding significant temperature fluctuations, providing adequate ventilation without causing drafts (Schreiber and Thacker, 2012), maintaining stable humidity levels, and ensuring sufficient food and water availability (Leuwerke, 2009). Additionally, prior infections with swine influenza virus, PRRS virus, or PCV2 may predispose pigs to disease through immune modulation, making pigs more susceptible to an outbreak of *M. hyorhinis*-related illness (Leuwerke, 2009). Vaccines are available for all above mentioned swine diseases, so it is advisable for farmers to consider vaccinating their pigs.

No specific vaccine has been licensed for *M. hyorhinis* in Europe yet. However, clinical vaccination trials have shown a reduction in cases of pericarditis and lameness (Martinson et al., 2018). Potential future approvals for vaccines appear promising based on current experimental data. Autogenous vaccines tailored to individual herds have demonstrated successful application in the field.

1.6. Core Genome Multi-Locus Sequence Typing

Core genome multi-locus sequence typing (cgMLST) represents a significant advancement in molecular infection epidemiology, providing a robust framework for high-resolution typing of bacterial species. This method extends the traditional MLST by including a larger set of genes, often several hundred, from the core genome of a bacterial species. As a result, cgMLST provides deeper insight into the genetic relationships and evolution of pathogens, enhancing epidemiological investigations, outbreak tracking, and identification of infection sources (Ghanem and El-Gazzar, 2018; Menghwar et al., 2022).

The cgMLST builds upon the principles of MLST, which targets a small number of housekeeping genes, usually 6-7 genes, to characterize bacterial isolates (Maiden et al., 1998). In contrast, cgMLST encompasses a much broader array of genes that are conserved across the core genome of the species being studied. The core genome consists of genes that are present in all strains of a species, providing a stable genetic foundation for comparative analyses (Ruppitsch et al. 2015). By examining hundreds of these conserved loci, cgMLST achieves higher resolution in distinguishing between closely related strains (Dyer et al., 2025).

The process of cgMLST involves several key steps:

1. **Genome Sequencing:** Whole genome sequencing is performed on the bacterial isolates to obtain comprehensive genetic data (Leopold et al., 2014).
2. **Gene Identification:** Using bioinformatics tools, genes from the core genome are identified and extracted from the whole genome sequencing data (Ruppitsch et al., 2015).
3. **Allele Assignment:** Each core target gene is analyzed to determine its specific allele type. This involves comparing the gene sequences to a reference database of known alleles (Dyer et al., 2025).
4. **Allelic Profiling:** The combination of allele types across all core genes constitutes the allelic profile of the isolate (Leopold et al., 2014).
5. **Data Analysis:** Allelic profiles are compared to assess genetic relatedness among isolates. Phylogenetic trees and clustering algorithms are often used to visualize these relationships (Moura et al., 2016).

The comprehensive nature of cgMLST makes it particularly useful for studying bacterial pathogens with significant genetic diversity. It not only provides a high-resolution typing scheme but also facilitates the identification of genetic variations that may contribute to virulence, antibiotic resistance, and other clinically relevant traits (Ruppitsch et al., 2015).

The cgMLST approach offers several advantages over traditional typing methods. Its high resolution enables the discrimination of closely related strains, which is crucial for outbreak investigations and transmission studies. Additionally, the standardized nature of cgMLST allows for easy comparison of data across different laboratories and studies, fostering collaboration and data sharing within the scientific community (Moura et al., 2016).

However, cgMLST is not without its challenges. The requirement for whole-genome sequencing and advanced bioinformatics tools can be resource-intensive, which may limit its use in laboratories with restricted funding. Furthermore, the generation and maintenance of comprehensive allele databases necessitate ongoing efforts in data curation and quality control (Uelze et al., 2020).

2. Original Publication

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A core genome multilocus sequence typing scheme for *Mycoplasma hyorhinis*

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ABSTRACT

Mycoplasma (M.) hyorhinis is a commensal and pathobiont residing in the upper respiratory tract in swine and with the ability to spread systemically, mainly causing polyserositis and polyarthritis in nursery pigs. Since little is known on the epidemiology of *M. hyorhinis* infection, whole genome sequences of 73 strains isolated from pigs in Austria ($n = 71$) and Germany ($n = 2$), that have been isolated from clinically affected pigs during routine diagnostics, and publicly available genomes of eight *M. hyorhinis* strains were analyzed in the presented study. For this purpose, a core genome multi locus sequence typing (cgMLST) scheme encompassing 453 target genes was developed using the Ridom® SeqSphere + software. Results were compared to two previously described conventional MLST schemes and to a core genome single nucleotide polymorphism (cgSNP) analysis approach. Core genome MLST showed high diversity among the *M. hyorhinis* strains studied and while certain isolates from one farm or a single animal formed cgMLST clusters (≤ 8 allele differences), no isolates with identical allele profiles were identified. In addition, cgMLST had superior discriminatory power (Simpson's ID = 0.995) over conventional MLST (Simpson's ID = 0.952 and 0.905), while demonstrating a lack of congruence between conventional MLST and genome-wide relationship. Core genome SNP results were highly congruent with cgMLST results but lacked in resolution when comparing closely related isolates. Thus, cgMLST is the most suitable method for epidemiological investigations such as outbreak analysis, and to gain insights into *M. hyorhinis* population structure.

1. Introduction

Mycoplasma (M.) hyorhinis is a ubiquitously occurring commensal and pathobiont in swine that resides in the upper airways (Friis and Feenstra, 1994; Kobisch and Friis, 1996; Rovira, 2009). *M. hyorhinis* colonizes the respiratory epithelium, where it is suspected to damage the cilia and reduce epithelial thickness, which may favor systemic spread (Lee et al., 2016), although mechanisms underlying systemic infection are only poorly understood (Pieters and Maes, 2019). *M. hyorhinis* commonly causes polyserositis and polyarthritis in piglets ranging from 3 to 10 weeks of age, while older piglets most often only develop mild arthritis (Friis and Feenstra, 1994; Kobisch and Friis, 1996; Rovira, 2009). *M. hyorhinis* has also been shown to be an important factor in the

Porcine Respiratory Disease Complex (PRDC) where it acts as a secondary pathogen (Lee et al., 2016) but is also capable of inducing lung lesions after experimental infection (Lin et al., 2006; Lee et al., 2018). Other clinical manifestations such as otitis and eustachitis (Morita et al., 1995, 1999), abortion (Shin et al., 2003), conjunctivitis (Resende et al., 2019) and meningitis (Bünger et al., 2020) have also been reported in conjunction with *M. hyorhinis* infection. However, the role and importance of *M. hyorhinis* in the pathogenesis of these diseases remain uncertain until further investigated.

In general, *M. hyorhinis* infection is difficult to control and to eliminate from affected farms. Since no commercial vaccines against *M. hyorhinis* are currently available in Europe, control of infection relies on antibiotic treatment and/or measures preventing further spread of

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the pathogen and interrupting infection chains. For monitoring *Mycoplasma* infections, and to identify infection sources and modes of transmission, sequence-based genotyping methods such as multilocus sequence typing (MLST) (Mayor et al., 2008; Manso-Silván et al., 2012), multiple locus variable number of tandem repeat (VNTR) analysis (MLVA) (Nathues et al., 2011; Dos Santos et al., 2015), single nucleotide variant (SNV) analysis (Kinnear et al., 2021) and single nucleotide polymorphism (SNP) analysis (Ghanem and El-Gazzar, 2018; Kovács et al., 2020; Yair et al., 2020) have been described. The first MLST protocol for *M. hyorhinis* was developed by Tocqueville et al. (2014) based on the housekeeping genes *dnaA*, *rpoB*, *gyrB*, *glx*, *adk* and *gmk*, which had a higher power of discrimination than gene sequencing of the high-affinity transport system protein p37. This protocol was enhanced and simplified by Trüeb et al. (2016), by designing new primers for the six MLST genes allowing amplification and sequencing with one protocol. Clavijo et al. (2019) designed a MLST-s protocol that included two genes encoding surface proteins (*p95*, *mtlD*) next to two housekeeping genes (*pdhB*, *ung*) to further improve discriminatory power (Clavijo et al., 2019). Very recently, Földi et al. (2020) developed a new MLST for *M. hyorhinis* utilizing the housekeeping genes *lepA*, *rpoB*, *rpoC*, *glx*, *uvrA* and *valS* and compared it to the previously described MLST (Tocqueville et al., 2014; Trüeb et al., 2016) and MLST-s (Clavijo et al., 2019), resulting in a higher discriminatory power for the new MLST scheme; nevertheless, the authors recommended combining MLST with MLVA for fine-scale typing of *M. hyorhinis* (Földi et al., 2020). Overall, the resolution of conventional MLST seemed to be rather moderate for the differentiation of closely related *M. hyorhinis* strains in previous studies. In contrast, core genome multi locus sequence typing (cgMLST) based on whole genome sequencing (WGS) data has been proven to be a superior typing tool highly suited for epidemiologic studies including outbreak analyses. Core genome MLST schemes for several microbial pathogens including mycoplasmas like *M. gallisepticum*, *M. synoviae*, and *M. anserisalpiginidis* (Ghanem and El-Gazzar, 2018; Ghanem et al., 2018; Kovács et al., 2020) have been established, providing accurate, reproducible, portable, and comparable typing results, and therefore exhibiting sufficient discriminatory power for outbreak investigations.

Here, we report the development and evaluation of a standardized cgMLST scheme for the accurate typing of *M. hyorhinis* strains by characterizing isolates predominantly recovered from Austrian pig herds and by comparing cgMLST results with those of conventional MLST schemes.

2. Material and methods

2.1. *Mycoplasma hyorhinis* strains and whole genome sequencing

Whole genome sequences of 81 *M. hyorhinis* strains were analyzed in the study including 71 strains isolated from pigs in Austria, two strains isolated from pigs in Germany, and eight *M. hyorhinis* strains for which finished genomes were publicly available (PG42^T [IMT49388], SK76, HUB-1, JF5820, GDL-1, DBS1050, MCLD, MDBK-IPV) (Genbank accession numbers provided in Table S1). All Austrian and the two German *M. hyorhinis* strains were obtained from pigs between 2009 and 2020 during routine diagnostic examinations of porcine samples taken from different body sites including the nasal cavity, lungs, serous membranes (serosa), joints (synovia), eyes and the brain. All sampled animals showed clinical disease and/or pathologic lesions associated with *M. hyorhinis* infection (Table S1). All piglets and samples had been referred to the University Clinic for Swine for diagnostic purposes by the owner and herd veterinarian. The two German strains originated from different farms, while the 71 Austrian *M. hyorhinis* isolates originated from a total of 48 pig farms. More than one *M. hyorhinis* strain were derived from 14 farms ($n = 37$ strains); on 10 of these farms, multiple isolates were derived from one individual pig ($n = 22$ strains) (Table S1). *M. hyorhinis* strains were cultured and identified to the species level using 16S rRNA gene sequencing (isolates collected before 2016) or by MALDI-TOF mass spectrometry (MS) (isolates from 2016 on) as described previously

(Spergser et al., 2019; Bünge et al., 2020). All isolates were stored at -80°C until further investigation.

For WGS, Austrian and German *M. hyorhinis* isolates were thawed and streaked onto SP4 agar plates (Bünge et al., 2020). After incubation at 37°C under 5% CO_2 atmosphere for up to 7 days, a single colony was picked from each isolate and cultured in 3 mL SP4 medium to the late logarithmic growth phase. Cultures were rechecked for species affiliation using MALDI-TOF MS and DNA was then extracted using MagAttract[®] HMW DNA Kit according to the protocol of the manufacturer (Qiagen, Hilden, Germany). Quantity and quality of extracted DNA were determined by NanoDrop[™] 2000 (Thermo Scientific, Vienna, Austria). Whole genome sequencing was performed on ready-to-sequence libraries prepared by the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, USA) and the Illumina MiSeq platform (Illumina, San Diego, USA) was used for paired-end sequencing. Raw reads were quality checked, trimmed, and *de novo* assembled using SPAdes 3.10, with default parameters (Bankevich et al., 2012). Whole genome sequences of all 73 sequenced *M. hyorhinis* strains were deposited at Genbank and are accessible under the accession numbers provided in Table S1.

2.2. Core genome MLST

A cgMLST scheme for *M. hyorhinis* was developed using Ridom[®] SeqSphere + version 7.0 (Ridom[®] GmbH, Münster, Germany; <https://www.ridom.de/seqsphere/>) as described previously for two other *Mycoplasma* species (Ghanem and El-Gazzar, 2018; Ghanem et al., 2018). Briefly, the reference genome of *M. hyorhinis* type strain PG42^T (CP064323) and 20 query genomes were utilized for the development of an *ad hoc* cgMLST scheme. Query genomes were selected based on MLST analysis of 80 *M. hyorhinis* genomes blasted (Altschul et al., 1990) against core genes of the reference genome identified after filtration. Afterwards, a minimum spanning tree (MST) was generated including all the blasted *M. hyorhinis* isolates. Of those, 20 *M. hyorhinis* isolates exhibiting >100 allele differences to their closest relatives and representing the overall diversity of the studied *M. hyorhinis* population (geography, year of isolation, source) were selected to be included as query genomes (Table S1). As first step in cgMLST development, a target gene set appropriate for cgMLST was defined using the cgMLST Target Definer tool of SeqSphere + with default parameters that filtered the reference genome to exclude unfit gene targets. Filters included a homologous gene filter (excluding genes with > 90 % identity and > 100 bp overlap), a start codon filter (excluding genes missing a start codon), a minimum length filter (excluding genes < 50 bp), a stop codon filter (excluding genes devoid of a stop codon, with multiple stop codons, or with a stop codon not located at the end of the gene), and a gene overlap filter (excluding the shorter of two genes that overlap by > 4 bp) (Ghanem and El-Gazzar, 2018; Ghanem et al., 2018). Then the 20 query genomes were blasted pairwise against the reference genome's target gene set to select shared fit targets. All reference genome-filtered genes that were found in all query genomes with a sequence identity of > 90 % and 100 % overlap formed the final targets of the cgMLST scheme. For cgMLST evaluation, the remaining 60 genomes (Table S1) were used to identify the percentage of cgMLST targets present in the *M. hyorhinis* strain cohort (good cgMLST target genes). Finally, all 81 *M. hyorhinis* strains were analyzed by the developed cgMLST scheme to define individual allelic profiles utilized to construct an MST by pairwise ignoring missing values. To determine allelic distances between closely related *M. hyorhinis* strains, isolates recovered from the lung and inner organs of individual pigs were compared and the threshold for clonal cluster definition was set based on the results of the cgMLST MST within individual pigs. Furthermore, a phylogenetic tree was calculated using the neighbor joining (NJ) algorithm implemented in the SeqSphere + software and visualized by applying iTOL (v. 5.7; <https://itol.embl.de/>) (Letunic and Bork, 2019).

2.3. Core genome SNP typing

For comparative purposes and to evaluate the resolution of the *M. hyorhinis* cgMLST, a single-nucleotide polymorphism (SNP) analysis was performed using the Find Nucleotide Variants tool of SeqSphere + with default parameters excluding insertions/deletions and neighboring SNPs located within 300 bp or less to minimize effects of recombination induced polymorphisms. For each *M. hyorhinis* strain, a concatenated sequence of cgMLST target genes was aligned to the reference sequence, and a neighbor-joining phylogenetic tree was generated based on the core genome SNPs after exclusion of target genes that were not found in all 81 *M. hyorhinis* strains.

2.4. Conventional MLST

MLST analyses were performed on *M. hyorhinis* strains according to two published MLST schemes, termed here as MLSTa (Tocqueville et al., 2014; Trüeb et al., 2016) and MLSTb (Földi et al., 2020), either by amplifying gene fragments from *dnaA*, *rpoB*, *gyrB*, *gltX*, *adk* and *gmk* (Trüeb et al., 2016) or by analyzing gene fragments from *lepA*, *rpoB*, *rpoC*, *gltX*, *uvrA*, and *valS* (Földi et al., 2020) extracted from WGS. For MLSTa, resulting allele sequences were compared with those published at PubMLST (<https://pubmlst.org/mhyorhinis/>) and field strains were assigned to sequence types (STs) according to their MLST profiles. In case of field strains harboring novel allele sequences, sequences were deposited at the PubMLST database and new STs were defined. For MLSTb, sequences extracted from WGS were compared to entries in the Genbank database (<https://www.ncbi.nlm.nih.gov/nucleotide/>; *lepA*: MT090880-MT090918, *rpoB*: MT090841-MT090879, *rpoC*: MT090802-MT090840, *gltX*: MT090919-MT090957, *uvrA*: MT090763-MT090801, *valS*: MT106126-MT106164). Since all investigated field strains either showed novel allele sequences in at least one of the analyzed house-keeping genes or new allelic profile combinations (STs), an arbitrary number (starting from the already defined allele and ST numbers) was assigned to each new allelic type or allele combination with identical sequences or combinations being assigned the same allele or ST number. Minimum spanning trees (MSTs) based on allelic profiles (STs) obtained from investigated *M. hyorhinis* strains were generated using GrapeTree (Zhou et al., 2017) including profiles available at the PubMLST database (MLSTa) or published by Földi et al. (2020) (MLSTb).

The discriminatory power of cgMLST and conventional MLST schemes were determined and compared based on the Simpson's index of diversity (ID) (Hunter and Gaston, 1988) by applying the online tool Comparing Partitions (<http://www.comparingpartitions.info/>) using default parameters. To assess the overall concordance between cgMLST, MLSTa and MLSTb, Adjusted Rand coefficients (Hubert and Arabie, 1985) also using default parameters were calculated by Comparing Partitions.

3. Results

3.1. Whole genome sequencing

All 73 *M. hyorhinis* isolates were successfully sequenced using an Illumina MiSeq platform. Resulting raw reads (mean number 375,200) were quality filtered and assembled *de novo* generating assemblies with a mean size of 823.025 Kb (all representing full genomes of *M. hyorhinis*), a mean number of 63.1 contigs (>300 bases), and an average calculated coverage of 40.6. Raw reads and genome assemblies were deposited at GenBank databases (accession numbers are presented in Table S1).

3.2. Development and evaluation of the *M. hyorhinis* cgMLST scheme

Based on the *M. hyorhinis* PG42^T reference genome (CP064323)

containing 708 genes with coding DNA sequences, a reference task template was generated that retained a total of 656 genes after filtering. Using seven finished genomes and WGS of 13 isolates sequenced in our study as query genomes, a cgMLST scheme with 453 target genes (495,930 bases) was defined, covering 59 % of the reference genome sequence. The number of accessory genes was 203 (207,861 bases), representing 25.7 % of the reference genome sequence. Core target genes defined are listed in Table S2. For evaluation purposes, WGS of 60 *M. hyorhinis* isolates were loaded into SeqSphere + and typed according to the 453 cgMLST target scheme. At least 97 % (mean 98.9 % $\pm$ 0.92 standard deviation) good cgMLST target genes were found in all WGS used for evaluation. The average number of alleles for 453 targets was 15 alleles (range 2–60 alleles). To determine clonal cluster distances, a subset of 18 isolates recovered from the lung and inner organs of eight pigs was used. The isolates 1366L18, 1366N18, 3051L16 and 3051S16 from the two pigs g and k (Table S1) were excluded from cluster threshold determination as they showed numbers of allele differences that are not expected for a clonal cluster (Fig. 1, Ghanem et al., 2018). The maximum number of allele differences observed among these strains was eight alleles, thus we defined a difference of eight alleles as the threshold for distinct clonal clusters. Based on this threshold, 10 different clonal clusters comprising 18 isolates from eight pigs (eight farms) and five isolates from different animals kept in two farms (farms B and N) were identified (Figs. 1, 2, 3 and Fig. S1).

3.3. Typing of *M. hyorhinis* strains using cgMLST

The cgMLST scheme was tested on all 81 strains to investigate the epidemiology of *M. hyorhinis*, to obtain insights into its population structure, and to evaluate the relatedness of the 71 included Austrian strains. Allelic profiles for all strains are listed in Table S2. Overall, results of cgMLST showed a high degree of diversity among epidemiologically unrelated *M. hyorhinis* strains including those isolated in Austria, illustrated by a high number of allele differences between unrelated strains (Fig. 1). Moreover, there was a clear separation between the Austrian and German strain cohort and the eight strains with publicly available genome sequences that originated from different parts of the world (Figs. 2 and 3). On the other hand, epidemiologically related strains grouped together or were even part of a clonal cluster as shown for strains concurrently isolated either from individual pigs (from different body sites) or from different animals in farm A and C–N (Figs. 2 and 3). Even isolates recovered at different time points from animals in farm B, D, F, H, J, K, and N, with time intervals between isolations ranging from three months (farm J, 450L20/450S20 – 450N20) to four years (farm H, 2731S16/2731J16 – 2731N20; farm K, 3051L16/3051S16 – 3051N20) grouped together (Figs. 2 and 3), albeit expressing higher numbers of allele differences (Fig. 1). Although several strains isolated from individual pigs or from different animals per farm were typed into the same clonal cluster, distances between other isolates from individual pigs or from different animals per farm were > 60 different alleles indicating a diverse population of *M. hyorhinis* existing in certain farms (C, G, I, K) or even in individual animals (no. 1366, 3051) (Figs. 1 and 2). Furthermore, four strains (1474S18, 560N15, 3933N18, 87_4N17) isolated from four different farms clustered close to farm A-, B- or N-related strains (Fig. 2) which could be explained by the fact that corresponding farms were located in the same federal state and in three of the four cases in the same district (Table S1). No further correlation was identified between source, year of isolation, federal state, district, and the distinct clonal clusters defined (Table S1, Fig. 3). Altogether, cgMLST typed the 81 *M. hyorhinis* strains into 68 different STs based on > 8 allele differences (threshold for clonal clusters) resulting in a Simpson's ID of 0.995.

3.4. Comparison between cgMLST and cgSNP typing

To evaluate the resolution of the newly defined *M. hyorhinis* cgMLST,

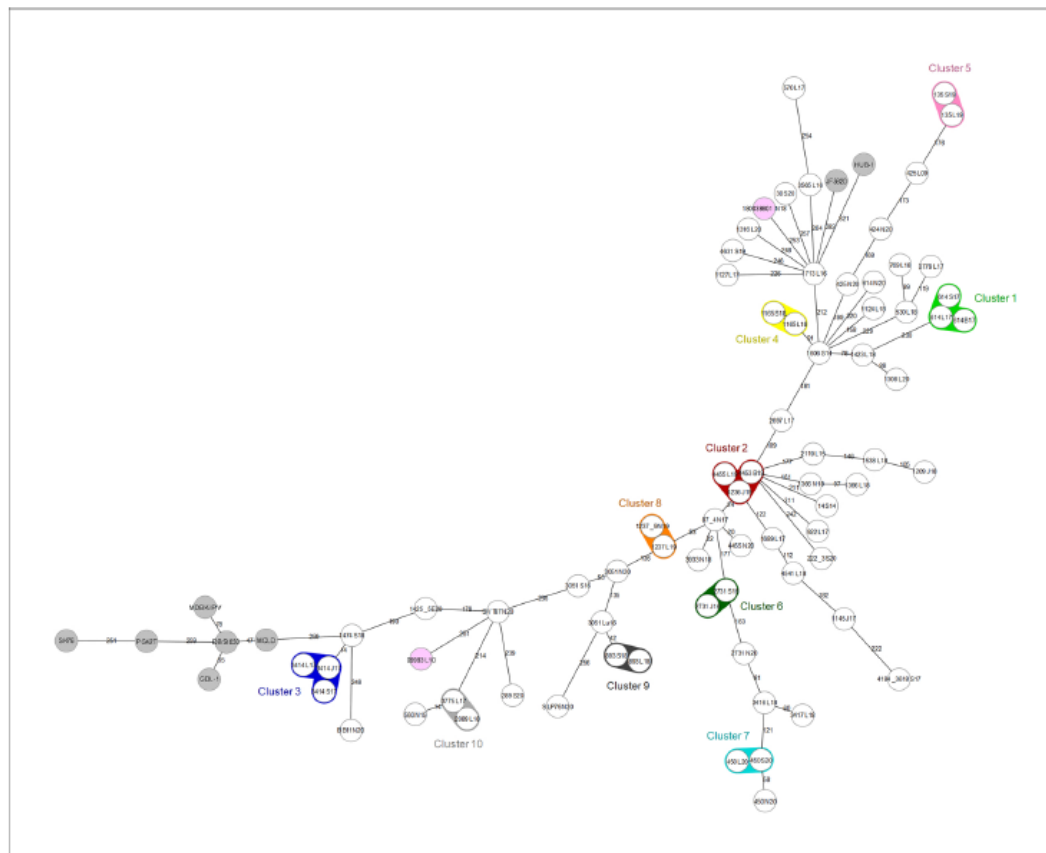


Fig. 1. Minimum spanning tree based on allelic profiles of 453 core genome targets from 81 *M. hyorhinis* strains.

The distance matrix underlying the network was built from all pairwise allelic profile comparisons of 453 cgMLST targets using the pairwise ignoring-missing-values option in SeqSphere+ software. Ten distinct clonal clusters with fewer than 8 allele differences are color coded. Strain origin is indicated by the color of the circles: pink = Germany; grey = publicly available genome, white = Austria. The number on connecting lines illustrates the number of differing alleles in a pairwise comparison.

results obtained were compared with a cgSNP typing approach. Two hundred and eighty-eight cgMLST target genes were present in all 81 strains and therefore included in cgSNP analysis. A total of 3822 filtered SNP positions were identified and considered for tree calculation. The resulting neighbor joining (NJ) tree (Fig. S1) was compared to the NJ tree built from cgMLST profiles of the 81 *M. hyorhinis* strains (Fig. 2). This demonstrated a high degree of congruence between the topologies of closely related strains and concordant clustering according to cgMLST clonal cluster definition with a weaker resolution observed for cgSNP typing. More distantly related strains, however, performed differently with the two typing methods applied.

3.5. Typing of *M. hyorhinis* strains using conventional MLST schemes

All 81 *M. hyorhinis* strains were also tested by two previously described MLST schemes, here termed as MLSTa (Tocqueville et al., 2014; Tr ub et al., 2016) and MLSTb (F ldi et al., 2020). By applying MLSTa resulting STs were compared with those published in the PubMLST database (231 strains in total, including the 81 *M. hyorhinis* strains) (Fig. S2A). The 81 *M. hyorhinis* strains were typed into 42 STs (Simpson's ID of 0.952) with ST40 being the most prevalent ($n = 15$) followed by ST92 ($n = 6$), ST95, and ST98 (both $n = 4$). By utilizing the recently published MLSTb, the analyzed 81 *M. hyorhinis* strains belonged to 54 different STs (Simpson's ID of 0.985) predominantly assigned to

ST49 ($n = 5$), ST60, ST67, ST73, and ST75 (all $n = 4$). Comparison of resulting STs was limited to *M. hyorhinis* strains from Hungary ($n = 38$) and Slovakia ($n = 1$) which were published by F ldi et al. (2020, Fig. S2B), as no MLSTb results for other strains are available to date. When STs obtained from MLSTa and MLSTb were compared to cgMLST results and clonal cluster formation, lower resolution capabilities of both conventional MLST schemes were evident (Fig. 2) and the Adjusted Rand coefficients defining the proportion of congruence between the typing methods used were moderate (0.496 for cgMLST-MLSTb) to extremely low (0.278 for MLSTa-MLSTb, and 0.179 for cgMLST-MLSTa) indicating moderate to very low concordance between the utilized typing methods.

4. Discussion

This study describes the first WGS based typing of *M. hyorhinis* that yields high discriminatory power, differentiating 81 strains into 68 cgMLST STs. Comparison with conventional MLST (Fig. 2) confirms the higher discriminatory power of cgMLST over conventional MLST previously described for other *Mycoplasma* species (Ghanem and El-Gazzar, 2018; Kinnear et al., 2021). Additionally, some results of conventional MLSTa also contradict cgMLST data, as isolates typed into STs 40, 80, 92 and 98 each contained groups of isolates that were only distantly related when genome-wide allele calling was performed (Fig. 2). The more

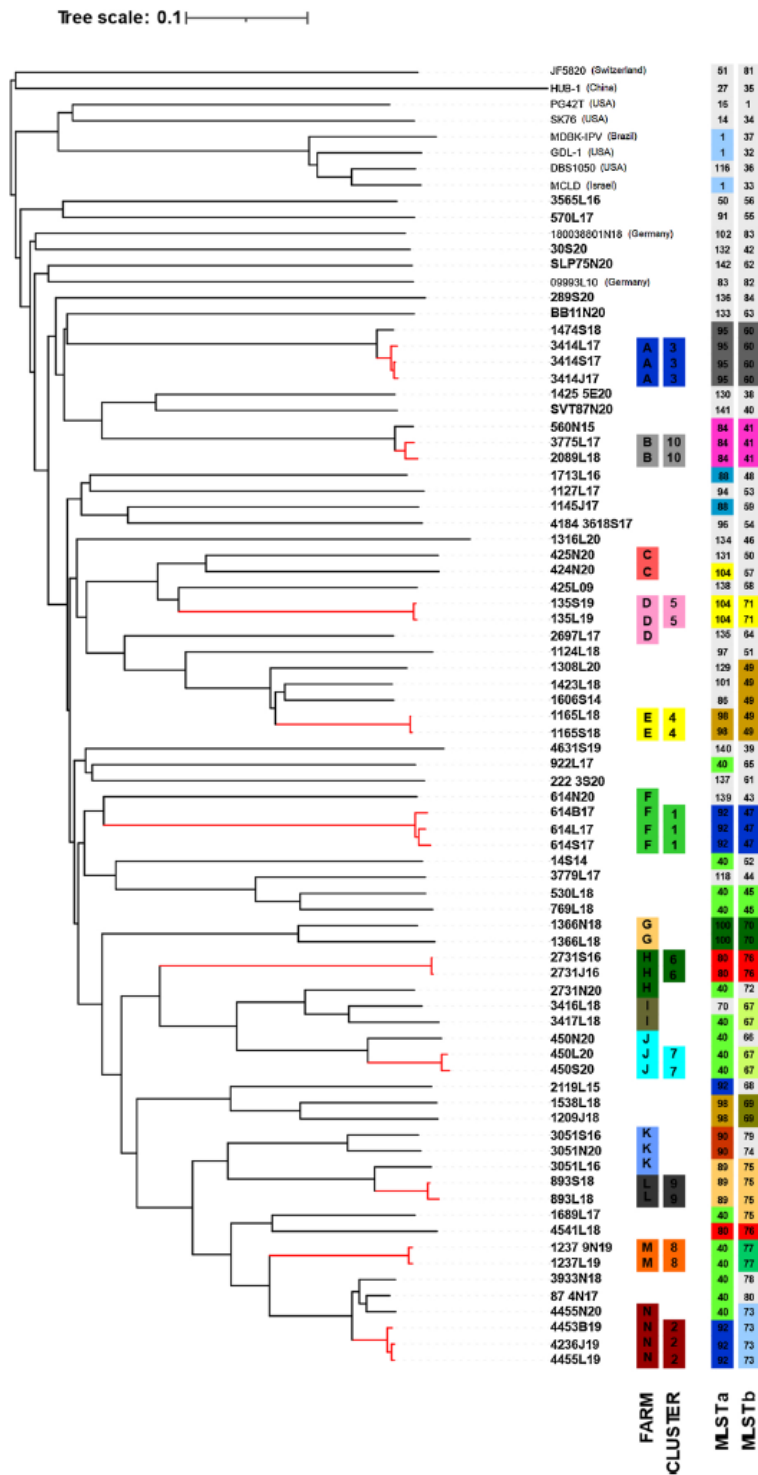


Fig. 2. Phylogenetic tree based on allelic profiles of 453 core genome targets from 81 *M. hyorhinis* strains.

The tree was built using the neighbor-joining algorithm and pairwise ignoring-missing-values option in SeqSphere+. Clades of clonal clusters are highlighted in red. Data on farm, clonal cluster and STs resulting from MLSTa and MLSTb are depicted opposed to the tree. Visualization was realized using iTOL. Origin of strains not from Austria are indicated behind the strain designation.

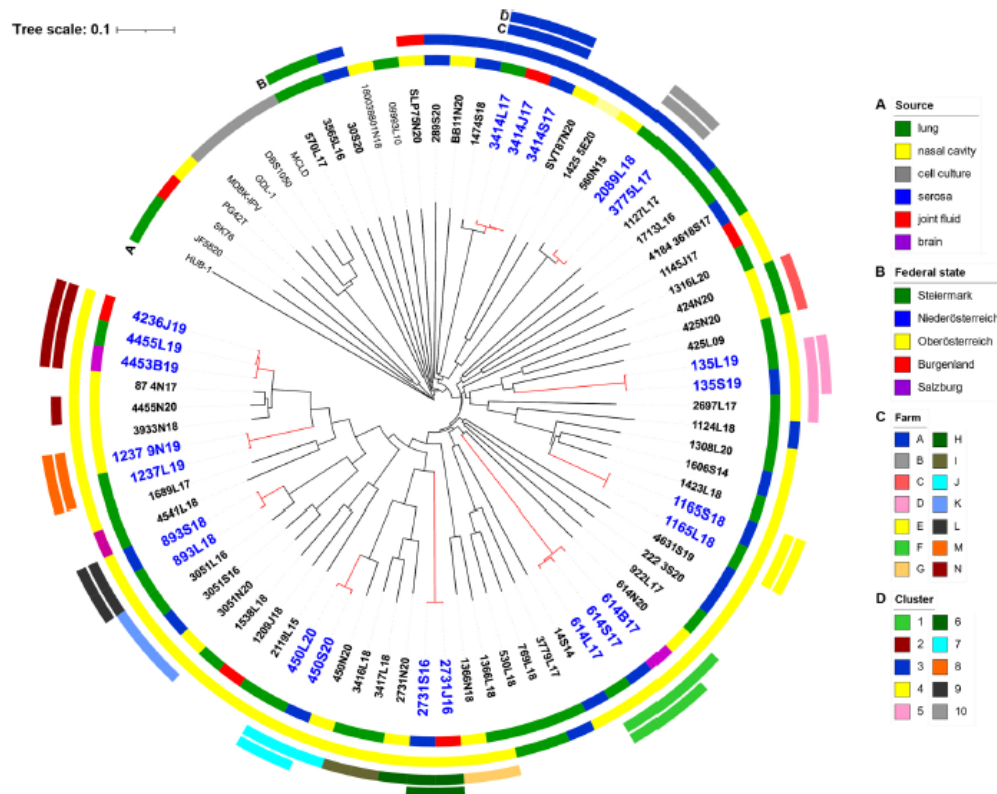


Fig. 3. Neighbor joining phylogenetic tree based on cgMLST analysis of 81 *M. hyorhinis* strains.

cgMLST was calculated with SeqSphere+ (pairwise ignoring-missing-values option). Clades of clonal clusters are colored in red and strains comprising distinct clusters highlighted in blue. Available metadata for source or sample type (A), Austrian federal state (B), farm (C), and clonal cluster (D) are depicted as colored circles. Visualization was realized using iTOL.

recent MLSTb seemed to reflect genome-wide relationship much better (Fig. 2), although the moderate Adjusted Rand coefficients indicate that sequence typing of only few selected housekeeping genes does not always reflect the genomic relationship between isolates correctly, limiting the suitability of conventional MLST protocols for epidemiological studies and outbreak analyses. Higher efforts in both cost and time are the main downsides of cgMLST compared to conventional MLST and MLST-s protocols (Trüeb et al., 2016; Clavijo et al., 2019; Foldi et al., 2020). However, isolation of *M. hyorhinis* is less laborious and time-consuming than for certain other *Mycoplasma* species and next generation sequencing is developing quickly, making WGS faster and more affordable and, therefore, more suitable for epidemiologic investigations. Compliant with previous studies (Ghanem and El-Gazzar, 2018; Ghanem et al., 2018; Kovács et al., 2020), comparison of cgSNP and cgMLST typing results in our study revealed high congruence in topology and concordant clustering (Fig. 2, Fig. S1) of closely related strains with a small advantage for cgMLST in resolution especially for closely related isolates. The latter is due to cgSNP being limited to target genes that are present in all strains, while cgMLST includes all genes that were pairwise compared ignoring missing values.

In the present study, the threshold for clonal clusters was determined at ≤ 8 allele differences, which is similar to the cluster typing threshold of ≤ 10 allele differences that was previously described for *M. gallisepticum* (Ghanem et al., 2018) and should also account for nucleotide variability within one strain that might occur after repeated passages (Ghanem and El-Gazzar, 2018). Clonal clusters were only formed by isolates from one farm and/or individual pigs. However, this was not

true for all isolates from one farm or pig as some showed distinctly larger allele differences (farms C, G, I, K; Fig. 1,2) indicating diverse *M. hyorhinis* populations within single farms and individual animals that consist of several subpopulations more distantly related to each other. On the other hand, as differences of ≥ 58 alleles occurred after only three months on farm J (450S20 – 450N20), another possible explanation for those larger differences is rapid evolution of *M. hyorhinis* strains within one system which has already been shown for *M. gallisepticum* (Delaney et al., 2012). Another finding of note is the relatedness of isolates from farm K, where two isolates taken from the same animal in 2016 (3051L16, 3051S16) were both closer related to a nasal isolate from 2020 (3051N20) than to each other (Fig. 1), supporting the theory of diverse subpopulations that are related but undergo constant and rapid changes. It also suggests populations on single farms being relatively stable over time, which is reinforced by two isolates from farm B that formed a clonal cluster (Fig. 1, Cluster 10) despite the samples being taken six months apart. In general, pig farms in Austria are concentrated in certain regions, resulting in a high number of isolates from these areas in our sample pool, but information about relations in pig flow or pig trade relations were mainly unavailable for this study. At large, the correlation of geographical origin of isolates and their relationship seems to be low for isolates in the present study. These findings are compliant with findings from Kovács et al. (2020) that could not show any correlation between geographical distances and cgMLST results for *M. anserisalpiginidis*, while isolates from the same integration were more closely related, probably due to host movement or contact. Thus, for our study, close relationship between isolates from different farms (Fig. 2

and 3) can most likely be explained by an epidemiological relationship such as a related pig flow. In most cases, isolates from one system were closer related than isolates that presumably were not epidemiologically related, even if the isolates did not form a clonal cluster and/or were collected at different time points. Furthermore, cgMLST was able to differentiate between isolates from individual animals and did not find isolates with identical allele profiles. Thus, this study confirms previous conclusions on cgMLST as a highly suitable tool for epidemiological studies such as outbreak investigations and pathogen tracing of *Mycoplasma* species (Ghanem and El-Gazzar, 2018; Ghanem et al., 2018; Kovács et al., 2020), now including *M. hyorhinis*.

As of our findings, cgMLST does not seem to reflect any aspects of pathogenicity, such as virulence, likelihood of systemic spread or affinity to certain organs, currently precluding the utility of this method for risk-based diagnostics. However, when analyzing *M. pneumoniae* strains, SNP analysis has proven to be superior to MLST while typing non-housekeeping genes (Touati et al., 2015) and has been used to determine macrolide susceptibility (Ji et al., 2014). Further, MLST-s added surface protein encoding genes to improve discriminatory power over the original MLST protocol (Clavijo et al., 2019), so did MLVA when combined with the recently described MLSTb (Földi et al., 2020). It is to be expected, that using the combination of MLSTb and MLVA on the data set of this study would yield a discriminatory power much closer to that of cgMLST. However, as this study was focused on the development of a standalone method that can be operated *in silico* on WGS data, a direct comparison of cgMLST with MLSTb combined with MLVA was not included in this study. In conclusion, adding MLVA, explorative SNP or accessory gene typing on top of the cgMLST scheme might improve the discriminatory power in differentiating closely related *M. hyorhinis* strains even further which might allow drawing conclusions on pathogenicity.

Finally, this study only contains isolates originating from farms with clinical disease which has been the reason for the initiation of diagnostics including *M. hyorhinis* detection. Thus, the isolates analyzed in this study are neither representative for the entire *M. hyorhinis* population in the respective farms, nor do the samples reflect the existing *M. hyorhinis* population in Austria correctly. To improve the understanding of *M. hyorhinis* population structure and epidemiology as well as pathogenesis of infection, analyzing a more representative sample pool that includes commensal isolates from apparently unaffected farms is required, as is documentation of pig flow or other potential pathogen transmission modes. Also, as our findings suggest the possible occurrence of multiple subpopulations of *M. hyorhinis* within a farm, analyzing larger sample sizes from each farm is advisable.

5. Conclusion

In summary, the cgMLST scheme developed here demonstrated to be highly discriminatory for the accurate differentiation of *M. hyorhinis* strains, which can be as well used in an outbreak investigation context as a complement to epidemiological data.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetmic.2021.109249>.

References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
- Bankovich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V. M., Nikolenko, S.I., Pham, S., Pribelski, A.D., Pyshkin, A.V., Sirotdin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. <https://doi.org/10.1089/cmb.2012.0021>.
- Bünger, M., Brunthaler, R., Unterwiesing, C., Loncaric, I., Dippel, M., Ruczkicka, U., Schwarz, L., Griessler, A., Voglmayr, T., Verhovsek, D., Ladinig, A., Spersger, J., 2020. *Mycoplasma hyorhinis* as a possible cause of fibrinopurulent meningitis in pigs? - a case series. *Porc. Heal. Manag.* 6, 1–8. <https://doi.org/10.1186/s40813-020-00178-8>.
- Clavijo, M.J., Sreevatsan, S., Johnson, T.J., Rovira, A., 2019. Molecular epidemiology of *Mycoplasma hyorhinis* porcine field isolates in the United States. *PLoS One* 14, e0223653. <https://doi.org/10.1371/journal.pone.0223653>.
- Delaney, N.F., Balenger, S., Bonneaud, C., Marx, C.J., Hill, G.E., Ferguson-Noel, N., Tsai, P., Rodrigo, A., Edwards, S.V., 2012. Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, *Mycoplasma gallisepticum*. *PLoS Genet.* 8 <https://doi.org/10.1371/journal.pgen.1002511>.
- Dos Santos, L.F., Clavijo, M.J., Sreevatsan, S., Rovira, A., Moreira, M.A.S., Pieters, M., 2015. Genotyping of *Mycoplasma hyorhinis* using multiple-locus variable number tandem repeat analysis. *J. Microbiol. Methods* 111, 87–92. <https://doi.org/10.1016/j.jmimet.2015.02.003>.
- Földi, D., Bekő, K., Felde, O., Kreisinger, Z., Kovács, Á.B., Tóth, F., Bányai, K., Kiss, K., Biksi, I., Gyuranecz, M., 2020. Genotyping *Mycoplasma hyorhinis* by multi-locus sequence typing and multiple-locus variable-number tandem-repeat analysis. *Vet. Microbiol.* 249 <https://doi.org/10.1016/j.vetmic.2020.108836>.
- Friis, N.F., Feenstra, A.A., 1994. *Mycoplasma hyorhinis* in the etiology of serositis among piglets. *Acta Vet. Scand.* 35, 93–98. <https://doi.org/10.1186/BF03548359>.
- Ghanem, M., El-Gazzar, M., 2018. Development of *Mycoplasma synoviae* (MS) core genome multilocus sequence typing (cgMLST) scheme. *Vet. Microbiol.* 218, 84–89. <https://doi.org/10.1016/j.vetmic.2018.03.021>.
- Ghanem, M., Wang, L., Zhang, Y., Edwards, S., Lu, A., Ley, D., El-Gazzar, M., 2018. Core genome multilocus sequence typing: a standardized approach for molecular typing of *Mycoplasma gallisepticum*. *J. Clin. Microbiol.* 56, 1–11. <https://doi.org/10.1128/JCM.01145-17>.
- Hubert, L., Arabie, P., 1985. Comparing partitions. *J. Classif.* 193–218.
- Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26, 2465–2466. <https://doi.org/10.1128/jcm.26.11.2465-2466.1988>.
- Ji, M., Lee, N.S., Oh, J.M., Jo, J.Y., Choi, E.H., Yoo, S.J., Kim, H.B., Hwang, S.H., Choi, S.H., Lee, S.O., Kim, M.N., Sung, H., 2014. Single-nucleotide polymorphism PCR for the detection of *Mycoplasma pneumoniae* and determination of macrolide resistance in respiratory samples. *J. Microbiol. Methods* 102, 32–36. <https://doi.org/10.1016/j.jmimet.2014.04.009>.
- Kinnear, A., Waldner, M., McAllister, T.A., Zaheer, R., Register, K., Jelinski, M., 2021. Application of four genotyping methods to *Mycoplasma bovis* isolates derived from western Canadian feedlot cattle. *J. Clin. Microbiol.* 59 <https://doi.org/10.1128/jcm.00044-21>.
- Kobisch, M., Friis, N.F., 1996. Swine mycoplasmoses. *OIE Rev. Sci. Tech.* 15, 1569–1605. <https://doi.org/10.20506/rst.15.4.983>.
- Kovács, Á.B., Kreisinger, Z., Forró, B., Gröszner, D., Mitter, A., Marton, S., Bali, K., Sawicka, A., Tomczyk, G., Bányai, K., Gyuranecz, M., Gyuranecz, M., 2020. The core genome multi-locus sequence typing of *Mycoplasma anseris/pingitidis*. *BMC Genomics* 21, 1–8. <https://doi.org/10.1186/s12864-020-06817-2>.
- Lee, J.A., Oh, Y.R., Hwang, M.A., Lee, J.B., Park, S.Y., Song, C.S., Choi, I.S., Lee, S.W., 2016. *Mycoplasma hyorhinis* is a potential pathogen of porcine respiratory disease complex that aggravates pneumonia caused by porcine reproductive and respiratory syndrome virus. *Vet. Immunol. Immunopathol.* 177, 48–51. <https://doi.org/10.1016/j.vetimm.2016.06.008>.
- Lee, J.A., Hwang, M.A., Han, J.H., Cho, E.H., Lee, J.B., Park, S.Y., Song, C.S., Choi, I.S., Lee, S.W., 2018. Reduction of mycoplasmal lesions and clinical signs by vaccination against *Mycoplasma hyorhinis*. *Vet. Immunol. Immunopathol.* 196, 14–17. <https://doi.org/10.1016/j.vetimm.2017.12.001>.
- Letunic, I., Bork, P., 2019. Interactive Tree of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res.* 47, 256–259. <https://doi.org/10.1093/nar/gkz239>.
- Lin, J.H., Chen, S.P., Yeh, K.S., Weng, C.N., 2006. *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. *Vet. Microbiol.* 115, 111–116. <https://doi.org/10.1016/j.vetmic.2006.02.004>.
- Manso-Silvén, L., Dupuy, V., Lysnyansky, I., Ozdemir, U., Thiaucourt, F., 2012. Phylogeny and molecular typing of *Mycoplasma agalactiae* and *Mycoplasma bovis* by

- multilocus sequencing. *Vet. Microbiol.* 161, 104–112. <https://doi.org/10.1016/j.vetmic.2012.07.015>.
- Mayor, D., Jores, J., Korczak, B.M., Kuhnert, P., 2008. Multilocus sequence typing (MLST) of *Mycoplasma hyopneumoniae*: a diverse pathogen with limited clonality. *Vet. Microbiol.* 127, 63–72. <https://doi.org/10.1016/j.vetmic.2007.08.010>.
- Morita, T., Fukuda, H., Awakura, T., Shimada, A., Umemura, T., Kazama, S., Yagihashi, T., 1995. Demonstration of *Mycoplasma hyorhinis* as a possible primary pathogen for porcine otitis media. *Vet. Pathol.* 32, 107–111. <https://doi.org/10.1177/030098589503200202>.
- Morita, T., Ohiwa, S., Shimada, A., Kazama, S., Yagihashi, T., Umemura, T., 1999. Intranasally inoculated *Mycoplasma hyorhinis* causes eustachitis in pigs. *Vet. Pathol.* 36, 174–178. <https://doi.org/10.1354/vp.36-2-174>.
- Nathues, H., Beilage, Egrosse, Kreienbrock, L., Rosengarten, R., Spersger, J., 2011. RAPD and VNTR analyses demonstrate genotypic heterogeneity of *Mycoplasma hyopneumoniae* isolates from pigs housed in a region with high pig density. *Vet. Microbiol.* 152, 339–345. <https://doi.org/10.1016/j.vetmic.2011.05.029>.
- Pieters, M., Maes, D., 2019. *Mycoplasmosis. Diseases of Swine*, 12th edition, pp. 863–883.
- Resende, T.P., Pieters, M., Vannucci, F.A., 2019. Swine conjunctivitis outbreaks associated with *Mycoplasma hyorhinis*. *J. Vet. Diagn. Invest.* 31, 766–769. <https://doi.org/10.1177/1040638719865767>.
- Rovira, A., 2009. Review of *Mycoplasma hyorhinis*. *Allen D. Leman Swine Conf.* 87–88.
- Shin, J.H., Joo, H.S., Lee, W.H., Seok, H.B., Calsamig, M., Pijoan, C., Molitor, T.W., 2003. Identification and characterization of cytopathogenic *Mycoplasma hyorhinis* from swine farms with a history of abortions. *J. Vet. Med. Sci.* 65, 501–509. <https://doi.org/10.1292/jvms.65.501>.
- Spersger, J., Hess, C., Loncaric, I., Ramirez, A.S., 2019. Matrix-assisted laser desorption ionization-time of flight mass spectrometry is a superior diagnostic tool for the identification and differentiation of mycoplasmas isolated from animals. *J. Clin. Microbiol.* 57, 1–16. <https://doi.org/10.1128/JCM.00316-19>.
- Tocqueville, V., Ferré, S., Nguyen, N.H.P., Kempf, I., Marois-Créhana, C., 2014. Multilocus sequence typing of *Mycoplasma hyorhinis* strains identified by a real-time TaqMan PCR assay. *J. Clin. Microbiol.* 52, 1664–1671. <https://doi.org/10.1128/JCM.03437-13>.
- Touati, A., Blouin, Y., Sirand-Pugnet, P., Renaudin, H., Oishi, T., Vergnaud, G., Bébér, C., Pereyrea, S., 2015. Molecular epidemiology of *Mycoplasma pneumoniae*: genotyping using single nucleotide polymorphisms and snapshot technology. *J. Clin. Microbiol.* 53, 3182–3194. <https://doi.org/10.1128/JCM.01156-15>.
- Trüeb, B., Catelli, E., Luehrs, A., Nathues, H., Kuhnert, P., 2016. Genetic variability and limited clonality of *Mycoplasma hyorhinis* in pig herds. *Vet. Microbiol.* 191, 9–14. <https://doi.org/10.1016/j.vetmic.2016.05.015>.
- Yair, Y., Borovok, I., Mikula, I., Falk, R., Fox, L.K., Gophna, U., Lysnyansky, I., 2020. Genomics-based epidemiology of bovine *Mycoplasma bovis* strains in Israel. *BMC Genomics* 21, 1–11. <https://doi.org/10.1186/s12864-020-6460-0>.
- Zhou, Z., Alikhan, N.F., Sergeant, M.J., Luhmann, N., Vaz, C., Francisco, A.P., Carriço, J. A., Achtman, M., 2017. GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens. *bioRxiv* 1395–1404. <https://doi.org/10.1101/216788>.

3. Discussion and Conclusion

In recent years, *M. hyorhinis* has increasingly been recognized as a significant pathogen in the swine industry, primarily due to its association with polyserositis, polyarthritis, and its contribution to the Porcine Respiratory Disease Complex (PRDC) (Friis and Feenstra, 1994). While once previously considered a commensal organism within the upper respiratory tract of pigs, *M. hyorhinis* has now been recognized as a significant cause of disease, particularly in younger pigs, resulting in economic losses due to reduced growth, increased mortality, and treatment cost (Friis and Feenstra 1994). Despite this recognition, much remains to be understood about the pathogen's epidemiology, the genetic diversity within its populations, and how these factors influence the spread of disease within pig herds (Bünger et al., 2021).

One of the key challenges in controlling *M. hyorhinis* infections is the absence of effective commercial vaccines. An autogenous vaccine specifically prepared for affected farm can compensate for the lack of commercially available vaccines in Europe. Though a cultivation of *M. hyorhinis* is necessary. Besides those options veterinarians and farmers are reliant on antibiotic therapies and strict biosecurity protocols to manage outbreaks. However, given the rising concerns about antibiotic resistance and the potential for antibiotics to lose effectiveness, alternative strategies for monitoring and controlling *M. hyorhinis* are required. This need has driven the development of more advanced diagnostic and genotyping methods, which allow for better tracking of outbreaks, understanding of pathogen transmission, and insights into the pathogen's evolutionary dynamics (Földi et al. 2023).

Traditional genotyping methods, such as MLST and variable number of tandem repeats (VNTR) analysis, have been employed for some time to study *M. hyorhinis* populations. These techniques have facilitated the identification of major genetic lineages and elucidation of transmission patterns (Földi et al., 2023; Tocqueville et al., 2014). However, these methods are limited in their discriminatory power among closely related strains. MLST, for instance, relies on a limited number of housekeeping genes, and while it can provide some insight into the genetic relationships between strains, its resolution is often insufficient for detailed epidemiological studies. Similarly, VNTR analysis, which targets short, repetitive DNA sequences, can sometimes fail to provide enough discriminatory power to differentiate between strains that are genetically very similar but may behave differently in terms of virulence or antibiotic resistance (Ruppitsch et al., 2015).

To overcome these limitations, this study introduced a cgMLST scheme for *M. hyorhinis*. By focusing on a much larger portion of the genome, cgMLST offers far greater resolution than traditional MLST, providing detailed insights into strain relationships. In particular, this study applied the cgMLST scheme to 81 *M. hyorhinis* strains from Austrian and German pig herds, revealing substantial genetic diversity among the strains and providing valuable insights into the population structure of the pathogen. The ability to detect genetic variation at a more granular level means that cgMLST can be particularly useful in identifying clonal clusters of *M. hyorhinis* and tracking their spread within and between herds.

One of the major findings of the study by Bünger et al. was the identification of distinct clonal clusters of *M. hyorhinis* within the sampled pig populations (Bünger et al., 2021). These clusters suggest that certain strains appear more prevalent in specific regions or farms. This finding has important implications for the control of *M. hyorhinis* outbreaks, as it suggests that targeted interventions could be designed to address specific clonal lineages that are particularly problematic in certain regions. Moreover, the ability to track the spread of these clonal clusters over time could help in understanding how the pathogen moves between herds, whether through direct contact between animals, environmental contamination, or the movement of farm personnel and equipment.

The study also compared the performance of cgMLST with core genome single nucleotide polymorphism (SNP) typing, another high-resolution genotyping method that has been used to study a wide range of bacterial pathogens (Bünger et al., 2021). While both methods provided high-resolution insights into the genetic diversity of *M. hyorhinis*, cgMLST has several advantages in terms of standardization and ease of use. SNP typing, while highly sensitive, can be more challenging to interpret and may not always be comparable between different studies or laboratories. In contrast, cgMLST enables standardized comparison across studies and laboratories, making it a more practical tool for large-scale epidemiological studies and for use in routine diagnostics.

One of the strengths of the cgMLST approach is its potential for long-term application in surveillance programs. As the scheme is based on a core set of genes that are present in all strains of *M. hyorhinis*, it provides a stable framework for comparing strains over time. This means that as more strains are collected and sequenced, they can be added to the existing cgMLST database, facilitates monitoring the emergence and evolution of strains. Over time,

this could provide valuable insights into the factors that drive the emergence of new pathogenic strains, such as changes in farming practices, antibiotic use, or pig genetics.

The application of cgMLST to *M. hyorhinis* also provides new opportunities to investigate virulence and resistance mechanisms. By correlating specific genetic changes with clinical outcomes or resistance to particular antibiotics, researchers can begin to identify the genetic factors that contribute to the pathogen's ability to cause disease. This could lead to the development of new diagnostic tests that are able to predict the virulence or resistance profile of a given strain based on its cgMLST profile, allowing for more targeted and effective treatment strategies.

However, there are also challenges and limitations to the cgMLST approach that must be acknowledged. While the scheme provides high-resolution data, it requires access to WGS, which may be resource-intensive and time-demanding, particularly for routine diagnostic use in the field (Bünger et al. 2021). Moreover, while cgMLST provides valuable insights into genetic relationships between strains, it does not directly reveal information about the pathogen's phenotype, such as its virulence or antibiotic resistance. These factors will need to be studied in conjunction with cgMLST data to fully understand the clinical implications of different *M. hyorhinis* strains.

Furthermore, while the study focused on pig herds in Austria and Germany, the generalizability of these findings to other regions remains to be seen how generalizable the findings are to other regions or production systems is uncertain. Different farming practices, biosecurity measures, and environmental factors could influence the population structure of *M. hyorhinis* in other parts of the world, and further studies will be needed to determine whether the same clonal clusters identified in this study are present in other regions. In addition, asymptomatic carriers are known to contribute to the transmission of *M. hyorhinis*, supporting its commonly unnoticed spread between herds.

In conclusion, the development of a cgMLST scheme for *M. hyorhinis* represents a significant advancement in our ability to study and track this important pathogen. By providing a high-resolution, standardized approach to genotyping, cgMLST offers new insights into the genetic diversity and population structure of *M. hyorhinis* and provides a valuable tool for outbreak investigation and surveillance. The findings of this study underscore the importance of continued research into the epidemiology of *M. hyorhinis* and the need for further development of tools and strategies to control its spread. Given ongoing challenges from infectious diseases

in swine production, the application of advanced genomic tools like cgMLST will be critical in improving animal health and reducing the economic impact of diseases like *M. hyorhinis*.

4. Summary

Mycoplasma hyorhinis is a common commensal and opportunistic pathogen in swine, particularly affecting piglets by causing polyserositis and polyarthritis, and contributing to the Porcine Respiratory Disease Complex. Its role in systemic infection and various clinical manifestations such as meningitis, conjunctivitis, and otitis remain poorly understood. Currently, there are no commercial vaccines in Europe, and management of infection largely relies on antibiotics and biosecurity measures. To better understand and track *M. hyorhinis* infections, advanced genotyping methods like multi-locus sequence typing and variable number tandem repeat analysis have been developed. However, these techniques show only moderate discriminatory power for differentiating closely related strains.

In this study, a core genome multi-locus sequence typing scheme was developed and applied to 81 *M. hyorhinis* strains from Austrian and German pig herds. The core genome multilocus sequence typing scheme demonstrated superior discriminatory power compared to traditional multi-locus sequence typing methods, revealing high genetic diversity among strains and differences in detection sites. Furthermore, core genome multilocus sequence typing allowing the identification of clonal clusters. The core genome multi-locus sequence typing approach was further compared with core genome single nucleotide polymorphism typing, confirming the high resolution of core genome multi-locus sequence typing for epidemiological studies. Overall, this study highlights the usefulness of core genome multi-locus sequence typing in tracking *M. hyorhinis* outbreaks and understanding its population structure in pig herds.

5. Zusammenfassung

Mycoplasma hyorhinis ist ein häufiger Kommensale und opportunistischer Erreger bei Schweinen, der insbesondere Ferkel betrifft und Polyserositis sowie Polyarthritits verursacht. und zum „Porcine Respiratory Disease Complex“ beiträgt. Seine Rolle bei systemischen Infektionen und verschiedenen klinischen Erscheinungsformen wie Meningitis, Konjunktivitis und Otitis ist bislang nur unzureichend verstanden. Derzeit sind in Europa keine kommerziellen Impfstoffe verfügbar, und die Bekämpfung von Infektionen beruht weitgehend auf dem Einsatz von Antibiotika und Maßnahmen der Biosicherheit. Um *M. hyorhinis* Infektionen besser zu verstehen und nachzuverfolgen, wurden fortgeschrittene Genotypisierungsmethoden wie Multi-Locus Sequence Typing und die Analyse von variablen Tandemwiederholungen entwickelt. Diese Techniken bieten jedoch nur eine moderate Fähigkeit, engverwandte Stämme zu differenzieren.

In dieser Studie wurde ein Core Genome Multi-Locus Sequence Typing Schema entwickelt und auf 81 *M. hyorhinis* Stämme aus österreichischen und deutschen Schweinebeständen angewendet. Das Core Genome Multi-Locus Sequence Typing Schema zeigte eine deutlich höhere Diskriminierungsfähigkeit im Vergleich zu herkömmlichen Multi-Locus Sequence Typing-Methoden und deckte damit eine hohe Diversität unter den Stämmen und Detektionslokalisationen auf. Des Weiteren ermöglichte Core Genome Multi-Locus Sequence Typing die Identifizierung klonaler Cluster. Das Core Genome Multi-Locus Sequence Typing wurde außerdem mit dem Core Genome Single Nucleotide Polymorphism Typing verglichen, wobei die hohe Auflösung des Core Genome Multi-Locus Sequence Typing für epidemiologische Studien bestätigt wurde. Diese Studie unterstreicht insgesamt den Nutzen des Core-Genome-Multi-Locus-Sequence-Typing bei der Nachverfolgung von *M. hyorhinis* Ausbrüchen und beim Verständnis der Populationsstruktur dieses Erregers in Schweinebeständen.

6. References

- Bisping W. 1979. Mycoplasma, in: Kompendium der veterinärmedizinischen Mikrobiologie. Ein Leitfaden für Studierende, Teil II. Spezielle Bakteriologie und Mykologie. M. & H. Schaper, p. 156.
- Brown R.J., Holden M.T.G., Spiller O.B., Chalker V.J. 2015. Development of a Multilocus Sequence Typing Scheme for Molecular Typing of *Mycoplasma pneumoniae*. Journal of Clinical Microbiology 53: 3195–3203.
- Bünger M., Brunthaler R., Unterweger C., Loncaric I., Dippel M., Ruczizka U., Schwarz L., Griessler A., Voglmayr T., Verhovsek D., Ladinig A., Spargser J. 2020. Mycoplasma hyorhinis as a possible cause of fibrinopurulent meningitis in pigs? - a case series. Porc Health Manag 6: 38.
- Bünger M., Posch M., Wiesauer J., Loncaric I., Cabal Rosel A., Ruppitsch W., Ladinig A., Spargser J. 2021. A core genome multilocus sequence typing scheme for *Mycoplasma hyorhinis*. Veterinary Microbiology 262: 109249.
- Calcutt M.J., Foecking M.F., Rosales R.S., Ellis R.J., Nicholas R.A.J. 2012. Genome Sequence of *Mycoplasma hyorhinis* Strain GDL-1. Journal of Bacteriology 194: 1848–1848.
- Caron J., Ouardani M., Dea S. 2000. Diagnosis and Differentiation of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* Infections in Pigs by PCR Amplification of the p36 and p46 Genes. Journal of Clinical Microbiology 38: 1390–1396.
- Clavijo M.J., Davies P., Morrison R., Bruner L., Olson S., Rosey E., Rovira A. 2019. Temporal patterns of colonization and infection with *Mycoplasma hyorhinis* in two swine production systems in the USA. Veterinary Microbiology 234: 110–118.
- Clavijo M.J., Oliveira S., Zimmerman J., Rendahl A., Rovira A. 2014. Field evaluation of a quantitative polymerase chain reaction assay for *Mycoplasma hyorhinis*. Journal of Veterinary Diagnostic Investigation 26: 755–760.

- Dyer N.P., Păuker B., Baxter L., Gupta A., Bunk B., Overmann J., Diricks M., Dreyer V., Niemann S., Holt K.E., Rahman M., Brown P.E., Stark R., Zhou Z., Ott S., Nübel U. 2025. EnteroBase in 2025: exploring the genomic epidemiology of bacterial pathogens. *Nucleic Acids Research* 53: D757–D762.
- El-Gazzar M., Ghanem M., McDonald K., Ferguson-Noel N., Raviv Z., Slemons R.D. 2016. Development of Multilocus Sequence Typing (MLST) for *Mycoplasma synoviae*. *Avian Diseases* 61: 25.
- Földi D., Bekő K., Felde O., Kreizinger Z., Kovács Á.B., Tóth F., Bányai K., Kiss K., Biksi I., Gyuranecz M. 2020. Genotyping *Mycoplasma hyorhinis* by multi-locus sequence typing and multiple-locus variable-number tandem-repeat analysis. *Veterinary Microbiology* 249: 108836.
- Földi D., Nagy Z.E., Belec N., Szeredi L., Földi J., Kollár A., Tenk M., Kreizinger Z., Gyuranecz M. 2023. Establishment of a *Mycoplasma hyorhinis* challenge model in 5-week-old piglets. *Frontiers in Microbiology* 14: 1209119.
- Fourour S., Marois-Créhan C., Martelet L., Fablet C., Kempf I., Gottschalk M., Segura M. 2019a. Intra-Species and Inter-Species Differences in Cytokine Production by Porcine Antigen-Presenting Cells Stimulated by *Mycoplasma hyopneumoniae*, *M. hyorhinis*, and *M. flocculare*. *Pathogens* 8: 34.
- Fourour S., Tocqueville V., Paboeuf F., Lediguerher G., Morin N., Kempf I., Marois-Créhan C. 2019b. Pathogenicity study of *Mycoplasma hyorhinis* and *M. flocculare* in specific-pathogen-free pigs pre-infected with *M. hyopneumoniae*. *Veterinary Microbiology* 232: 50–57.
- Friis N. 1976. A Serologic Variant of *Mycoplasma Hyorhinis* recovered from the Conjunctiva of Swine. *Acta Veterinaria Scandinavica* 343–353.
- Friis N.E., Feenstra A.A. 1994. *Mycoplasma Hyorhinis* in the Etiology of Serositis among Piglets. *Acta Veterinaria Scandinavica* 35: 93–98.

- Ghanem M., El-Gazzar M. 2018. Development of *Mycoplasma synoviae* (MS) core genome multilocus sequence typing (cgMLST) scheme. *Veterinary Microbiology* 2018: 84–89.
- Gomes Neto J.C., Bower L., Erickson B.Z., Wang C., Raymond M., Strait E.L. 2015. Quantitative real-time polymerase chain reaction for detecting *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* in pen-based oral, tonsillar, and nasal fluids. *Journal of Veterinary Science* 16: 195.
- Kobisch M., Friis N.F. 1996. Swine mycoplasmoses. *Revue Scientifique et Technique* 15: 1569–1605.
- Kornspan J.D., Tsur M., Tarshis M., Rottem S., Brenner T. 2015. *Mycoplasma hyorhinis* induces proinflammatory responses in mice lymphocytes. *Journal of Basic Microbiology* 55: 679–684.
- Lee J.-A., Oh Y.-R., Hwang M.-A., Lee J.-B., Park S.-Y., Song C.-S., Choi I.-S., Lee S.-W. 2016. *Mycoplasma hyorhinis* is a potential pathogen of porcine respiratory disease complex that aggravates pneumonia caused by porcine reproductive and respiratory syndrome virus. *Veterinary Immunology and Immunopathology* 177: 48–51.
- Leopold S.R., Goering R.V., Witten A., Harmsen D., Mellmann A. 2014. Bacterial Whole-Genome Sequencing Revisited: Portable, Scalable, and Standardized Analysis for Typing and Detection of Virulence and Antibiotic Resistance Genes. *Journal of Clinical Microbiology* 52: 2365–2370.
- Leuwerke B. 2009. *Mycoplasma hyorhinis*- Field experiences in diagnosis and control. Allen D. Leman Swine Conference 89–90.
- Luehrs A., Siegenthaler S., Grützner N., Grosse Beilage E., Kuhnert P., Nathues H. 2017. Occurrence of *Mycoplasma hyorhinis* infections in fattening pigs and association with clinical signs and pathological lesions of Enzootic Pneumonia. *Veterinary Microbiology* 203: 1–5.

- Maiden M.C.J., Bygraves J.A., Feil E., Morelli G., Russell J.E., Urwin R., Zhang Q., Zhou J., Zurth K., Caugant D.A., Feavers I.M., Achtman M., Spratt B.G. 1998. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences* 95: 3140–3145.
- Martinson B., Zoghby W., Barrett K., Bryson L., Christmas R., Minion F.C., Kroll J. 2018. Efficacy of an inactivated *Mycoplasma hyorhinis* vaccine in pigs. *Vaccine* 36: 408–412.
- Mayor D., Jores J., Korczak B.M., Kuhnert P. 2008. Multilocus sequence typing (MLST) of *Mycoplasma hyopneumoniae*: A diverse pathogen with limited clonality. *Veterinary Microbiology* 127: 63–72.
- Menghwar H., Guo A., Chen Y., Lysnyansky I., Parker A.M., Prysliak T., Perez-Casal J. 2022. A core genome multilocus sequence typing (cgMLST) analysis of *Mycoplasma bovis* isolates. *Veterinary Microbiology* 273: 109532.
- Moura A., Criscuolo A., Pouseele H., Maury M.M., Leclercq A., Tarr C., Björlman J.T., Dallmann T., Reimer A., Enouf V., Larssonneur E., Carleton H., Bracq-Dieye H., Katz L.S., Jones L., Touchon M., Tourdjman M., Walker M., Stroika S., Cantinelli T., Chenal-Francisque V., Kucerova Z., Rocha E.P.C., Nado C., Brisse S. 2016. Whole genome-based population biology and epidemiological surveillance of *Listeria monocytogenes*. *Nature Microbiology* 2: 16185.
- Palzer A., Ritzmann M., Spargser J. 2020. *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* in pig herds. In: Maes, D., Sibila, M., Pieters, M. (ed.). *Mycoplasmas in Swine*. Leuven: Uitgeverij ACCO 247–265.
- Petzoldt K., Kirchoff H. 1986. *Mycoplasma*, in: *Kompendium der veterinärmedizinischen Mikrobiologie. Ein Leitfaden für Studierende, Teil I. Allgemeine Bakteriologie, Mykologie Infektions- und Seuchenlehre*. M. & H. Schaper, p. 25.
- Razin S., Jacobs E. 1992. *Mycoplasma* adhesion. *Journal of General Microbiology* 138: 407–422.

- Razin S., Yogev D., Naot Y. 1998. Molecular Biology and Pathogenicity of Mycoplasmas. *Microbiology and Molecular Biology Reviews* 62: 1094–1156.
- Rottem S. 2003. Interaction of Mycoplasmas With Host Cells. *Physiological Reviews* 83: 417–432.
- Ruppitsch W., Pietzka A., Prior K., Bletz S., Fernandez H.L., Allerberger F., Harmsen D., Mellmann A. 2015. Defining and Evaluating a Core Genome Multilocus Sequence Typing Scheme for Whole-Genome Sequence-Based Typing of *Listeria monocytogenes*. *Journal of Clinical Microbiology* 53: 2869–2876.
- Schreiber T., Thacker B. 2012. *Mycoplasma hyosynoviae*, *Mycoplasma hyorhinis* and *Mycoplasma suis* overview: Disease basics, clinical presentations, diagnostics, treatments and prevention/ control strategies. Allen D. Leman Swine Conference 39: 73–76.
- Spergser J., Hess C., Loncaric I., Ramírez A.S. 2019. Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Is a Superior Diagnostic Tool for the Identification and Differentiation of Mycoplasmas Isolated from Animals. *Journal of Clinical Microbiology* 57: e00316-19.
- Switzer W.P. 1955. Studies on infectious atrophic rhinitis. IV. Characterization of a pleuropneumonia-like organism isolated from the nasal cavities of swine. *American Journal of Veterinary Research* 16: 540–544.
- Tocqueville V., Ferré S., Nguyen N.H.P., Kempf I., Marois-Créhan C. 2014. Multilocus Sequence Typing of *Mycoplasma hyorhinis* Strains Identified by a Real-Time TaqMan PCR Assay. *Journal of Clinical Microbiology* 52: 1664–1671.
- Uelze L., Grützke J., Borowiak M., Hammerl J.A., Juraschek K., Deneke C., Tausch S.H., Malorny B. 2020. Typing methods based on whole genome sequencing data. *One Health Outlook* 2: 3.