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Comparative analysis of *Mycoplasma hyorhinis* isolates and studying the pathogenesis of infection

Ph.D. thesis

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Copy … of eight.

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Dorottya Földi

Table of contents

Abbreviations

AA	amino acid
adk	adenylate kinase
ADWG	average daily weight gain
bp	base pair
CCU	colour changing unit
cgMLST	core genome multi-locus sequence typing
DNA	deoxyribonucleic acid
dnaA	chromosomal replication initiation protein
dNTP	deoxynucleoside triphosphate
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
gltX	glutamyl-tRNA synthetase
gmk	guanylate kinase
GT	genotype
gyrB	DNA gyrase subunit B
H&E	haematoxylin and eosin
IP	intraperitoneal
IV	intravenous
lepA	leader peptidase A
MIC	minimal inhibitory concentration
MIC_{50}	minimum inhibitory concentration that inhibits 50% of isolates
MIC_{90}	minimum inhibitory concentration that inhibits 90% of isolates
MLST/ MLSTn, MLSTp, MLSTs	multi-locus sequence typing /n: here described, p: previously published, s: with surface protein genes
MLVA	multiple-locus variable-number tandem-repeat analysis
mtlD	mannitol-1-phosphate 5-dehydrogenase
p37	extracytoplasmic thiamine-binding lipoprotein
p95	outer membrane protein p95
PCR	polymerase chain reaction
PCV2	porcine circovirus 2
pdhB	pyruvate dehydrogenase-E beta-1 subunit
PRRSV	porcine respiratory and reproductive syndrome virus
RNA	ribonucleic acid
rpoB, rpoC	DNA-directed RNA polymerase β and β' subunit
rRNA	ribosomal ribonucleic acid
SNP	single nucleotide polymorphism
ST	sequence type
ung	uracil-DNA glycosylase
uvrA	UvrABC system protein A
valS	valine-tRNA ligase
Vlp	variable lipoprotein

1. Summary

Mycoplasma hyorhinis is a facultative swine pathogen distributed worldwide. The bacterium causes polyarthritis and polyserositis in weaners and finishers, and also has important role in the porcine respiratory disease complex as a secondary pathogen. Sows are asymptomatic carriers of the bacterium and infect the piglets during farrowing. After weaning, *M. hyorhinis* spreads quickly among piglets. Decrease of maternal antibodies, stress and co-infection with other, mainly viral pathogens enhance the spread of the disease and contributes to the appearance of clinical signs. Up to day, there is no commercially available vaccine in Europe for the long term protection against *M. hyorhinis.* Therefore, adequate housing conditions and control of other infections are crucial in the prevention of clinical signs, while targeted antimicrobial therapy can mitigate their severeness.

In this study, minimal inhibitory concentrations (MIC) of ten frequently used antibiotics were determined by broth microdilution method against recent (2019-2021) *M. hyorhinis* isolates originating from five European countries (Belgium, Germany, Hungary, Italy and Poland). Low concentrations of tetracyclines (doxycycline: MIC_{90} 0.078 µg/ml, oxytetracycline: MIC_{90} 0.25 µg/ml), tiamulin (MIC_{90} 0.312 µg/ml) and florfenicol (MIC_{90} 2 µg/ml) inhibited the growth of the examined isolates, while moderately higher concentrations of enrofloxacin (MIC_{90} 1.25 µg/ml) were required for the same effect. For the tested macrolides (tylosin: MIC_{90} >64 µg/ml; tylvalosin: MIC_{90} 5 µg/ml; tilmicosin: MIC_{90} >64 µg/ml; tulathromycin: MIC_{90} >64 µg/ml) and lincomycin (MIC_{90} >64 µg/ml) bimodal MIC patterns were detected. While approximately half of the examined strains were susceptible to low concentrations of macrolides and lincomycin, these antibiotics were inefficient on the other half of the samples. The use of the antibiotics that were found efficient at low concetrations (complemented with repeated susceptibility testing within certain intervals) should improve therapeutic success and prevent the development of antibiotic resistance. The results provide essential information for the treatment of the disease, as antibiotic susceptibility testing of mycoplasmas is usually not part of the routine diagnostic work.

The slow growth characteristics and complex media requirement of the pathogen make the test time consuming and expensive. Therefore, a mismatch amplification mutation assay (MAMA) for the detection of macrolide and lincomycin susceptibility was also developed. The assay targets a point mutation in the 23S rRNA gene [at position 2066 according to the 23S rRNA gene of the *M. hyorhinis* type strain (NCTC 10130)] associated with antibiotic susceptibility, thus enables testing even without isolation. With three exceptions, the results of the MAMA were in line with the determined MIC in the broth microdilution method in the tested 123 isolates and 15 clinical samples. The developed molecular biological assay can be a rapid and cost-effective alternative for the conventional broth microdilution susceptibility testing.

Polymerase chain reaction (PCR)-based multi-locus sequence typing (MLST) is wildly used among bacteria for phylogenetic studies. The benefits of the assay are reliability, high resolution, repeatability and easy comparison of the results. The other frequently used PCR-based typing assay is the multiple-locus variable-number tandem-repeat analysis (MLVA), which is suitable for the separation of closely related isolates. Previously published *M. hyorhinis* MLST systems included regions which decreased either the reproducibility or the reliability of the assays. In the present study, a novel MLST was developed, targeting the most variable regions of six house-keeping genes (*lepA*, *rpoB*, *rpoC*, *gltX*, *uvrA* and *valS*). In addition, a six-locus-based MLVA scheme of high resolution (39 genotypes distinguished among 41 isolates) was established for the genotyping of *M. hyorhinis*. Analyses of the same set of samples with all existing typing systems revealed that the new MLST system has high resolution (38 sequence types/47 isolates) and improved reliability and reproducibility compared to the previous MLST assays. The combination of the new MLST and MLVA is suitable for the robust differentiation of closely related isolates, and represents an appropriate tool for phylogenetic studies; moreover, the MLVA is an easily accessible technique which can be used alone in epidemiological investigations.

A reliable challenge model is essential for conducting vaccine efficacy experiments. In this study, we aimed to establish such a challenge model by comparing two challenge routes using the same pathogenic clinical isolate. One of the challenge groups was inoculated with *M. hyorhinis* by intravenous (IV) route on two consecutive days (Group IV-IV), while the other group was challenged IV on the first day, and next day intraperitoneally (IP; Group IV-IP). The observed clinical signs, lesions and serological responses were compared to a negative control group and between the challenge groups. After inoculation with *M. hyorhinis,* clinical signs were recorded, blood was taken twice a week for four weeks, then at necropsy gross pathological examination was carried out and samples were collected for the detection of mycoplasmas, general bacteriology and histopathology. All pathognomonic signs of *M. hyorhinis* infection were observed in both challenge groups, with more severe lesions of the serosa detected in Group IV-IV. Antibody response after inoculation was tested by enzyme linked immunosorbent assay (ELISA). Significant differences were detected between the challenge groups and the control group in the case of ELISA titres, average daily weight gain and pathological scores, while only Group IV-IV differed significantly from the control group in the case of histopathological scores. Based on our results both challenge routes are suitable for the induction of pathognomonic lesions of *M. hyorhinis*, yet the use of double dose IV inoculation is suggested by us due to the more pronounced pathological and histopathological lesions and supposed resemblance to the natural route of infection.

Összefoglalás

A *Mycoplasma hyorhinis* egy világszerte elterjedt, fakultatív patogén baktérium. Választáskori és növendék malacokban okoz savóshártya- és több ízületre kiterjedő gyulladással járó kórképet. Emellett másodlagos kórokozóként fontos szerepe van a sertések légzőszervi betegségkomplexében. A kocák tünetmentes hordozók, a malacok tőlük fertőződnek, majd a betegség gyorsan terjed a battérián. A fertőzés terjedésben és a klinikai tünetek megjelenésében fontos szerepe van a stressznek, az anyai ellenanyagok csökkenésének és a társfertőzések jelenlétének. Jelenleg Európában nincs elérhető vakcina, megelőzés csak a tartási körülmények javításával és más kórokozókkal szembeni védekezéssel lehetséges. A tünetek megjelenésük után antimikrobiális kezeléssel enyhíthetőek.

Munkánk során leves mikro-hígításos módszerrel meghatároztuk tíz, a sertés ágazatban gyakran használt antibiotikum minimális gátló koncentrációját (MIC) a közelmúltban gyűjtött (2019–2021), öt európai országból származó (Belgium, Lengyelország, Magyarország, Németország és Olaszország) *M. hyorhinis* izolátummal szemben. A vizsgált tetraciklinek (doxiciklin: MIC_{90} 0,078 µg/ml, oxitetraciklin: MIC_{90} 0,25 µg/ml), a tiamulin (pleuromutilin; MIC_{90} 0,312 µg/ml) és a florfenikol (fenikol; MIC_{90} 2 µg/ml) alacsony koncentrációban gátolták a törzsek növekedését. Míg az enrofloxacin esetében ehhez mérsékelten emelkedett koncentrációra volt szükség (MIC_{90} 1,25 µg/ml). A vizsgált makrolidok (tilozin: MIC_{90} >64 µg/ml, tilvalozin: MIC_{90} 5 µg/ml, tilmikozin: MIC_{90} >64 µg/ml, tulatromicin: MIC_{90} >64 µg/ml) és linkomicin (MIC_{90} >64 µg/ml) esetében bimodális MIC–eloszlást tapasztaltunk. Míg az izolátumok közel felének növekedését alacsony koncentrációban gátolták ezek a szerek, az izolátumok másik felével szemben hatástalannak bizonyultak. Az alacsony koncentrációban is hatásos antibiotikumok szakszerű használata (kiegészítve megfelelő időközönként elvégzett, ismételt érzékenység vizsgálattal) javíthatja a kezelés hatékonyságát és megelőzheti a rezisztencia kialakulását. A vizsgálattal értékes adatokkal szolgáltunk a gyakorlat számára, mivel a *Mycoplasma* fajok antibiotikumérzékenységének meghatározása nem rutin diagnosztikai feladat.

A kórokozó lassú növekedése és összetett tápközegigénye miatt ez a vizsgálat költséges és időigényes. Éppen ezért a makrolid- és linkomicinérzékenység gyors kimutatására kifejlesztettünk egy *mismatch amplification mutation* tesztet (MAMA). Ennek segítségével gyorsan, akár a baktérium izolálása nélkül lehetséges az antibiotikumérzékenység meghatározása a 23S rRNS gén egy antibiotikumérzékenységgel összefüggő pontmutációja [2066 pozícióban a *M. hyorhinis* típustörzs (NCTC 10130) 23S rRNS génje szerint] alapján. Három eset kivételével a MAMA eredménye megegyezett a leves mikro-hígításos módszerrel kapott MIC–eredménnyel a vizsgált 123 izolátum és 15 klinikai minta esetében. A fejlesztett

molekuláris biológiai teszt költséghatékony és gyors alternatívát kínál a hagyományos antibiotikumérzékenység meghatározás mellett.

A polimeráz láncreakció (PCR) alapú multi-lókusz szekvenciatipizálás (MLST) tesztek széles körben elterjedtek baktériumok rokonsági kapcsolatainak feltérképezésére. Az MLST előnye, hogy megbízható és könnyen összehasonlítható eredményt biztosít, jó felbontóképesség mellett. A másik elterjedt módszer a tandem ismétlődő régiók ismétlés számának meghatározása (MLVA), melynek segítségével közel-rokon törzsek is elkülöníthetőek. *M. hyorhinis* esetében korábban fejlesztettek már több MLST rendszert, melyek olyan régiókat tartalmaznak, amik rontották a rendszerek felbontóképességét vagy ismételhetőségét. Munkánk során hat háztartási gén (*lepA*, *rpoB*, *rpoC*, *gltX*, *uvrA* és *valS*) legvariábilisabb szakaszait vizsgáló MLST rendszert hoztunk létre, melyet kiegészít a fejlesztett, hat lókusz vizsgálatán alapuló, nagy felbontóképességű (39 genotípust különböztetett meg a vizsgált 41 izolátum esetében) MLVA rendszer. Ugyanazt a mintasort összehasonlítottuk a már publikált és az itt leírt MLST rendszerekben, ez alapján az általunk fejlesztett MLST rendszer felbontóképessége volt a legnagyobb (38 szekvencia típus/47 izolátum), miközben a megbízhatósága és az ismételhetősége is javult. A kidolgozott MLST és MLVA kombinációja alkalmas közeli rokon minták elkülönítésére és megfelelő eszköz filogenetikai vizsgálatok elvégzésére. Emellett az MLVA, egy széles körben hozzáférhető eljárás, önmagában alkalmas járványtani vizsgálatokra.

Célunk volt egy megfelelő fertőzési modell kidolgozása, melyet később vakcina-hatékonysági vizsgálatokhoz használhatunk. Malacokat két különböző módon fertőztünk egy virulens klinikai izolátummal. Az egyik csoportot két egymást követő nap intravénásan (IV) fertőztük (IV-IV csoport), míg a másik csoport egyik nap IV, másik nap intraperitoneálisan (IP) kapta a fertőző anyagot (IV-IP csoport). Az elváltozásokat negatív kontroll csoporthoz és egymáshoz hasonlítottuk. Fertőzés után a klinikai tüneteket rögzítettük, illetve rendszeresen vért vettünk az állatoktól négy héten keresztül, majd a boncolás során vizsgáltuk a kórbonctani elváltozásokat és mintát gyűjtöttünk *Mycoplasma*-kimutatás, általános bakteriológiai és kórszövettani vizsgálatok elvégzésére. Mindkét csoportban kiváltottuk a *M. hyorhinis* fertőzés minden jellegzetes tünetét, azonban az IV-IV csoportban súlyosabb elváltozásokat tapasztaltunk a savóshártyákon. Az ellenanyagválasz mértéke mindkét csoportban szignifikánsan magasabb volt a kontroll csoporthoz képest. Hasonló eltérést tapasztaltunk a napi átlagos súlygyarapodás és a makroszkópos kórbonctani elváltozások tekintetében is, azonban a kórszövettani vizsgálat során csak az IV-IV csoportban mutattak szignifikáns eltérést a malacok a kontroll csoporthoz képest. Vizsgálatunk alapján mindkét fertőzési mód alkalmas a tünetek kiváltására, de mivel az IV-IV fertőzés feltehetően jobban hasonlít a fertőzés természetes módjához és a kiváltott elváltozások is súlyosabbak voltak, ezt a fertőzési módot javasoljuk használni a további vizsgálatok során.

2. Introduction

2.1 Aetiology

Mycoplasma (Mesomycoplasma) hyorhinis is a facultative pathogen, which was first described by Switzer in 1955. A pleuropneumonia-like organism was isolated from the nasal cavities of healthy pigs and pigs with atrophic rhinitis. Clinical signs after experimental inoculation via the intraperitoneal (IP) route were characterised as a syndrome similar to Glässer's-disease with fibrinous polyserositis and arthritis (Switzer, 1955). Nowadays, besides polyserositis and arthritis *M. hyorhinis* infection is associated with ear infections (Morita *et al.*, 1995), conjunctivitis (Resende *et al.*, 2019) and meningitis (Bünger *et al.*, 2020). *M. hyorhinis* is also a frequent cell culture contaminant (Kong *et al.*, 2001) and is associated with human cancer formation and metastasis (Huang *et al.*, 2001).

M. hyorhinis is a member of the Mollicutes class, *Mycoplasmataceae* family. Mycoplasmas are the smallest free living bacteria with pleomorphic cell morphology due to the lack of cell wall. *M. hyorhinis* is characterized by small genome size (806 507 bases for the type strain), low guanidine-cytosine content (GC% 25.9 for the type strain) and a low number of protein coding genes (689 for the type strain; Razin, 1985). Based on 16S rRNA sequences *M. hyorhinis* belongs to the hyopneumoniae cluster in close relationship with *M. hyopneumoniae* and *M. flocculare*, the other swine respiratory tract mycoplasmas. However, *M. hyorhinis* is only distantly related to *M. hyosynoviae,* a swine pathogen *Mycoplasma* sp. causing arthritis and belonging to the hominis clade (Blank *et al.*, 1996; Thompson *et al.*, 2011).

2.2 Epidemiology

Recent data about the role of *M. hyorhinis* in arthritis and polyserositis cases and its prevalence in oral fluid samples shows the importance and abundance of this pathogen. In the United States, after examining oral fluid samples from 37 geographically distant farms 97% prevalence was found on herd level (Pillman *et al.*, 2019). In 2015, *M. hyorhinis* was detected in 44% of polyserositis and 12% of arthritis cases in the Minnesota Veterinary Diagnostic Laboratory (Clavijo *et al.*, 2017). In Korea, *M. hyorhinis* was found in 66.1% of examined farms based on oral fluid samples (Cheong *et al.*, 2017). In Italy, *M. hyorhinis* was detected in 61.6% of post-weaning pigs with polyarthritis and was the most common bacterium detected in these cases (Salogni *et al.*, 2022).

As Switzer observed during the first isolation, *M. hyorhinis* is present in the nasal microbiota of healthy pigs (Switzer, 1955). Both cross-sectional and longitudinal studies on herds indicate

that the prevalence of *M. hyorhinis* in nasal samples of sows and pre-weaning piglets is low, in these studies prevalence between 0-10% was detected. However, after weaning and during the nursery phase, the prevalence of *M. hyorhinis* rises rapidly and by the age of 45 days more than 90% of the piglets are tested positive by real-time polymerase chain reaction (PCR; Clavijo *et al.*, 2017; 2019). In another study, higher positivity rates among sows were found; 72% of the examined tonsillar samples were positive for *M. hyorhinis*. Even though the prevalence was low (only 8.3%) in piglets one week after farrowing, it increased to 50% by the time of weaning (Roos *et al.*, 2019). Correlation between the parity of sows and the presence of *M. hyorhinis* in nasal or tonsillar samples was found, indicating that lower parity sows are more likely to be carriers (Clavijo *et al.*, 2017; Roos *et al.*, 2019). Additionally, in the longitudinal study, signs of intermittent shedding were found as PCR-positive piglets came from PCR-negative sows. Intermittent shedding of bacteria is not without precedent among mycoplasmas as it was described earlier in the case of *M. hyopneumoniae* and *M. bovis* as well (Clavijo *et al.*, 2019).

Clinical signs of *M. hyorhinis* infection typically appear in three to ten-week-old piglets. Though the susceptibility decreases drastically after ten weeks of age piglets up to 16 weeks of age can develop polyserositis and arthritis (Martinson *et al.*, 2017). Interestingly, eustachitis caused by *M. hyorhinis* appear earlier, mostly around the first week of age (Morita *et al.*, 1995), while conjunctivitis usually manifests in eight to ten weeks old pigs, towards the end of the susceptible period (Resende *et al.*, 2019). Predisposing factors such as stress caused by weaning or the comingling with other piglets, decay of colostral immunity or presence of viral infections like porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV2) or swine influenza A virus are considered important for the appearance and severity of the clinical signs (Palzer *et al.*, 2015; Chen *et al.*, 2016b; Clavijo *et al.*, 2019). *M. hyorhinis* is frequently associated with the porcine respiratory disease complex, co-infections with PCV2, *Glaesserella (*former *Haemophilus) parasuis* or *M. hyopneumoniae* aggravates the course of the disease (Palzer *et al.*, 2015; Luehrs *et al.*, 2017; Lee *et al.*, 2018).

2.3 Pathogenesis

After infection, *M. hyorhinis* first colonises the upper and lower ciliated respiratory epithelium. To induce the lesions characteristic of *M. hyorhinis* infection the bacteria need to spread systemically from the respiratory tract. Neither the mode of dissemination from the respiratory tract nor the route of systemic spread (blood or lymphatic system) has been revealed yet in this species. Based on observations of cell cultures, *M. hyorhinis* not only adheres to the surface of the host cells but is capable to enter the cells and become an intracellular pathogen (Kornspan *et al.*, 2015). There are several membrane proteins which

are known to participate in adherence. The most studied proteins belong to the variable lipoprotein (Vlp) family; in addition, the p37 protein and glycolysis enzymes with moonlighting functions also have been associated with this characteristic.

The Vlp family proteins are coded by seven genes (Vlp A-G) and, besides adherence, their role in immune evasion was also described. Each Vlp protein has three regions with unique structures and functions. These proteins are described by high-frequency phase variation in expression and size variation (Xiong *et al.*, 2016a, 2016b). In the promoter of each gene a poly-adenine tract regulates the expression of the genes. While the size variation is probably regulated by DNA slippage, which results in changes of the length of region III (Citti *et al.*, 2000). The length of region III affects the capability to bind extracellular matrix components (ECM) and plasminogen (J. Li *et al.*, 2022a) and the protection from the complement independent (i.e. antibody-dependent) immune response. Thus, it is hypothesised that the Vlp proteins act as a flexible shield and by changing the length of these proteins adherence sites or molecules in the membrane can be masked (Citti *et al.*, 1997; Xiong *et al.*, 2016b). Expression changes of the Vlp proteins were also connected to morphology changes of the colonies (Rosengarten and Wise, 1990).

The p37 protein or extracytoplasmic thiamine-binding lipoprotein was originally described in mouse sarcoma cells as a factor inducing invasiveness (Dudler *et al.*, 1988). Since then, the p37 protein has been associated with several human cancer formations, malignancy, metastasis and invasiveness (Sippel *et al.*, 2009) besides being a recognised adhesion molecule in *M. hyorhinis* (Clavijo *et al.*, 2019).

Moonlighting proteins are described as proteins with more than one biological action. Many of these molecules are highly conserved in prokaryotes, often metabolic regulators, enzymes or molecular chaperones (Henderson and Martin, 2011). In *M. hyorhinis* three proteins, two glycolysis enzymes and a molecular chaperone have been described as moonlighting proteins and have roles in cell adherence. These are the enolase, glyceraldehyde-3-phosphate dehydrogenase and the heat-shock protein 70 family member DnaK (Wang *et al.*, 2021; Y. Li *et al.*, 2022; Wang *et al.*, 2022b). The pyruvate dehydrogenase E1 complex subunit alpha was also proved to have membrane localization, although in this case adherence-related function has not been described yet (Chen *et al.*, 2016a).

The basement membrane (*membrana basalis*) is a specialized ECM under the epithelial cells, this layer is a tissue barrier preventing the dissemination of pathogens, like *M. hyorhinis* from the respiratory tract. Essential components of the basement membrane are laminin and collagen type IV. The binding of plasminogen is also an important factor in tissue invasion in many invasive bacteria. The fibrinolytic system of the host is responsible for degrading fibrin clots and various ECM and connective tissue components. By binding plasminogen, the bacteria activate this system which leads to tissue and structure damage. Interaction with

fibronectin, collagen type IV, laminin and plasminogen was described in the case of Vlp proteins, enolase, glyceraldehyde-3-phosphate dehydrogenase and DnaK (Wang *et al.*, 2021; J. Li *et al.*, 2022a; Y. Li *et al.*, 2022; Wang *et al.*, 2022b).

Hydrogen peroxide (H_2O_2) production is another important factor causing tissue damage. *M. hyorhinis* has a glycerol-3-phosphate oxidase enzyme which turns glycerol-3-phosphate into dihydroxyacetone-phosphate and H_2O_2 in the presence of molecular oxygen (Rosengarten and Kirchhoff, 2010; Ferrarini *et al.*, 2018). *M. hyorhinis* infection is often followed by production of pro-inflammatory cytokines, activation of mononuclear phagocytic cells and B-cell proliferation. Besides causing tissue damage, the exaggerated inflammatory response can potentially suppress T-cell response (Mühlradt *et al.*, 1998; Trueeb *et al.*, 2020; Zhang *et al.*, 2022). Apoptosis inducing feature of *M. hyorhinis* was also described on cell lines (Liu and Shou, 2011).

2.4 Clinical signs

The first signs of infection typically appear between three to ten days after exposure. These are lethargy, listlessness, fever, loss of appetite and rough hair coat (Barden and Decker, 1971; Gomes Neto *et al.*, 2012; Lee *et al.*, 2018). Joint swelling can also appear shortly after infection around the fourth or fifth day as an indicator of arthritis (Figure 1). The joint swelling or lameness usually becomes more pronounced in the first two weeks, arthritis can be so painful that the pigs are reluctant to move or unable to stand. About two weeks after the infection, the clinical signs of arthritis can gradually start to alleviate (Barden and Decker, 1971; Wang *et al.*, 2022a). Generally, the swelling is first observed in the tarsus and tends to be symmetrical (Barden and Decker, 1971). The number of affected joints may be associated with the age of the pig: by the end of the susceptible age arthritis is normally detected in fewer sites (Martinson *et al.*, 2017). Clinical signs of polyserositis or pneumonia can appear as dyspnoea and coughing (Lee *et al.*, 2018).

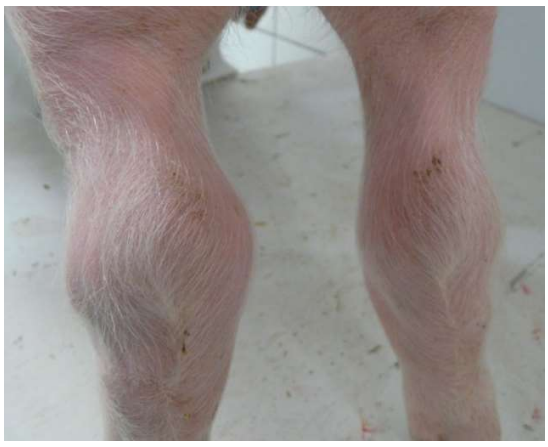

Figure 1: Swollen tarsal joint caused by *Mycoplasma hyorhinis* infection.

Besides the general clinical signs, less frequently, *M. hyorhinis* can cause ear infections, conjunctivitis and meningitis. In the ear, *M. hyorhinis* can cause eustachitis, which manifests as head shaking or tilting of the head to one side (Morita *et al.*, 1995). The first clinical signs of conjunctivitis are conjunctival redness, oedema and serous ocular discharge, then later hyperaemia and persisted oedema can be observed with mucopurulent discharge (Resende *et al.*, 2019). In pigs with meningitis besides coughing and stiff movements central nervous signs like head tilt and locomotive disorders are noticeable (Bünger *et al.*, 2020).

Loss of appetite and failure to thrive are both major concerns of pig producers, regarding *M. hyorhinis* infection. The average daily weight gain of infected animals can decrease by up to 200 grams (Martinson *et al.*, 2018a) and the weight difference between healthy and infected pigs can persist throughout the fattening period (Barden and Decker, 1971).

2.5 Pathological lesions

As the *M. hyorhinis* infection progresses three types of joint lesions can be observed. At first, increased, turbid, serosanguinous synovial fluid appears with an oedematous and hyperaemic synovial membrane (Figure 2). Then the viscosity of the synovial fluid decreases and the synovial membrane proliferates and reddens, at this stage brownish discolouration of the cartilage and mild erosions can appear. At last, at the chronic stage, the thickening of the joint capsule and pericapsular tissue can be observed, the synovial fluid becomes thin and watery, while more severe cartilage erosions are detectable and pannus formation can appear (Ennis *et al.*, 1971; Gomes Neto *et al.*, 2012; Bünger *et al.*, 2020; Wang *et al.*, 2022a).

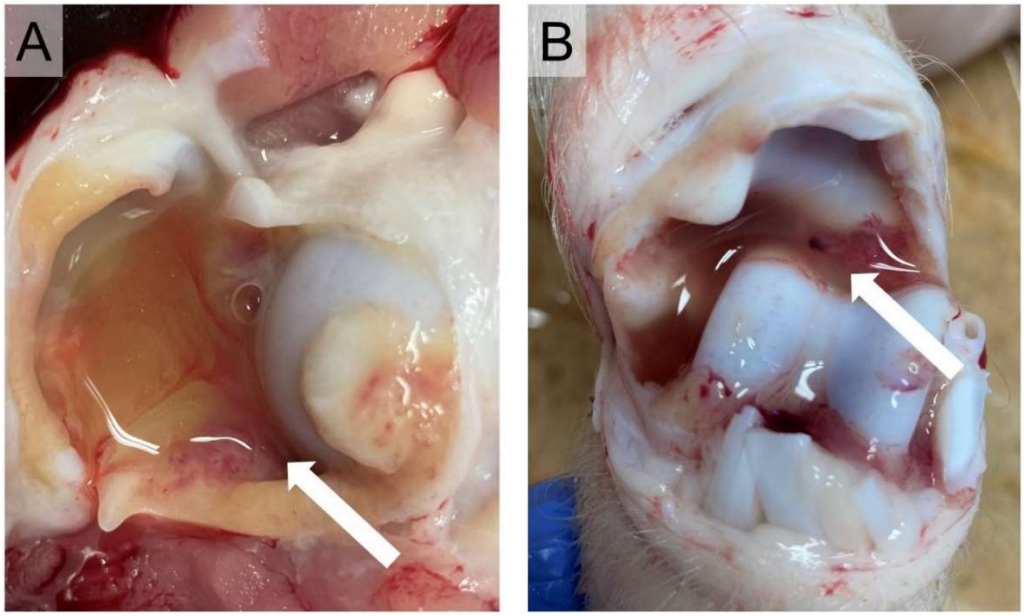

Figure 2: Pathological lesions of the joints caused by *Mycoplasma hyorhinis* infection.
A: Increased serosanguinous synovial fluid, B: Turbid, slightly reddish, serosanguinous, increased synovial fluid

The polyserositis caused by the infection appear on the pleura, pericardium and peritoneum of the animals. First diffuse, serofibrinous inflammation can appear in these membranes, which progresses to organized, adhered serositis with thickening of the affected serosa at the chronic stage (Figure 3). The amounts of pericardial and ascites fluid can increase. In the lungs dark red to purple areas can appear in the caudal, middle and accessory lobes (Ennis *et al.*, 1971; Gomes Neto *et al.*, 2012; Lee *et al.*, 2018; Bünger *et al.*, 2020; Wang *et al.*, 2022a).

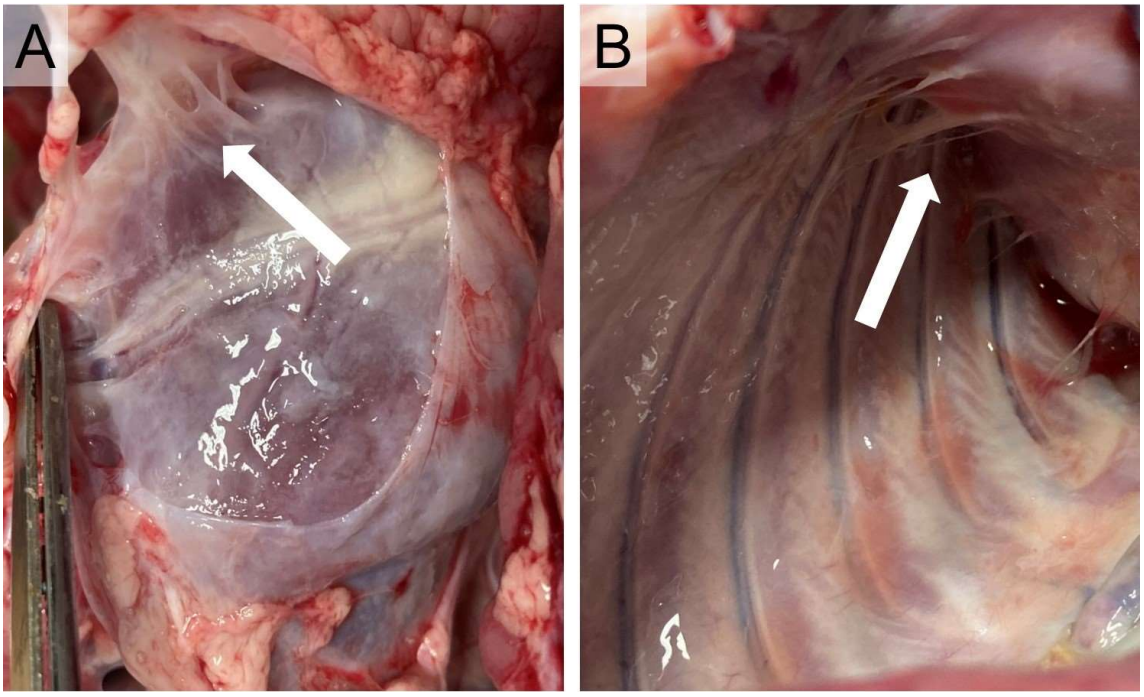

Figure 3: Polyserositis caused by *Mycoplasma hyorhinis* infection.
A: Chronic fibrinous pericarditis, B: Chronic fibrinous pleuritis

Eustachitis usually appears with inflammation of the tympanic cavity, otitis interna or otitis externa with preceded inflammation to other sites of the ear (Morita *et al.*, 1995). In the case of meningitis turbid meninges were detected in some cases (Bünger *et al.*, 2020).

During the histopathological examinations of the lesions infiltration of mononuclear cells, mostly lymphocytes and macrophages in every affected membrane (serosal and synovial membranes, conjunctiva and mucosa of the ear) are evident (Figure 4). The inflammatory infiltration with immune cells is responsible for the thickening of these membranes (Ennis *et al.*, 1971; Morita *et al.*, 1995; Resende *et al.*, 2019). Besides, in the serosa fibrinopurulent pleuritis, pericarditis and peritonitis can be recognized and in the synovial membrane generalized perivascular cuffing can be detected (Ennis *et al.*, 1971; Gomes Neto *et al.*, 2012; Bünger *et al.*, 2020; Wang *et al.*, 2022a). Typical histopathological lesions in the lungs are loss of microcilia, bronchopneumonia, intralobular interstitial pneumonia and peribronchiolar lymphoid cuffing (Lee *et al.*, 2018; Bünger *et al.*, 2020). In the case of conjunctivitis, the ocular conjunctiva can become eroded and ulcerated (Resende *et al.*, 2019). Lymphatic and partly fibrinopurulent leptomeninges can be observed during meningitis (Bünger *et al.*, 2020).

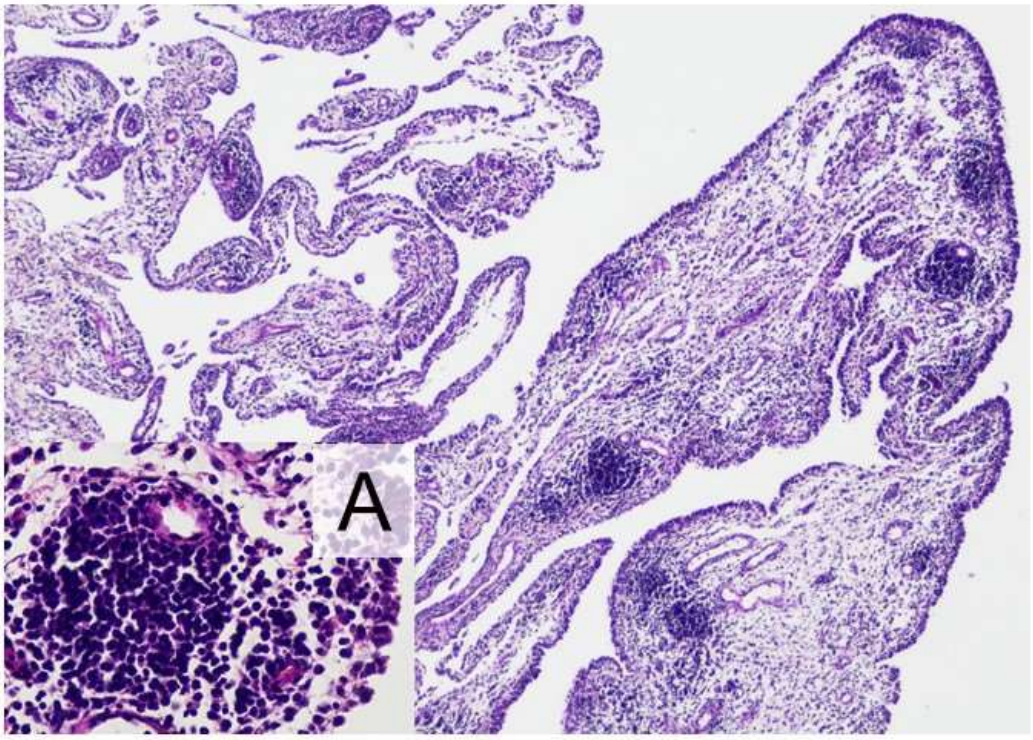

Figure 4: Histopathological lesions caused by *Mycoplasma hyorhinis* infection.
Severe lympho-histiocytic inflammation of the synovial membrane associated with the formation of lymphoid follicle around blood vessels 40×, A: Perivascular follicle 400×; H&E stain

2.6 Diagnosis

M. hyorhinis is part of the normal microbiota in the upper respiratory tract and tonsils and the presence of *M. hyorhinis* in oral fluid samples did not show any correlation with the clinical manifestation of lameness (Pillman *et al.*, 2019). Consequently, these samples are not sufficient for proper diagnosis, although oral fluid or nasal swab samples can be useful in epidemiological studies (Gomes Neto *et al.*, 2012; Makhanon *et al.*, 2012). Samples from characteristic lesions are required for diagnostic purposes, such as synovial fluid, joint swabs, and pieces of synovium, lung tissue, heart tissue or pericardial, thoracic and peritoneal fluid. Samples from acute infection prior to antibiotic treatment are preferred for diagnostic testing.

Clinical signs and pathological lesions of *M. hyorhinis* infection are not pathognomic, therefore detection of the pathogen from the lesions is essential for proper diagnosis. *Glaesserella parasuis* and *Streptococcus suis* both induce similar macroscopic lesions, while lesions of the lung and pleura can originate from swine influenza A virus, PRRSV, PCV2, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *M. hyopneumoniae*. Involvement of *M. hyosynoviae*, *Erysipelothrix rhusiopathiae* and *Actinobacillus suis* should be excluded in the case of joint lesions (Gomes Neto *et al.*, 2012; Palzer *et al.*, 2015; Lee *et al.*, 2016; Pillman *et al.*, 2019)

2.6.1 Conventional methods

Isolation of mycoplasmas can be difficult, due to the small genome size, mycoplasmas have major gaps in most metabolic pathways therefore they require complex, nutrient-rich media for isolation and laboratory growth. However, based on genomic studies *M. hyorhinis* has a wider carbohydrate uptake than the other swine respiratory tract *Mycoplasma* species, which can explain the reduced isolation time and even the role of *M. hyorhinis* as a frequent cell culture contaminant (Ferrarini *et al.*, 2016). *M. hyorhinis* ferments glucose and does not hydrolyse arginine or urea as energy source. During primer isolation typical fried-egg shaped colonies (Figure 5) appear after two to five days and grow up to 0.5 – 1 mm in diameter (Kobisch and Friis, 1996).

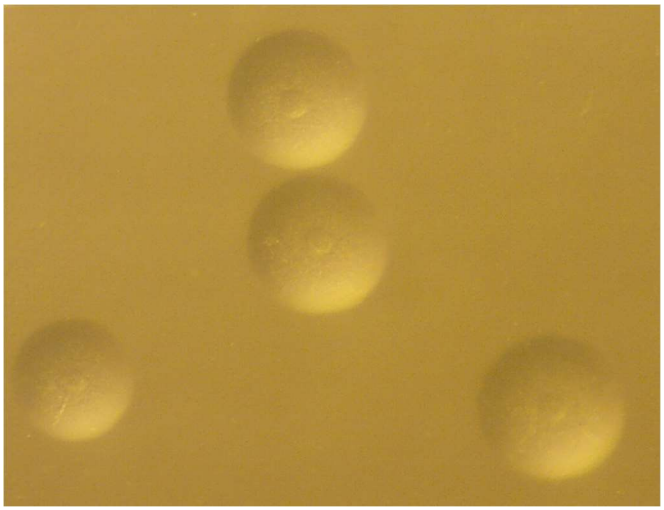

Figure 5: Typical fried-egg shape morphology of *Mycoplasma hyorhinis* on solid media.

Detection of antibody response after infection by enzyme linked immunosorbent assay (ELISA) could be an easy and cost effective monitoring method. However, no tests are available commercially to detect the antibodies against *M. hyorhinis*. Nevertheless, there are several ELISAs published using whole cell antigen (Gomes Neto *et al.*, 2014; Lee *et al.*, 2016) or recombinant versions of the p37 protein (Bumgardner *et al.*, 2018) and the Vlp proteins (Giménez-Lirola *et al.*, 2019; Merodio *et al.*, 2021).

For the detection of *M. hyorhinis* in formalin-fixed, paraffin-wax-embedded tissues *in situ* hybridization techniques are also available. The advantage of these methods is that the bacteria can be observed in their natural environment in cellular detail with high sensitivity and specificity. On the other hand, hybridization techniques are time consuming and require specific laboratory equipment, which hinders their use in routine diagnosis (Boye *et al.*, 2001; Kim *et al.*, 2010).

2.6.2 Polymerase chain reaction based methods and genotyping assays

Due to their fastidious nature, PCR became the gold standard in diagnostic testing for the detection of mycoplasmas. Several conventional and real-time PCR assays have been developed over the years targeting the p37, 16S rRNA or 16S-23S rRNA internal transcribed spacer region of the genome (Assunção *et al.*, 2005; Barate *et al.*, 2012; Clavijo *et al.*, 2014; Tocqueville *et al.*, 2014; Gomes Neto *et al.*, 2015; Hong *et al.*, 2015; Fourour *et al.*, 2018; Resende *et al.*, 2019; Wu *et al.*, 2019).

Genome based typing methods are widely used for the analyses of mycoplasmas, because the results are easily transferred and comparable between laboratories and some of the techniques do not even require isolation prior testing. Multi-locus sequence typing (MLST) is one of these typing methods, where relationships are determined based on polymorphisms in house-keeping gene fragments. For the *M. hyorhinis* MLST gene fragments of six house-keeping genes, the chromosomal replication initiation protein (*dnaA*), the DNA-directed RNA polymerase β subunit (*rpoB*), the DNA gyrase subunit B (*gyrB*), the glutamyl-tRNA synthetase (*gltX*), the adenylate kinase (*adk*) and the guanylate kinase (*gmk*) were selected (Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016). To increase the resolution of this assay a new MLST scheme (MLSTs) was introduced (Clavijo *et al.*, 2019), which targeted two house-keeping [pyruvate dehydrogenase-E beta-1 subunit (*pdhB*) and uracil-DNA glycosylase (*ung*)] genes complemented with two surface protein coding genes [outer membrane protein p95 (*p95*) and mannitol-1-phosphate 5-dehydrogenase (*mtlD*)]. Recently, a core genome MLST (cgMLST) has been developed for epidemiologic studies and outbreak analysis. The advantage and the main disadvantage of the latter assay are the same, the use of whole genome sequences for the analysis. Increasing the number of house-keeping genes in the assay can increase the resolution. On the other hand, whole genome sequencing requires prior isolation, excess time and it is costly (Bünger *et al.*, 2021).

As the MLST systems are based on a limited number of house-keeping gene fragments, the assays are not typically applicable to differentiate closely related isolates. Another sequence based genotyping method is the multiple-locus variable-number tandem-repeat analysis (MLVA). This method examines the number of tandem repeat units in the genome. The advantages of the MLVA are that no sequencing is required, the results are reproducible and achieved rapidly using only a conventional PCR. A two-allele (MHR_0152 and MHR_0298) based MLVA was developed for the characterization of *M. hyorhinis* isolates. As tandem repeat regions of the genome are highly variable, typing based on these regions are suitable to detect short term evolutionary events (Dos Santos *et al.*, 2015).

2.7 Treatment and control

Control of *Mycoplasma* species has three general approaches: maintaining pathogen free-populations, treatment with medication, and vaccination. While vaccination and maintaining pathogen free-populations provide long term control, medication is only a short term solution. (Kleven, 2008).

2.7.1 Control of *Mycoplasma hyorhinis* at herd level

Maintaining pathogen free herds is not feasible in the field for several reasons. First, adult animals are asymptomatic carriers of *M. hyorhinis* and the sows are not routinely screened for the presence of this pathogen. Second, due to the presumed intermittent shedding of the bacteria even with regular screening the presence of carrier/infected animals can be over looked (Clavijo *et al.*, 2019). Thirdly, due to the high prevalence of this pathogen, newly introduced gilts are potential carriers, which was confirmed in epidemiological studies (Clavijo *et al.*, 2017; Roos *et al.*, 2019). Lastly, the piglets are transported at the age when the pathogen is transmitted with the highest frequency, spreading the disease between farms (Clavijo *et al.*, 2017; 2019).

Good herd health management is essential to provide animal welfare. By optimizing housing conditions like: humidity, light intensity and duration, dust, noise, concentration of chemicals like ammonia and carbon dioxide and stocking density; important stress factors can be excluded (von-Borell *et al.*, 2001). As stress factors have a vital role in *M. hyorhinis* infection, good health practices are fundamental in prevention. Co-infections, mainly with viral pathogens are also proven to have part in the appearance of clinical signs. Accordingly, vaccination against pathogens like PCV2 and PRRSV are key factors in controlling economical losses due to *M. hyorhinis* infection also (Palzer *et al.*, 2015; Lee *et al.*, 2018; Clavijo *et al.*, 2019).

2.7.2 Vaccination

Infections that pose threat to young piglets can be prevented by vaccination as early as the maternal antibodies decrease, or by vaccination of the sows and boosting maternal immunity (Tizard, 2021). Age of vaccination can be critical in case of *M. hyorhinis* as this pathogen can cause clinical signs from three to 16 weeks old piglets (Kobisch, Friis, 1996; Martinson *et al.*, 2017) and decrease of maternal antibodies is a predisposing factor in the formation of clinical signs (Clavijo *et al.*, 2019). Due to the early onset of the disease, vaccination of sows should be considered; on the other hand proper protection needed for prolonged period, therefore vaccination of the piglets seems necessary. When determining the age of piglets at vaccination

it should be considered that vaccination during weaning (around three to four weeks of age) can be less effective due to the altered immune reaction caused by the stress of the weaning, as it was observed in case of *M. hyopneumoniae* (Arsenakis *et al.*, 2017). As boosting the mucosal immunity can prevent colonisation and do not interfere with maternal antibodies intranasal vaccines can be applied earlier than intramuscular or intradermal vaccines (Tizard, 2021). Live-attenuated vaccines are frequently used in controlling avian mycoplasmas by boosting mucosal immunity (Yadav *et al.*, 2021) and proved to be effective against *M. hyopneumonaie* as well (Feng *et al.*, 2013).

Up to date, there are no commercially available vaccines against *M. hyorhinis* in Europe., Nevertheless, there is an inactivated vaccine in the United States (Ingelvac MycoMAX™, Boehringer Ingelheim Animal Health Inc. USA; Martinson *et al.*, 2018b) and there are two other inactivated vaccine candidates and a recombinant vaccine under development these days also (Lee *et al.*, 2018; Wei *et al.*, 2020; WO2018027526A1). Moreover, considering the emerging concerns about *M. hyorhinis* infections and the “one health” concept, the development of further vaccine candidates is expected.

2.7.3 Antibiotic therapy

The third option for the control of mycoplasmas is medication. In the lack of effective vaccines, medication is the primary control option in the case of *M. hyorhinis*. Although antibiotic treatment can ease the clinical signs, it is not suitable for the elimination of the pathogen and long term control of the infection. One reason for that is antibiotics that are applicable against mycoplasmas are mostly bacteriostatic. The other reason is that *M. hyorhinis* can enter the host cells, where antibiotics can hardly reach them (Kornspan *et al.*, 2015; Gautier-Bouchardon, 2018). Apart from this, excessive use of antibiotics can lead to the emergence of resistant strains (Kleven, 2008).

Mycoplasmas are cell wall less organisms, accordingly, antibiotics inhibiting the cell wall synthesis (β-lactams, glycopeptides, fosfomycin) are ineffective against them. Furthermore, mycoplasmas are naturally resistant to rifampicin due to a mutation in the *rpoB* gene, and polymixins, sulphonamides and first-generation quinolones, because of the lack of metabolic pathways (Gautier-Bouchardon, 2018). Antimicrobials widely used against mycoplasmas are the protein synthesis inhibiting macrolides, lincosamides, tetracyclines, aminoglycosides, pleuromutilins, phenicols and DNA synthesis inhibiting fluoroquinolones (Hannan *et al.*, 1989, 1997; Ter Laak *et al.*, 1991; Kobayashi *et al.*, 1996b, 1996a, 2005; Hannan, 2000; Wu *et al.*, 2000; Jin *et al.*, 2014; Jang *et al.*, 2016; Bekő *et al.*, 2019a; Rosales *et al.*, 2020). Moreover, *M. hyorhinis* is naturally resistant to 14-membered macrolides, for example, erythromycin (Kobayashi *et al.*, 1996b). Antibiotic susceptibility testing of mycoplasmas can be done by broth

microdilution or agar dilution technique. However, the complex media requirement and the long isolation and culturing time results in expensive and time consuming susceptibility testing (Hannan, 2000).

M. hyorhinis isolates are usually susceptible to tetracyclines, pleuromutilins and fluoroquinolones (Hannan *et al.*, 1989, 1997; Ter Laak *et al.*, 1991; Kobayashi *et al.*, 1996b, 1996a, 2005; Hannan, 2000; Wu *et al.*, 2000; Jin *et al.*, 2014; Jang *et al.*, 2016; Bekő *et al.*, 2019a; Rosales *et al.*, 2020). Even so, isolates with decreased susceptibility against chlortetracycline were detected in samples isolated between 2009 and 2011 in Korea (Jang *et al.*, 2016). Decreased susceptibility against macrolides and lincomycin is more common among isolates worldwide. In Japan, around 10% of the strains isolated between 1991 and 1994 had decreased susceptibility, which increased to 40% by 2005 (Kobayashi *et al.*, 1996b, 2005). Among Hungarian isolates from 2014 to 2017, decreased susceptibility was detected in around 30% (Bekő *et al.*, 2019a). Macrolide and lincosamide antibiotics share similar mode of action and bind within the tunnel of the 50S ribosomal subunit. Bacteria in general can become resistant to these antibiotics by: (1) target site modification either through methylation or mutation, (2) efflux mechanism, (3) drug inactivation. Target site modification has effect on a wide-range of these antibiotics whereas efflux and drug inactivation have more targeted action (Gautier-Bouchardon, 2018). A point mutation in the 23S rRNA gene at position 2059 (numbered according to *Escherichia coli*) is associated with decreased susceptibility to macrolides and lincomycin in case of *M. hyorhinis* (Kobayashi *et al.*, 2005).

3. Aims of the study

The aims of the study were:

1. To determine the MICs of ten frequently used antimicrobials against *M. hyorhinis* isolates collected in Europe between 2019 and 2021.

2. To identify resistance markers in the genome of *M. hyorhinis* isolates and to develop a molecular typing method for the detection of the identified markers.

3. To develop genotyping systems for *M. hyorhinis* isolates with higher resolution capacities.

4. To establish a *M. hyorhinis* challenge model for future vaccine efficacy studies.

4. Materials and methods

4.1 Samples

4.1.1 Sample collection and isolation

During the present study, the *M. hyorhinis* collection established in 2014 has been expanded with 133 novel isolates of diverse origin regarding time of isolation, country of origin and organ of origin. Hungarian isolates originated from clinical samples like: synovial fluid, pericardial content, pleural exudate, serosa, lung, meninges, tonsillar and nasal swabs, and were collected between 2014 and 2021 (Supplementary table 1). The samples were taken during routine diagnostic examinations or with the consent of the owners in slaughterhouses, therefore - according to the ethics committee of the Veterinary Research Institute, Budapest, Hungary - ethical approval was not required.

The isolates were cultured in *Mycoplasma* Liquid media (Mycoplasma Experience Ltd., Bletchingley, United Kingdom). Briefly, 200 µl fluid sample, small pieces of the tissues or swabs were inoculated into 2 ml liquid media, then the broths were filtered by 0.45 µm pore size cellulose acetate membrane filter (Sartorius Stedim Biotech GmBH., Göttingen, Germany). Then the broths were incubated at 37°C until colour change (red to yellow shift in the presence of phenol red indicator). After colour change, samples were transferred to *Mycoplasma* Solid media (Mycoplasma Experience) and incubated at 37°C with 5% CO_2 until colonies appeared. Pure cultures were obtained by filter-cloning technique, one colony was cut from the agar plates, inoculated into fresh liquid media and incubated until colour change.

Samples from other countries were provided in pure culture form*:* two isolates from Belgium, 15 isolates from Germany, 20 isolates from Italy and 19 isolates from Poland (Supplementary table 1).

4.1.2 Identification of the isolates

The QIAamp DNA Mini Kit (Qiagen GmBH, Hilden, Germany) was used for DNA extraction from clinical samples or isolates (submitting 200 µl of pure cultures for use in PCR assays or 10 ml cultures for whole genome sequencing) according to the manufacturers' instructions for Gram-negative bacteria. The prepared DNA was further tested for the presence of *M. hyorhinis* and contaminant *Mycoplasma* species.

4.1.2.1 *Mycoplasma hyorhinis* specific polymerase chain reactions

To detect the presence of *M. hyorhinis* both conventional and real-time PCRs were used. For the conventional PCR previously published primers targeting the p37 gene were applied (Assunção *et al.*, 2005). For the real-time PCR previously published primers targeting the 16S rRNA gene (Resende *et al.*, 2019) were used and further optimized.

The original primers in the species-specific real-time PCR (Resende *et al.*, 2019) showed tendencies for self dimerization and inadequate melting temperatures, which limited the sensitivity of the assay. By shortening the forward primer, adding four nucleotides to the probe and completely redesigning the reverse primer to a different position, the oligos complied more with the rules of TaqMan design parameters (http://www.premierbiosoft.com/tech_notes/TaqMan.html) and the sensitivity of the assay improved. The new primers were designed based on the aligned sequences of the 16S rRNA gene from the whole genome sequences of the type strains of *M. hyorhinis* (NCTC 10130, GenBank accession number: LS991950.1), *M. hyopneumoniae* (NCTC 10110, GenBank: AE017243.1), *M. hyosynoviae* (NCTC 10167, GenBank: NZ_SOCH00000000.1) and *M. flocculare* (NCTC 10143, GenBank: CP007585.1) to limit the chance of cross-reactions. Melting temperatures and suitability of the designed primers were calculated using the NetPrimer software (Premier Biosoft International, San Francisco, California, USA) and the specificity of the primers was confirmed with BLASTN search (https://blast.ncbi.nl*M.*nih.gov/Blast.cgi). To test the sensitivity of the developed assay, tenfold dilutions of the type strain (NCTC 10130) were used in the range of 10^8-10^0 copy number/µl. Template copy number was calculated with the help of an online tool (https://cels.uri.edu/gsc/cndna.html) by measuring the concentration of DNA of pure *M. hyorhinis* cultures by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The lowest DNA concentrations detectable on the real-time platform were considered the detection limits of the methods. The specificity was tested by including *M. hyopneumoniae*, *M. hyosynoviae* and *M. flocculare* in the analyses.

The sensitivity of the reaction with the optimized primers was 10^1 copies/reaction, and no cross reactions were detected for *M. hyopneumoniae*, *M. hyosynoviae* and *M. flocculare*. Sequences of the *M. hyorhinis* specific primers are listed in Table 1.

Table 1: Primer and probe sequences of the *Mycoplasma hyorhinis* specific polymerase chain reactions.

Target		Sequences 5'-3'	Source
p37	F	GTA GTC AAG CAA GAG GAT GT	(Assunção *et al.*, 2005)
	R	GCT GGA GTT ATT ATA CCA GGA	
16S rRNA	F	CGT ACC TAA CCT ACC TTT AAG	this study
	R	TAA TGT TCC GCA CCC C	
	P	FAM-CCG GAT ATA GTT ATT TAT CGC ATG ATG AG-BHQ	

Abbreviations: F: forward, R: reverse, P: probe

The conventional PCRs were performed on a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, California, USA), while the real-time PCRs were performed using a Bio-Rad C1000 Touch™ Thermal Cycler, CFX96™ Real-Time system (Bio-Rad laboratories). Contents of the mastermix and thermocycling parameters for the *M. hyorhinis* specific PCRs are listed in Supplementary data 1a and 1b. The 346 bp long products of the p37 based conventional PCR were separated by agarose gel electrophoresis using 1% agarose gel (SeaKem® LE Agarose, Lonza Inc., Switzerland) with a 100 bp DNA ladder (GeneRuler 100 bp Plus, Thermo Fisher Scientific) as a molecular weight marker and visualized with ECO Safe Nucleic Acid Staining solution (Pacific Image Electronics Co., Ltd., Taiwan) under UV light.

4.1.2.2 Exclusion of contaminant *Mycoplasma* species

The presence of other porcine *Mycoplasma* species was checked by species-specific PCR. In the cases of *M. hyopneumoniae* and *M. hyosynoviae* real-time PCRs targeting the p97 and the 16S rRNA genes were used, respectively (Martinson *et al.*, 2018a; Wu *et al.*, 2019), with the same protocol as for *M. hyorhinis* (chapter 4.1.2.1, Supplementary data 1b). While for *M. flocculare* a conventional PCR targeting the 16S rRNA gene with the same protocol as for *M. hyorhinis* p37 PCR (chapter 4.1.2.1, Supplementary data 1a) was used (Assunção *et al.*, 2005). Primer sequences are listed in Table 2. The presence of other mycoplasmas was excluded with a universal *Mycoplasma* PCR system targeting the 16S/23S rRNA spacer region in Mollicutes (primer sequences listed in Table 2), where most species can be distinguished by product size (Lauerman *et al.*, 1995). The reaction mixture and thermocycling parameters for the universal *Mycoplasma* PCR are detailed in Supplementary data 1c. The products were separated and visualized with the method used for the *M. hyorhinis* specific PCR (chapter 4.1.2.1).

Table 2: Primer and probe sequences for the exclusion of contaminant species.

Target		Sequences 5'-3'	Source
M. hyopneumoniae	F	CCA GAA CCA AAT TCC TTC GCT G	(Wu et al., 2019)
	R	ACT GGC TGA ACT TCA TCT GGG CTA	
	P	FAM-AGC AGA TCT TAG TCA AAG TGC CCG TG-BHQ	
M. hyosynoviae	F	TTA ATG CCG GAT AAG TAT GAA	(Martinson et al., 2018a)
	R	GCA CCC TCA TCT CTT AG	
	P	FAM-AAG CAA ACG CTT CTT TCA TAA CGA AAT C-BHQ	
M. flocculare	F	ACG GGA TAG TAT TTT AGT TTT ACT A	(Assunção et al., 2005)
	R	TCT TCC CTT ACA ACA GCA GTT TAC A	
Universal *Mycoplasma*	F	ACA CCA TGG GAG CTG GTA AT	(Lauerman et al., 1995)
	R	CTT CAT CGA CTT TCA GAC CCA AGG CAT	

Abbreviations: F: forward, R: reverse, P: probe

4.1.3 Whole genome sequencing

For the whole genome sequencing 38 Hungarian isolates were selected based on their diverse origin (farm and organ of origin), however serial isolates from the same farm were also included (Bácsalmás: nine isolates, Hajdúszoboszló: four isolates, Jánoshalma: two isolates). The isolates were collected between 2014 and 2019 (Supplementary table 1 and 2).

For the whole genome sequencing first Illumina specific libraries were prepared with the Illumina® Nextera XT DNA Library Preparation Kit and Nextera XT Index Kit (Illumina Inc., San Diego, California, USA). The denatured library pool was loaded onto a NextSeq 500/550 Mid Output flowcell and sequenced using an Illumina® NextSeq 500 sequencer (Illumina) to gain short reads. The sequencing resulted in 150 bp long single reads, the average number of reads were 2 855 078. The reads were first quality checked with FastQC software version 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then the short reads was mapped to and annotated according to *M. hyorhinis* type strain (NCTC 10130, GenBank accession number: LS991950.1) by Geneious Prime software version 2019.2.1 (Kearse *et al.*, 2012) with the average coverage of 401.05.

4.2 Antibiotic susceptibility testing

Throughout the tests, *Mycoplasma* Liquid Media (*Mycoplasma* Experience) was used as a culture medium. The number of colour changing units (CCU) was calculated by plate microdilution from the highest dilution showing colour change (red to yellow shift) prior to susceptibility testing (Hannan, 2000).

The antimicrobials were selected based on their use in the swine industry and proven effect against mycoplasmas. Antibiotic susceptibility testing was performed on two major groups of samples: (1) isolates collected between 2019 and 2021 from five European countries (total of

76 isolates) were tested separately for ten antimicrobials in the Pan-European project (2) based on the published susceptibility data (Bekő *et al.*, 2019; Kobayashi *et al.*, 1996b; 2005) macrolides and lincomycin were tested to asses missing susceptibility data of isolates with whole genome sequences (MycSu60, MycSu127, MycSu128 and MycSu152) to be able to determine resistance markers.

Ten frequently used antibiotics were tested in this study: two tetracyclines (doxycycline and oxytetracycline), a pleuromutilin (tiamulin), a phenicol (florfenicol), a fluoroquinolone (enrofloxacin) four macrolides (tylosin, tilmicosin, tylvalosin and tulathromycin) and a lincosamide (lincomycin). All antibiotics used in the tests originated from Vetranal® (Merck KGaA, Darmstadt, Germany) except for tylvalosin and tulathromycin. Tylvalosin originated from ECO® Animal Health Ltd., London, UK and tulathromycin from Pfizer® Inc., New York, New York, USA. Stock solutions in the concentration of 1 mg/ml were prepared, aliquoted and stored frozen at -70°C until use. The antibiotic ranges for susceptibility testing were determined based on previous publications (Bekő *et al.*, 2019): tiamulin, doxycycline and enrofloxacin were tested in the range of 0.039-10 µg/ml, 0.125-32 µg/ml was used for florfenicol, and 0.25-64 µg/ml was used for the macrolides, lincomycin and oxytetracycline. For the Pan-European study the ranges of tylvalosin (to 0.039-10 µg/ml) and oxytetracycline (0.125-32 µg/ml) were changed considering the pharmacokinetics of these antimicrobials.

Twofold dilutions of the antibiotics were freshly prepared right before the tests. The broth microdilution test was performed in a 96-well microtiter plate containing the dilution series of the antibiotic, with sterility, pH and growth controls. The clinical isolates were tested in duplicates and the *M. hyorhinis* type strain (NCTC 10130) was included in the tests as quality control. All isolates were tested at a concentration of 10^5 CCU/ml. The minimal inhibitory concentration (MIC) of each isolate was defined as the lowest concentration of the antibiotic where no colour change (no growth) was recorded by the time the growth control changed colour (Hannan, 2000). MIC_{50} and MIC_{90} values were defined as the lowest concentrations that inhibited the growth of 50% and 90% of the tested isolates (Hannan, 2000).

4.3 Mismatch amplification mutation assay

Based on the results of available antibiotic susceptibility tests high MICs against growing numbers of isolates were found in the case of macrolides and lincomycin (Bekő *et al.*, 2019; Kobayashi *et al.*, 1996b; 2005). Since there are no official breakpoints for *M. hyorhinis* antibiotic susceptibility testing, previously published breakpoints for *M. hyopneumoniae* or for other porcine respiratory pathogens were used. Accordingly, MICs ≥ 4 µg/ml of tylosin, tylvalosin, gamithromycin and lincomycin, while MICs ≥ 16 µg/ml of tilmicosin and tulathromycin were considered high values (Hannan *et al.*, 1997; Felde *et al.*, 2020; Vilaró *et al.*, 2020). Single

nucleotide polymorphism (SNP) associated with high (genotype H) or low (genotype L) MIC values were described before based on whole genome sequence analyses. In *M. hyorhinis* or other mycoplasmas point mutations in the genes 23S rRNA, and 50S ribosomal protein L4 and L22 were previously connected with decreased macrolide and lincomycin susceptibility (Kobayashi *et al.*, 2005; Sulyok *et al.*, 2017; Bekő *et al.*, 2020; Felde *et al.*, 2020). In the present examination, whole genome sequences of the 38 isolates determined in this study were included (Supplementary table 1 and 2) and the antibiotic susceptibility-associated genes were extracted, aligned and analysed in Geneious Prime software version 2019.2.1 (Kearse *et al.*, 2012). In the protein coding genes only non-synonymous SNPs (resulting in amino acid change) were analysed, while in the 23S rRNA gene, all SNPs were investigated. To identify resistance-related mutations, the correlation between MIC values (Supplementary table 3) and the occurrence of the SNPs were studied. Mutations were considered resistance-related when occurred more frequently with increasing MIC values or exclusively in isolates with high MIC values. Nucleotide positions in this study are numbered according to the genome of *M. hyorhinis* type strain to avoid misunderstandings due to gaps in the alignment, however, for comparison with literature data numbering according to *E. coli* K-12 sub-strain MG1655 (GenBank accession number: NC_000913) is indicated in parentheses, where the given positions are first mentioned.

To detect the SNPs mismatch amplification mutation assays (MAMAs) were designed. Briefly, the assay is based on competing, allele specific primers, which are SNP-specific at the 3' end and contain a single destabilizing mismatch at the antepenultimate position of the 3' end to enhance discriminatory power. One of the allele specific primers is labelled with a 15-20 bp long GC clamp. This clamp increases the melting temperature and size of the allele specific product, which is the base of the detection either on a real-time platform (melt-MAMA) or by agarose gel electrophoresis (agarose-MAMA). On the real-time platform intercalating fluorescent DNA dye (EvaGreen) is used for detection (Birdsell *et al.*, 2012). Primers specific for resistance-related markers were designed based on the same alignments which were used for the SNP detection in the Geneious Prime software (Kerase *et al.*, 2012). The primers were designed as described before in chapter 4.1.2.1, for sensitivity testing beside the type strain (NCTC 10130; genotype L) DNA of a genotype H clinical isolate (MycSu24) was also included. Sensitivity limit of the melt-MAMA assay was determined as the lowest DNA concentration where genotype specific melting curves were detected. Primer sequences of the MAMA assay are described in Table 3.

Table 3: Primer sequences, amplicon sizes and melting temperatures of the amplicons used in the mismatch amplification mutation assay.

Primer name*	Primer sequence (5'-3')**	Amplicon size (bp)	Melting temperature range (°C)
23S-L	TTA CCC GCA TCA AGA CtA A	119	78.3-78.9
23S-H	*gggcgggcgggcgggcgggc* TTA CCC GCA TCA AGA CaA G	139	81.3-82.2
23S-C	TCC ACT AGA ACT AGC GTC CC		

*The letters in the primers name stand for: L: genotype-L, low minimal inhibitory concentration (MIC) associated marker specific primer; H: genotype-H, high MIC associated marker specific primer; C: consensus primer.
**Lower case nucleotides stand for the antepenultimate destabilizing mutations. Lower case nucleotides in italics stand for the GC clamp.

The agarose-MAMA PCRs were performed using Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories) reaction mixtures and thermocycling parameters are detailed in Supplementary data 1d. After amplification, 5 µl of each reaction mixture were submitted to electrophoresis in 3% agarose gel (MetaPhor Agarose, Lonza) using a 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific) as a molecular weight marker. The amplified PCR products were visualized with ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics) under UV light.The real-time PCRs were performed using Bio-Rad C1000 Touch™ Thermal Cycler, CFX96™ Real-Time System (Bio-Rad Laboratories). EvaGreen (intercalating DNA dye; Biotium Inc., Fremont, California USA) fluorescence intensity was measured at 525 nm at each ramp interval and plotted against the temperature. Reaction mixtures and thermocycling parameters for the melt-MAMA are listed in Supplementary data 1d.

For the validation of the developed assay a total of 138 samples (123 clinical isolate with MIC data and 15 DNA samples) were used (Supplementary table 1, 2 and 3). The corresponding isolates of some DNA samples (MycSu166, MycSu181, MycSu206 and MycSu212) were only tested for at least one macrolide or lincomycin during routine diagnostic work with the method described in chapter 4.2. When incongruence was observed between the results of the molecular assay and the MIC testing, analysis of the partial 23S rRNA gene sequence was performed with previously published primers Mhr-D5-1F and Mhr-D5-1R (Kobayashi *et al.*, 2005). The PCR conditions are detailed in Supplementary data 1e. The PCR products were sequenced on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Waltham, Massachusetts, USA).

4.4 Development of genotyping assays

4.4.1 Multi-locus sequence typing assay

House-keeping genes suitable for MLST were selected based on the whole genome sequences of the 38 clinical isolates determined in this study and seven publicly available *M. hyorhinis* whole genome sequences [*M. hyorhinis* type strain (NCTC 10130; GenBank: LS991950.1), DBS1050 (GenBank: CP006849.1), GDL-1 (GenBank: CP003231.1), HUB-1 (GenBank: CP002170.1), MCLD (GenBank: CP002669.1), MDBK-IPV (GenBank: CP016817.1), SK76 (GenBank: CP003914.1); Supplementary table 1 and 2] in Geneious Prime software version 2019.2.1 (Kearse *et al.*, 2012). At first, 17 genes were selected based on MLST schemes described in *Mycoplasma* sp. before (Manso-Silván *et al.*, 2012; Dijkman *et al.*, 2016; Ghanem and El-Gazzar, 2016; Bekő *et al.*, 2019b) including the ones from the published *M. hyorhinis* MLST assay (referred to MLSTp from here after; Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016). Further criteria for gene selection is summarised as follows: (1) the selected genes are present in all *M. hyorhinis* genomes, (2) the selected genes have highly diverse internal fragments surrounded by conserved regions, (3) the selected fragments have high Simpson's index of diversity (ID), (4) the amplicon sizes of the selected gene fragments are between 300 and 600 bp, suitable for Sanger sequencing and (5) the genes are evenly distributed in the genome. The primers flanking these regions were designed based on the alignments used for gene selection. Primer design, sensitivity and specificity testing was performed as described in chapter 4.1.2.1. Primer sequences are summarized in Table 4 and the PCR conditions are detailed in Supplementary data 1f. The MLST developed by us will be referred as MLSTn throughout the study. The PCRs were performed on a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories).

Table 4: Primer sequences used for multi-locus sequence typing.

Gene		Primer sequences 5'-3'	Product length
lepA	F	GCT GCT ACT GAA GGT GCT TTA TTA	444 bp
	R	TGT GAA TCA ACA GGA TAA AAC CC	
rpoB	F	GAT CTT CAA AAG CAG GTA ATG ATG	470 bp
	R	ATA TAG CCG ATT GAA ACA TCA TTG	
rpoC	F	GTG TAC TCT ACA AAT TCT GGT GTT TCT	565 bp
	R	TGA CAA GAT AAA ATG GAA CGG AT	
gltX	F	GTC AGA ATA GCT GTT TAT GAA AGT GAG	410 bp
	R	GGA CCT CTT ACT ATA TCA TTT CAA GC	
uvrA	F	GTA GTT GAA CAC GAT GAA GAA ACA	517 bp
	R	CCA CCC GAT AAA GTA GTT GCT G	
valS	F	ATT CAA CTC CAG GAC AAG ACA TT	657 bp
	R	TGT AAC TTT TTC TGC TTG TGG G	

Abbreviations: F: forward, R: reverse

For the diversity analysis the sequences of the gene fragments were concatenated (3,063 bp total). A total of 47 samples were used for the validation of the assay (38 whole genome sequences, seven GenBank sequences and two other isolates from the same animal as MycSu32t; Supplementary table 2). In the case of isolates without whole genome sequences (MycSu32i and MycSu32s) each MLST gene fragment was amplified individually and their sequences were determined on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems). The phylogenetic analysis was performed with the Maximum Likelihood method, using the Hasegawa-Kishino-Yano model in the MegaX software (Kumar *et al.*, 2018).

4.4.2 Multi-locus variable-number tandem-repeat analysis assay

Tandem-repeat regions were identified in the *M. hyorhinis* type strain (NCTC 10130) genome, and in six other publicly available *M. hyorhinis* (HUB-1, GDL-1, SK76, DBS1050, MCLD, MDBK-IPV) genomes using the Tandem Repeat Finder program (Benson, 1999). At first, 23 loci were selected based on period size (at least 12 bp) and the percent of insertions/deletions (0%). Primers were designed and sensitivity and specificity of the assays were performed as described in chapter 4.1.2.1. The developed MLVA system was applied to characterize the same samples which was used for the validation of the MLSTn system, except for the samples with only GenBank sequences which are not available at our laboratory (Supplementary table 2). The designed primers are listed in Table 5 and the PCR conditions are detailed in Supplementary data 1g.

Table 5: Primer sequences of the multiple-locus variable-number tandem-repeat alleles.

Allele	Position in *M. hyorhinis* type strain (NCTC 10130)		Primer sequences 5'-3'
Mhr205	205,528 - 205,320	F	GCA GAA TCC ACT CTA GCT CAA ACT A
		R	TTT GTA TTG TGT TCC TTT TTT TAA TCC
Mhr396	396,104 - 396,142	F	TAA TTT GGA TAA AAA TAC TCA ATA TGA AGT AG
		R	AAA CTA TAC TCA ACT GTA TAT TTT GAA CCA T
Mhr438	438,283 - 438,867	F	CTT TAT CAA TGA ATT TTT ACA AAT GGA A
		R	CAA ATC AAT CTG GTT CAG CAT CA
Mhr441	441,069 - 441,914	F	TGT GTT CTT GAT CTT TTG AGG TTT T
		R	TCC CAA TCA CAA CAA CCA GGA
Mhr442	442,537 - 442,591	F	TGG TTC ATC TAC AAG CGG AGG
		R	TGA ATC TGA TCC TGT TCC AGT CTG
Mhr444	444,088 - 444,479	F	ATA TTG GGT TTT TTG TTT GTA ATT TCT T
		R	ATC AGG AAC ATC TAC AAG CGG AG

Abbreviations: F: forward, R: reverse

The PCRs were performed on Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories). After amplification, 5-10 µl of each reaction mixture were submitted to electrophoresis. Electrophoresis of amplicons above 200 bp length was carried out in 2% (SeaKem® LE Agarose, Lonza) agarose gel with a 100-bp DNA ladder (GeneRuler 100 bp Plus, Thermo Fisher Scientific), while amplicons below 200 bp were detected in 3% (MetaPhor Agarose, Lonza) agarose gel using a 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific) as a molecular weight marker. The amplified PCR products were visualized with ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics) under UV light. Stained gels were photographically documented (Kodak Inc., Rochester, New York, USA) and band sizes were estimated with the Kodak MI SE software package (Kodak). The estimated band sizes were converted to the number of repeat units based on a formula given for each allele in Table 6. The clustering analysis was performed with the Neighbor-Joining method based on pairwise distances in the MegaX software (Kumar *et al.*, 2018).

Table 6: Length of repeat units, product length in the *Mycoplasma hyorhinis* type strain (NCTC 10130) and formula for the calculation of repeat numbers.

Allele	Repeat unit (bp)	Product length in *M. hyorhinis* type strain (NCTC 10130)	Product size
Mhr205	21	183	120+21n
Mhr396	19	372	334+19n
Mhr438	39	741	178+39n
Mhr441	66	920	75+66n
Mhr442	24	151	97+24n
Mhr444	36	546	118+36n

*The product size is the size of the flanking region plus n×repeat size, with n being the number of repeats.

4.4.3 Comparison of genotyping methods

The MLST analysis of 45 strains (the whole genome sequences of the 38 clinical isolates from the present study and the publicly available sequences; Supplementary table 2) was performed according to the previously published MLST systems [MLSTp (Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016) and MLSTs (Clavijo *et al.*, 2019)]. The gene fragments from *dnaA*, *rpoB*, *gyrB*, *gltX*, *adk* and *gmk* for MLSTp and *pdhB*, *p95*, *mtlD* and *ung* for MLSTs were extracted from the genome sequences, concatenated, aligned and analysed in Geneious Prime software version 2019.2.1 (Kearse *et al.*, 2012). The allele profiles and sequence types of isolates from the present study were compared to the available data for both MLSTp and MLSTs. In case of MLSTp sequences (173 sequences in total) from the PubMLST database (https://pubmlst.org/mhyorhinis/) were submitted for phylogenetic analysis. While for MLSTs the published strains from the study of Clavijo and co-workers (148 sequences total) were included in the analyses. Phylogenetic analysis was performed with the Maximum Likelihood method, and Tamura-3 model in the MegaX software (Kumar *et al.*, 2018) for both MLSTp and MLSTs.

The discriminatory power of the MLST systems was compared based on Simpson's ID and the variable nucleotides based on parsimony-informative sites for the same sample set (45 samples, Supplementary table 2). The variable nucleotides based on parsimony-informative sites were determined for the genes of the MLST systems individually and as concatenated sequences in MegaX software (Kumar *et al.*, 2018). The Simpson's ID was determined using the online tool ComparingPartitions (http://www.comparingpartitions.info/). Phylogenetic trees and MLST profiles of the 45 isolates used in this study and determined by the three MLST-based systems were also compared (Figure 13, Supplementary table 1 and 4). The diversity of the MLVA system described here was determined based on the same 38 clinical isolates by calculating the Simpson's ID with the online tool ComparingPartitions. In order to define the proportion of congruence between the MLST described here, the previously published MLST systems (Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016; Clavijo *et al.*, 2019) and the MLVA described here, the adjusted Rand coefficients were calculated by the online tool ComparingPartitions.

4.5 Challenge model

4.5.1 Experimental animals

Sixteen, four-week-old Choice hybrid piglets were transported to the animal house of the Veterinary Medical Research Institute six days prior to infection. The animals were obtained from a farm with low *M. hyorhinis* prevalence and high health status (free from: brucellosis, leptospirosis, Aujeszky's disease, porcine reproductive and respiratory syndrome, swine dysentery, atrophic rhinitis, *Actinobacillus pleuropneumoniae*, *M. hyopneumoniae*, lice and mange). The *M. hyorhinis* free status of the piglets was checked before the challenge by real-time PCR testing and culture of nasal swabs as described in chapters 4.1.1 and 4.1.2.1. DNA from the samples of the challenge model was extracted by ReliaPrep gDNA Tissue Miniprep System (Promega Inc., Madison, Wisconsin, USA) according to the manufacturers' instruction.

Upon arrival, the animals were weighed and randomly divided into three groups with similar average weights. The groups were housed in separate pens, and feed and water were provided *ad libitum.* The experiment was approved by the National Scientific Ethical Committee on Animal Experimentation under the reference number: PE/EA/746-7/2021.

4.5.2 Challenge material and challenge routes

The *M. hyorhinis* isolate (MycSu160) used during this study was isolated from the pericardium of an affected pig, collected in Hungary, 2019 (Supplementary table 1). The challenge material was prepared freshly for each challenge day by inoculating the *M. hyorhinis* isolate MycSu160 into *Mycoplasma* Liquid media (Mycoplasma Experience) 48 hours prior to challenge and incubating at 37°C. Determination of colour changing units of the inoculated broth was carried out on the day of the challenge the same way as described before (chapter 4.2).

Group IV-IV (n=6) was inoculated by intravenous (IV) route on days 0 and 1 (D0, D1) with 10 ml challenge material. Group IV-IP (n=6) was challenged IV on D0 with 10 ml challenge material and by intraperitoneal (IP) route on D1 with 20 ml challenge material. On D0 the copy number of the challenge strain was 4.6×10^5 CCU/ml, while on D1 it was 1.2×10^6 CCU/ml. The total challenge dose was 1.66×10^7 CCU/pig and 2.86×10^7 CCU/pig in Group IV-IV and IV-IP, respectively. Animals in the Control Group (n=4) received sterile liquid media in the same volume as the challenge material. The controls were inoculated by the IV route on D0. Two of these animals were inoculated by the IV route and the remaining two pigs by the IP route on D1.

4.5.3 Clinical observations

The animals were observed daily from D0 until the end of the study at D28. Clinical signs of arthritis (swollen joints, lameness) and respiratory disease (coughing or laboured breath) were recorded. Body temperatures were measured daily from D-2. Body weight measurement, blood and nasal swab sampling for PCR and isolation (chapters 4.1.1 and 4.1.2.1) were carried out twice a week. Blood were collected from the external jugular vein (*vena externa jugularis*), and transferred into two ml Eppendorf tubes. The schedule of events is summarized in Table 7. Average daily weight gain (ADWG) was calculated by subtracting the weight measured at D-6 from the weight measured at D27 and dividing it by the number of days past (n=33).

Table 7: Schedule of the events.

Time	Event
D-6	The arrival of 16 four-week-old piglets Body weight measurement Blood sampling
D-2	Collection of nasal swabs for PCR and *Mycoplasma* isolation Body temperature measurement
D-1	Body temperature measurement
D0	IV challenge of all groups
D1	IV challenge of Group IV-IV and two animals from Control Group IP challenge of Group IV-IP and two animals from Control Group
D0-D27	Daily body temperature measurement and clinical observations Twice a week body weight measurement Collection of nasal swabs for PCR and *Mycoplasma* isolation Blood sampling
D28	Euthanasia (after electrical stunning exsanguination was performed) Pathological examination Sample collection for PCR, histopathology and bacteriology

4.5.4 Gross pathological examination

Joints of the carpus, elbow, tarsus and stifle on both sides were opened and examined for signs of arthritis. The thoracic and abdominal cavities (pleura, pericardium, peritoneum) were checked for serositis. Body condition, skin, subcutaneous tissues, musculoskeletal system, eyes and conjunctiva, nasal, and oral cavity, trachea, lungs, heart, lymph nodes, gastrointestinal system, liver, spleen, kidney and brain were also checked for lesions. The scoring system of the gross pathological examination is detailed in Table 8. Lesions of joints and serosa were scored to reflect severity based on previously described criteria (Martinson *et al.*, 2018b). Total scores were calculated by summarizing all organ scores.

Table 8: Scoring system of the gross pathological examination

Organ	Score	Description
Eyes/ocular mucosa Nose/nasal cavity Oral cavity Skin Subcutaneous areas Musculoskeletal system Trachea Lungs Cardia Lymph nodes, tonsil Stomach, intestine Liver Spleen Kidney Brain	0-1	0: no lesion 1: the lesion is present
Body condition	0-2	0: Vertebrae, ribs and bony protuberances are only detectable with firm pressure. 1: Vertebrae, ribs and bony protuberances prominent. 2: Vertebrae, ribs and bony protuberances are very prominent
Joints	0-2	0: no lesion 1: mild serous arthritis with mild joint swelling 2: severe fibrinopurulent arthritis with pronounced joint swelling
Thoracic cavity Pericardial cavity Peritoneal cavity	0-2	0: no lesion 1: serofibrinous serositis in a circumscribed area 2: diffuse/multifocal serofibrinous serositis

Swab samples for bacterial culture, *M. hyorhinis* isolation and *M. hyorhinis* specific PCR (chapters 4.1.1 and 4.1.2.1) were taken from the conjunctiva, lung, serosa, the four examined joints and the brain. Joints on both sides were sampled with the same swab. Necropsy samples were also tested for the presence of *M. hyopneumoniae* and *M. hyosynoviae* (chapter 4.1.2.2). *M. hyorhinis* positive re-isolates (Supplementary table 1 and 2) were genetically characterized by MLST and MLVA (chapters 4.4.1 and 4.4.2).

4.5.5 Bacteriology

The presence of bacterial pathogens other than *Mycoplasma* sp. was tested by culturing the necropsy samples on Columbia sheep blood agar (Biolab Inc., Budapest, Hungary) and sheep blood agar supplemented with nicotinamide adenine dinucleotide (Merck) at the final concentration of 20 µg/ml. The agar plates were incubated in the presence of 5% CO_2 at 37°C for 48 hours.

4.5.6 Histological examination

Samples for histopathology were collected from the conjunctiva, choana, tonsilla, trachea, lungs (7 lobes), pericardium, heart, mediastinal and mesenteric lymph nodes, liver, spleen, kidney, joints and brain (cerebrum, cerebellum, brain stem). Tissue samples were fixed in 10% neutral, buffered formaldehyde for 24 hours at 4°C. After trimming, tissue samples were dehydrated in xylene and in a dilution series of ethanol, and embedded in paraffin. Four µm thick sections were cut, mounted on glass slides, and stained with haematoxylin and eosin (H&E). The slides were examined by brightfield microscope at 40× to 400× magnification. Scoring system of the histopathological lesions are detailed in Table 9 and illustrated in Figures 6-9. Total scores were calculated by summarizing all organ scores.

Table 9: Histopathological score system

Organs	Score 0	Score 1	Score 2	Score 3
Joint	Synoviocytes are normal, no inflammation	Synoviocytes are normal, mild infiltration of mononuclear cells (lymphocytes, plasmacells, macrophages) in the subintima	Hyperplasia of synoviocytes is present, mild to moderate infiltration of mononuclear cells (lymphocytes, plasmacells, macrophages) and scant neutrophyl granulocytes in the subintima, few perivascular lymphoid follicle, leucocytic extravasation	Hyperplasia of synoviocytes is present, moderate to severe infiltration of mononuclear cells (lymphocytes, plasmacells, macrophages) and moderate infiltration of neutrophyl granulocytes in the subintima, several perivascular lymphoid follicle, leucocytic extravasation
Lung	Pleura normal, no inflammation	Scant filamentous projections consisting of connective tissue	Moderate number of filamentous projections consisting of connective tissue, and multifocal, moderate thickening of the pleura by proliferating connective tissue	Large number of filamentous projections consisting of connective tissue, and multifocal, moderate thickening of the pleura by proliferating connective tissue
Spleen, liver	Peritoneum normal, no inflammation	Scant filamentous projections consisting of connective tissue	Moderate number of filamentous projections consisting of connective tissue	Large number of filamentous projections consisting of connective tissue
Pericardium	Pericardium normal, no inflammation	Scant filamentous projections consisting of connective tissue	Moderate number of filamentous projections consisting of connective tissue, and moderate thickening of the pericardium/epicardium by proliferating connective tissue	Large number of filamentous projections consisting of connective tissue, and pronounced thickening of the pericardium/epicardium by proliferating connective tissue
Other organs	No lesions	Mild inflammation	Moderate inflammation	Severe inflammation

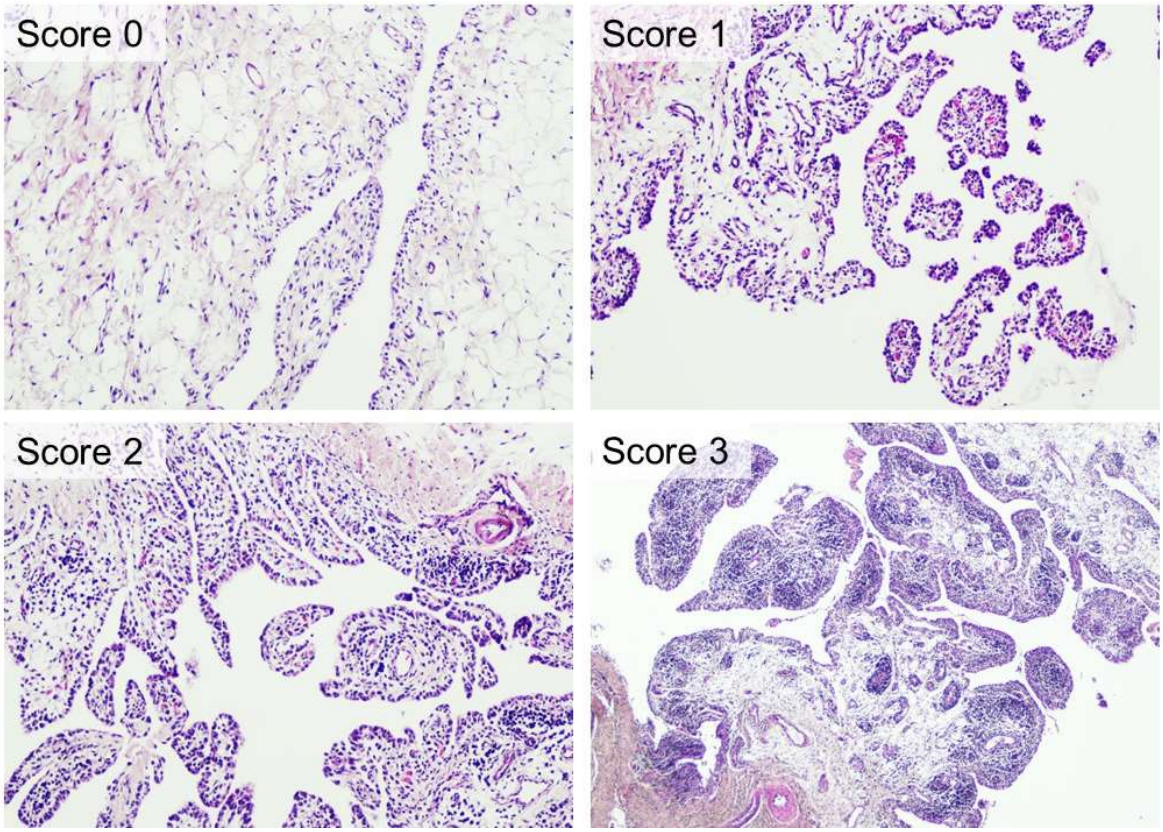

Figure 6: Representations of histopathological scores in the joints.
Scoring criteria is detailed in Table 9. Score 0-2: 100×; Score 3: 40×; H&E stained.

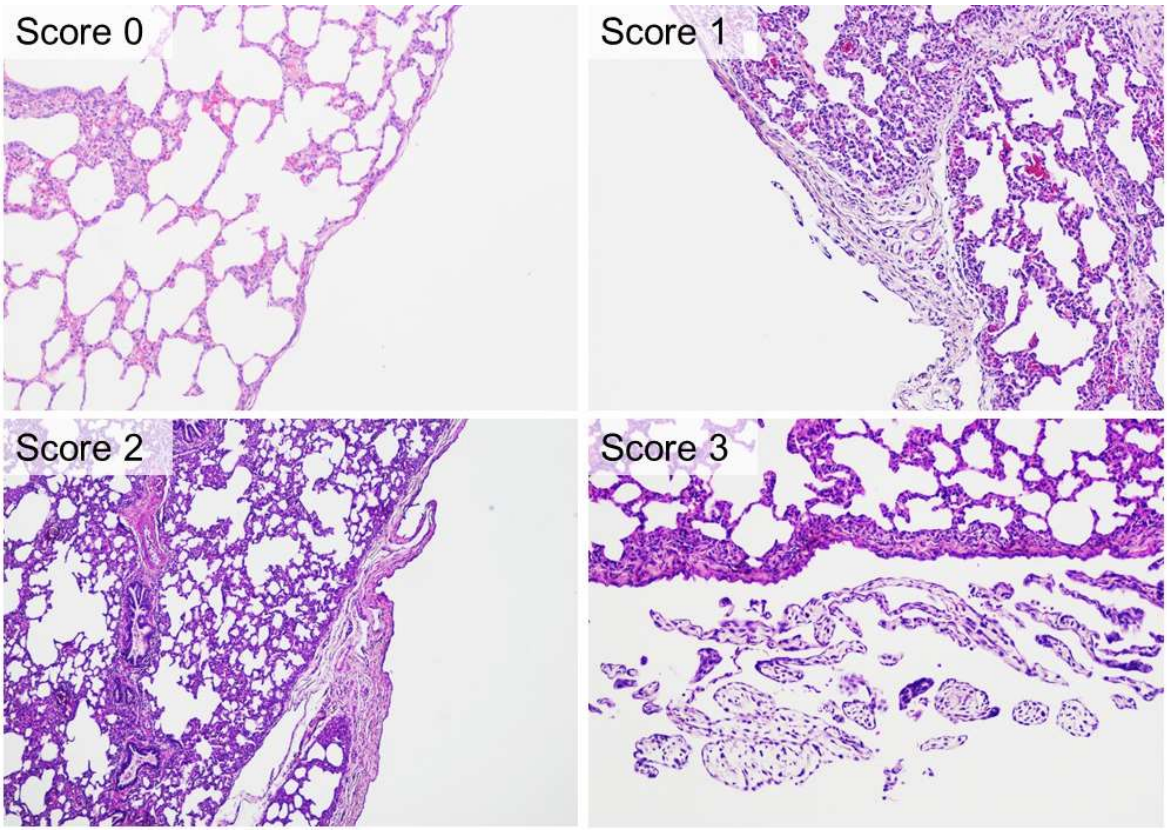

Figure 7: Representations of histopathological scores of the pleura.
Scoring criteria is detailed in Table 9. Score 0-1;3: 100×; Score 2: 40×; H&E stained.

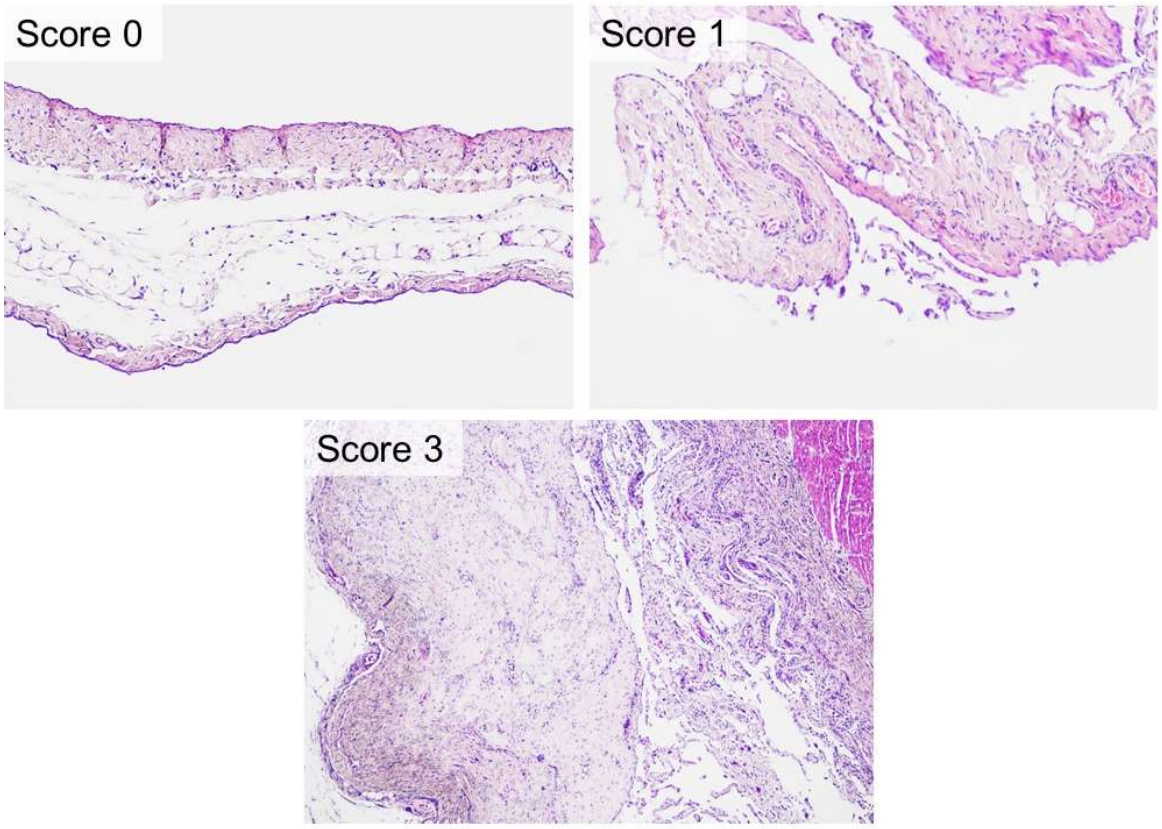

Figure 8: Representations of histopathological scores in the pericardium.
Scoring criteria is detailed in Table 9. Score 0-1: 100×; Score 3: 40×; H&E stained.

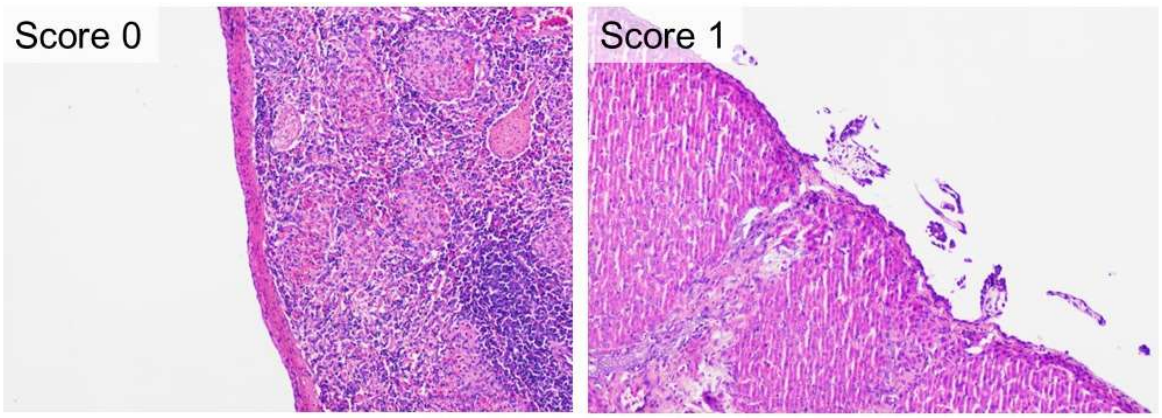

Figure 9: Representations of histopathological scores of the peritoneum (spleen, liver).
Scoring criteria is detailed in Table 9. Score 0-1: 100×; H&E stained.

4.5.7 Serology

Sera were tested in duplicates by an in-house ELISA, using an antigen prepared according to the sarcosyl assay previously described for *M. gallisepticum* (Stipkovits *et al.*, 1993). Briefly, to prepare the antigen, six clinical isolates of *M. hyorhinis* were propagated (Supplementary table 1 and 2). After colour change, the isolates were mixed, washed and treated with 0.5%

sarcosyl. The protein content of the antigen was determined with Coomassie (Bradford) Protein assay kit (Thermo Fisher Scientific) according to the manufacturers' instructions.

96-well ELISA plates were coated with the antigen diluted to the concentration of 1.25 µg/ml in phosphate buffered saline (PBS, pH 7.4). After blocking with 1% gelatine from cold water fish skin (Merck) each well was incubated with a serum sample diluted to 1:100 in PBS, followed by a horseradish peroxidase conjugated rabbit anti-swine immunoglobulin (Dako A/S, Glostrup, Denmark) diluted to 0.125 µg/ml in PBS. The reaction was visualized with tetramethylbenzidine (TMB, Diavet Ltd., Budapest, Hungary) substrate and the optical density (OD) of the solution was measured at 450 nm using a Multiscan FC reader (Thermo Fisher Scientific).

Blood samples were centrifuged after collection and the sera were kept at -70°C. Each serum sample was thawed only once. Each plate contained a negative control (mix of the sera of all Control animals taken at D28 from this study) a positive control (mix of the sera of all animals in Group IV-IV taken at D28 from this study) and a background control, where PBS was measured instead of the serum sample. The mean OD value of the background control was subtracted from the mean OD values of the samples and the controls (Terato *et al.*, 2016). The assay was considered valid if the negative to positive ratio of corrected OD values was under 40%. The sample to positive ratios (S/P%) were calculated and the sample was considered positive when S/P%>40% (Merodio *et al.*, 2021).

4.5.8 Statistical analysis

Statistical analyses were accomplished through the R programming language (R Core Team, 2021). To compare the effect of the different challenge routes statistical analysis of the ADWG, pathological scores (separately for the joints, serosa of the pericardium, pleura and peritoneum and summary of scores), histopathological scores (separately for the joints, serosa of the pericardium, pleura and peritoneum and summary of scores) and ELISA results from the last sampling were performed. In the case of the pathological and histopathological scores, first a Kruskal-Wallis non-parametric ANOVA test was carried out to determine whether the difference among the medians of the three study groups are statistically significant or not. If the results of the Kruskal-Wallis test were significant, a Dunn's test was performed to determine exactly which groups are different by making pairwise comparisons between each group. Since multiple groups were considered at the same time, p-values were adjusted for multiple comparisons by the Bonferroni method. In the case of the ADWG and the ELISA results, instead of the non-parametric test, first the normal distribution of the data was tested by the Shapiro-Wilk normality test, then a one-way ANOVA followed by Tukey multiple comparisons of means was performed.

5. Results

5.1 Antibiotic susceptibility testing of recent isolates

During the Pan-European antibiotic susceptibility study 76 *M. hyorhinis* isolates (two Belgian, 15 German, 20 Hungarian, 20 Italian and 19 Polish; Supplementary table 1) were tested for ten antimicrobials (doxycycline, oxytetracycline, tiamulin, enrofloxacin, florfenicol, tylosin, tylvalosin, tulathromycin, tilmicosin, lincomycin). Regardless of origin, two susceptibility patterns were detected. A bimodal MIC distribution for macrolides and lincomycin and a unimodal distribution for the other tested antimicrobials (Figure 10). The tested isolates were inhibited by low concentrations of tiamulin (MIC_{90} 0.312 µg/ml), doxycycline (MIC_{90} 0.078 µg/ml), oxytetracycline (MIC_{90} 0.25 µg/ml) and florfenicol (MIC_{90} 0.2 µg/ml). Moderate concentrations of enrofloxacin (MIC_{90} 1.25 µg/ml) inhibited the growth of the isolates except for one Hungarian isolate (MycSu230) where only the highest tested concentration (10 µg/ml) inhibited the growth. High MICs in the range of 32->64 µg/ml except for tylvalosin (5->10 µg/ml) were detected for the tested macrolides and lincomycin in 42-56% of the cases (32-43/76 isolates) in the Pan-European study.

Slight differences were observed when the number of isolates with high MICs for macrolides and lincomycin from different countries [Germany, Hungary, Italy and Poland (omitting Belgium due to the low number of isolates)] were compared. In the case of Hungary and Poland, less than half of the tested isolates (45% and 40% respectively) showed decreased susceptibility, while in Germany and Italy, the number of isolates with low susceptibility was higher, 73% and 65%, respectively. MIC ranges, MIC_{50} and MIC_{90} values of the 76 isolates tested in the Pan-European study are summarized in Table 10 separately by country of origin and for all tested isolates. All MIC values determined in the presented study can be found in Supplementary table 3.

MIC values of *M. hyorhinis* isolates from Europe over the course of approximately 35 years were compared. For the comparison studies using a similar method for antimicrobial testing [i.e using the guidelines of Hannan (2000)] and revealing MIC values of the type strain in the same range as in the present study were included. The detected MIC values are summarised in Table 11.

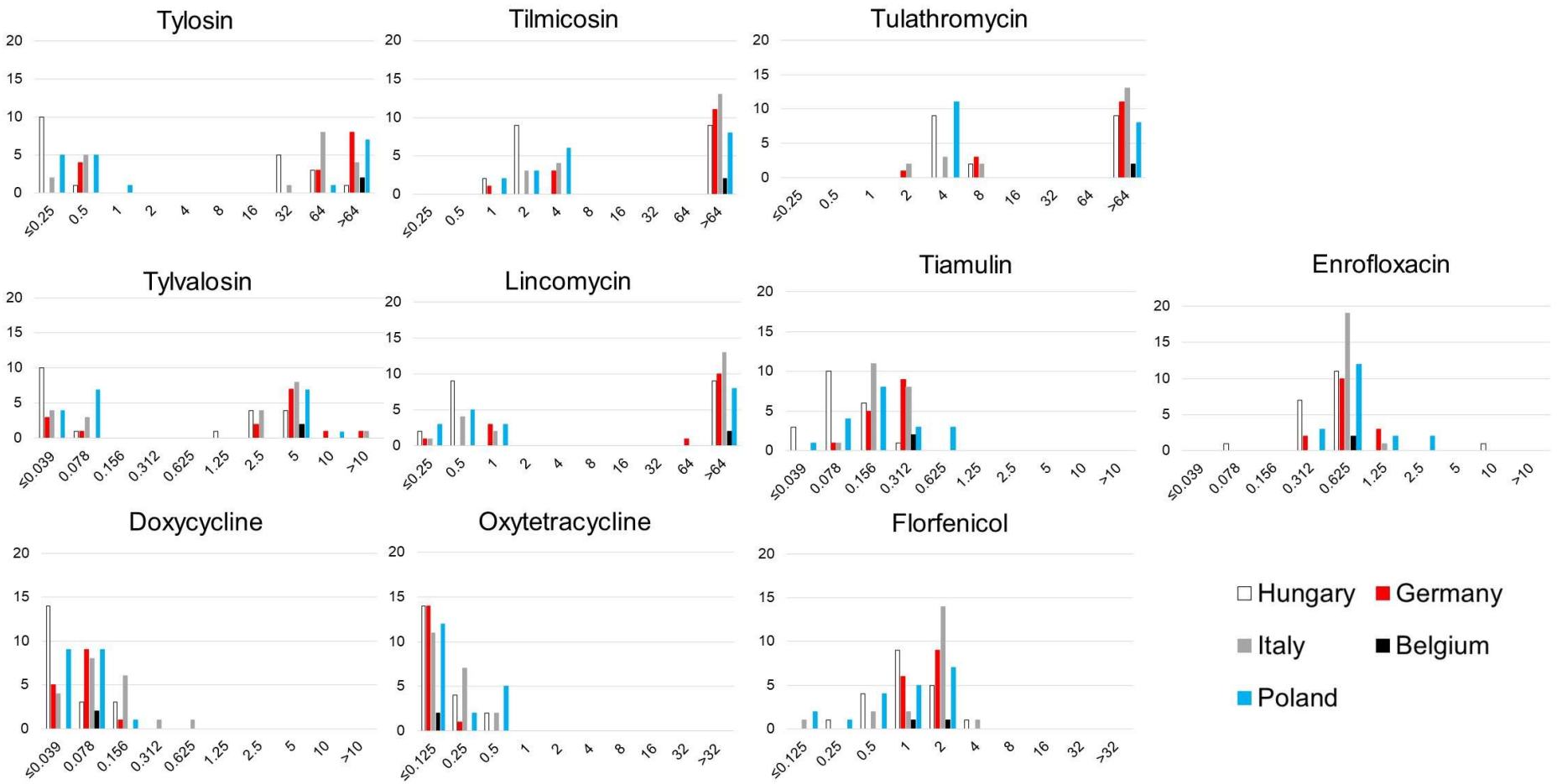

Figure 10: Minimal inhibitory concentration distribution of the 76 tested isolates by country of origin.
X-axis: concentration of the antimicrobials (µg/ml), Y-axis: number of isolates

Table 10: Minimal inhibitory concentration (MIC) values of ten antimicrobial agents.

Country of origin	MIC parameter	Tia	Dox	Oxy	Flo	Enr	Tyl	Til	Tyv	Tul	Lin
Belgium (2 isolates)	MIC	0.312	0.078	≤0.125	1 – 2	0.625	>64	>64	5	>64	>64
Germany (15 isolates)	MIC Range	0.078 – 0.312	≤0.039 – 0.156	≤0.125 – 0.25	1 – 2	0.312 – 1.25	0.5 – >64	1 – >64	≤0.039 – >10	2 – >64	≤0.25 – >64
	MIC_{50}	0.312	0.078	≤0.125	2	0.625	>64	>64	5	>64	>64
	MIC_{90}	0.312	0.078	≤0.125	2	1.25	>64	>64	10	>64	>64
Hungary (20 isolates)	MIC Range	≤0.039 – 0.312	≤0.039 – 0.156	≤0.125 – 0.5	0.25 – 4	0.078 – 10	≤0.25 – >64	1 – >64	≤0.039 – 5	4 – >64	≤0.25 – >64
	MIC_{50}	0.078	≤0.039	≤0.125	1	0.625	≤0.25	2	≤0.039	8	0.5
	MIC_{90}	0.156	0.156	0.25	2	0.625	64	>64	5	>64	>64
Italy (20 isolates)	MIC Range	0.078 – 0.312	≤0.039 – 0.625	≤0.125 – 0.5	≤0.125 – 4	0.625 – 1.25	≤0.25 – >64	2 – >64	≤0.039 – >10	2 – >64	≤0.25 – >64
	MIC_{50}	0.156	0.078	≤0.125	2	0.625	64	>64	2.5	>64	>64
	MIC_{90}	0.312	0.156	0.25	2	0.625	>64	>64	5	>64	>64
Poland (19 isolates)	MIC Range	≤0.039 – 0.625	≤0.039 – 0.156	≤0.125 – 0.5	≤0.125 – 2	0.312 – 2.5	≤0.25 – >64	1 – >64	≤0.039 – 10	4 – >64	≤0.25 – >64
	MIC_{50}	0.156	≤0.039	≤0.125	1	0.625	0.5	4	0.078	4	1
	MIC_{90}	0.625	0.078	0.5	2	1.25	>64	>64	5	>64	>64
All 76 isolates	MIC Range	≤0.039 – 0.625	≤0.039 – 0.625	≤0.125 – 0.5	≤0.125 – 4	≤0.039 – 10	≤0.25 – >64	1 – >64	≤0.039 – >10	2 – >64	≤0.25 – >64
	MIC_{50}	0.156	0.078	≤0.125	1	0.625	32	>64	2.5	>64	>64
	MIC_{90}	**0.312**	**0.078**	**0.25**	**2**	**1.25**	**>64**	**>64**	**5**	**>64**	**>64**

Abbreviations: Tia: tiamulin, Dox: doxycycline, Oxy: oxytetracycline, Flo: florfenicol, Enr: enrofloxacin, Tyl: tylosin, Til: tilmicosin, Tyv: tylvalosin, Tul: tulathromycin, Lin: lincomycin; MIC_{90} value of each tested antibiotic is highlighted in bold, MIC values over the here defined breakpoint for macrolides and lincomycin are highlighted in grey.

Table 11: Comparison of minimal inhibitory concentrations (MIC) of antimicrobial agents over the years.

	The Netherlands,1984-1989 (n=20)[a]			USA, Japan and Europe, before 1997 (n=20)[b]			Hungary, 2014-2017 (n=38)[c]			Belgium, Germany, Hungary, Italy, Poland 2019-2021 (n=76)[d]		
	MIC_{50}	MIC_{90}	Type strain MIC	MIC_{50}	MIC_{90}	Type strain MIC	MIC_{50}	MIC_{90}	Type strain MIC	MIC_{50}	MIC_{90}	Type strain MIC
Tia	0.06	0.12	≤0.03	0.1	0.25	0.05	0.156	0.312	0.078	0.156	0.312	0.078
Dox	≤0.03	0.12	≤0.03				0.078	0.312	≤0.039	0.078	0.078	≤0.039
Oxy	0.12	0.25	0.12	0.25	2.5	0.05	≤0.25	1	≤0.25	≤0.125	0.25	≤0.125
Flo							2	2	1	1	2	2
Enr				0.5	1	0.5	0.625	0.625	0.625	0.625	1.25	0.625
Tyl	0.12	0.25	0.06	0.5	2.5	0.5	0.5	>64	≤0.25	32	>64	≤0.25
Til							2	>64	1	>64	>64	2
Tyv							≤0.25	8	≤0.25*	2.5	5	≤0.039*
Tul							4	>64	2	>64	>64	4
Lin	0.5	1	0.5				0.5	>64	0.5	>64	>64	0.5

Abbreviations: Tia: tiamulin, Dox: doxycycline, Oxy: oxytetracycline, Flo: florfenicol, Enr: enrofloxacin, Tyl: tylosin, Til: tilmicosin, Tyv: tylvalosin, Tul: tulathromycin, Lin: lincomycin.

[a](Ter Laak *et al.*, 1991); [b](Hannan *et al.*, 1997); [c](Bekő *et al.*, 2019a); [d]this study; *minimal tested concentration changed due to changing the concentration range for tylvalosin

5.2 Mismatch amplification mutation assay

The MAMA was developed based on 38 isolates with available whole genome sequences and MIC data (tylosin, tilmicosin, tylvalosin, tulathromycin, gamithromycin and lincomycin; Bekő *et al.*, 2019), and further 85 isolates and 15 DNA samples were used for validation (Supplementary table 2). Macrolide and lincomycin susceptibility were decreased in 43% of these isolates (61/123). Typically, the same isolates showed lower susceptibility to all of these agents. In case of the few exceptions (Supplementary table 3, highlighted black), the isolates showed similar susceptibility to lincomycin and all but one macrolides: MycSu94 was highly susceptible to lincomycin and all macrolides except for tilmicosin (MIC 16 µg/ml, the here defined breakpoint), while MycSu128 showed decreased susceptibility to lincomycin and all macrolides except for gamithromycin (MIC 1 µg/ml). Eleven isolates showed decreased susceptibility to lincomycin and all macrolides except for tylvalosin (MIC 1.25-2.5 µg/ml). All MIC values for macrolides and lincomycin determined and/or analysed in the present study can be found in Supplementary table 3. To determine resistance related point mutations two 50S ribosomal proteins (L4, L22) and the 23S rRNA gene were analysed. While no SNP was detected on the ribosomal proteins, one SNP at nucleotide position A2066G (A2058G according to *E. coli* numbering), in the 23S rRNA gene was found to be related to decreased susceptibility to all tested macrolides and lincomycin. Both genotype L and H were successfully distinguished with the developed MAMA (Figure 11). The sensitivity of the assay was 10^3 copy numbers/reaction for both genotypes in the melt and agarose assays as well. No cross reactions were detected in the agarose-MAMA, however, *M. flocculare* showed a genotype L specific melting temperature in the melt-MAMA.

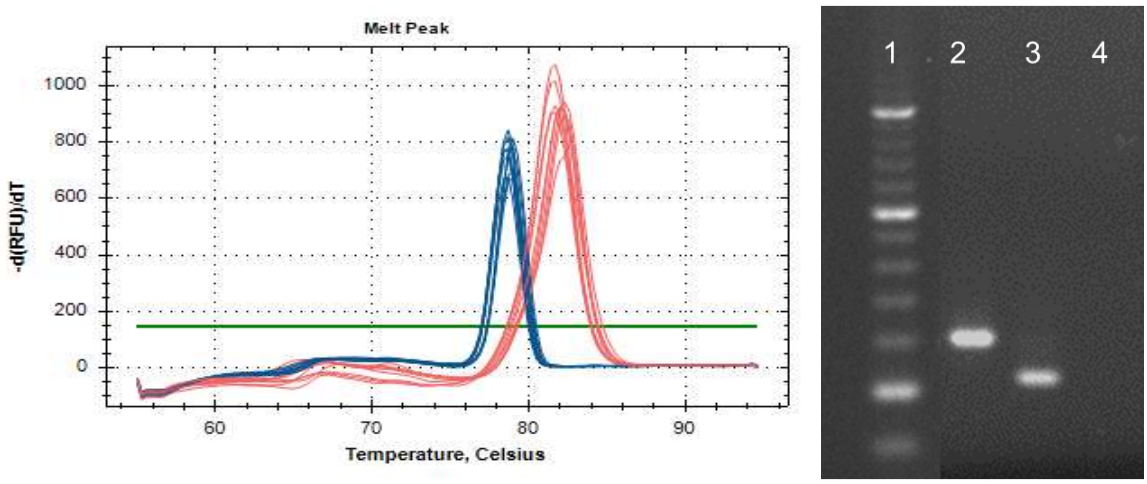

Figure 11: Results of the melt- (left) and agarose-MAMA (right).
Melting temperatures and sizes of the amplicons for genotype H (red line, 81.6°C, column 2, 139 bp) and genotype L (blue line, 78.6°C, column 3, 119 bp) were specific. Negative control (column 4) was not amplified. For the melting peak: X-axis: melting temperature (°C), Y-axis: a negative value of the change in relative fluorescence units (RFU) over the change in temperature. In the agarose gel column 1 is the DNA ladder.

In the case of clinical isolates where both agarose- and melt-MAMAs were conducted and both tests gave the same results. The results of the molecular assays were in line with the ones of the broth microdilution method for the majority of the tested macrolides. False reactions were detected in three cases (Ge-13, Po-4 and Po-9), where genotype L was detected in isolates which showed high MIC values with the broth microdilution method (Supplementary table 3). Partial sequencing of the 23S rRNA gene of these isolates revealed an adenine in the Polish isolates (Po-4 and Po-9) and thymine in Ge-13 instead of the expected guanine at position 2066. Results of the different methods were also incongruent in isolates with mixed susceptibility profiles to the tested macrolides (uniform susceptibility to all but one agents). Genotype H was detected in the isolates which showed high MICs for all macrolides except for gamithromycin and tylvalosin, while genotype L was observed in the isolate (MycSu94) which showed low MICs for all macrolides except for tilmicosin.

In the case of the clinical specimens, the test of one DNA sample (Su21) showed a mixed genotype by the agarose-MAMA and genotype L by the melt-MAMA, while genotype H was detected in the corresponding clinical isolate (MycSu101). Accordingly, at nucleotide position 2066 in the whole genome sequence of this isolate a guanidine was found (genotype H). The broth microdilution test showed elevated MIC values of the tested antibiotics against MycSu101.

5.3 Genotyping assays

5.3.1 Development of multi-locus sequence typing and multiple-locus variable-number tandem repeat analysis

Six gene fragments were selected for MLST development from the analysed 17 genes. These are the leader peptidase A (*lepA*), the DNA-directed RNA polymerase β subunit (*rpoB*), the DNA-directed RNA polymerase β' subunit (*rpoC*), the glutamyl-tRNA synthetase (*gltX*), the UvrABC system protein A (*uvrA*) and the valine-tRNA ligase (*valS*). The gene fragments of *rpoB* and *gltX* (genome positions are in Table 12) differed from those targeted in the previous MLST schemes where the gene fragments were positioned between 71,195-71,690 for *rpoB* and 418,819-419,188 for *gltX* (Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016). Gene fragment sequences of the here described MLST are available in the GenBank database (*lepA*: MT090880-MT090918, *rpoB*: MT090841-MT090879, *rpoC*: MT090802-MT090840, *gltX*: MT090919-MT090957, *uvrA*: MT090763-MT090801, *valS*: MT106126-MT106164). The sensitivity of the assays was between 10^5-10^2 copy numbers/reaction as detailed in Table 12. Cross-reaction was only detected in the case of *valS*, where *M. hyopneumoniae* gave a false positive result.

Table 12: Genome positions and sensitivity of gene fragments used for the developed multi-locus sequence typing.

Gene	Genome position in *Mycoplasma hyorhinis* type strain (GenBank: LS991950.1)	Sensitivity of the assay (copies/reaction)
gltX	417,808 – 418,217	10^3
lepA	92,731 – 93,174	10^3
rpoB	71,638 – 72,108	10^5
rpoC	75,490 – 76,054	10^3
uvrA	546,373 – 546,889	10^4
valS	9,426 – 10,081	10^2

For the development of the MLVA a total of 75 tandem repeats (TR) were found in the seven analysed *M. hyorhinis* whole genome sequences, out of which 23 TRs fulfilled the criteria for MLVA development. After preliminary amplifications, the following six alleles were selected: Mhr205, Mhr396, Mhr438, Mhr441, Mhr442 and Mhr444. Positions of these alleles in the type strain are listed in Table 13. The sensitivity of the MLVA PCRs was 10^3 copies/reaction and showed no cross-reaction with the tested *Mycoplasma* species.

Table 13: Genome positions of the alleles used in the multiple-locus variable-number tandem-repeat analysis.

Allele	Position in *M. hyorhinis* type strain (NCTC 10130)
Mhr205	205,528 – 205,320
Mhr396	396,104 – 396,142
Mhr438	438,283 – 438,867
Mhr441	441,069 – 441,914
Mhr442	442,537 – 442,591
Mhr444	444,088 – 444,479

5.3.2 Validation of the developed genotyping assays

The developed MLST and MLVA were validated using the same 41 isolates, in addition the six strains with publicly available whole genome sequences were also included in the MLST analysis (Supplementary table 1, 2 and 4). For the MLST analysis the seqences of the six gene fragments were concatenated (3,063 bp in total) and a phylogenetic tree was assessed (Figure 12). The analysed *M. hyorhinis* strains represented 38 sequence types (ST). The number of allele types by genes and the number of STs for the concatenated sequences are listed in Table 14. Allele types and STs of each isolate are listed in Supplementary table 4.

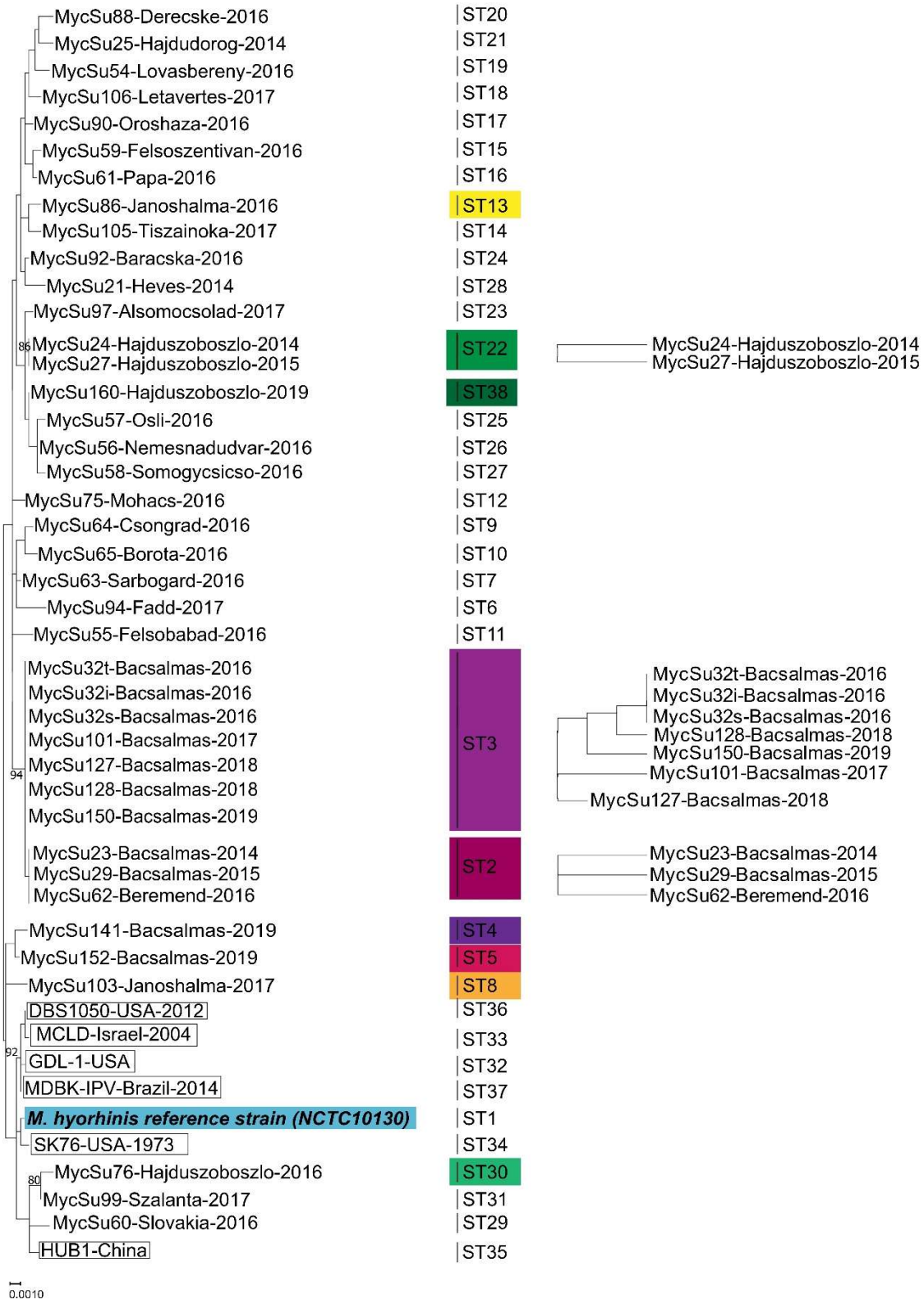

Figure 12: Molecular typing of *Mycoplasma hyorhinis* isolates by multi-locus sequence typing (left) and resolution of the identical sequence types by multiple-locus variable-number tandem-repeat analysis (right).

The MLST tree was constructed using the Maximum Likelihood method using gene fragments of *lepA*, *rpoB*, *rpoC*, *gltX*, *uvrA* and *valS* with 1000 bootstraps (only bootstraps>70% are presented). The resolution by MLVA is based on the alleles: Mhr205, Mhr396, Mhr438, Mhr441, Mhr442 and Mhr 444, the trees were constructed using the Neighbour-Joining method. The *M. hyorhinis* type strain is highlighted blue, and the strains with available whole genome sequences in the GenBank are framed. STs of the isolates from the same farm are highlighted with the shades of the same colour. Abbreviations: ST-sequence type, t-tonsil, s-serosa, i-synovial fluid.

Table 14: Parsimony informative sites (number and percent), number of allele or sequence types and Simpson index of diversity of the developed multi-locus sequence typing method.

Gene	Parsim-info	Parsim%	Allele type	Simpson's ID	CI (95%)
gltX	6/410	1.46	12	0.737	0.613-0.861
lepA	6/444	1.35	11	0.876	0.845-0.907
rpoB	7/470	1.48	15	0.685	0.534-0.836
rpoC	8/565	1.41	15	0.860	0.784-0.935
uvrA	6/517	1.16	12	0.705	0.578-0.832
valS	5/657	0.76	10	0.795	0.723-0.867
concatenated	38/3063	1.24	38	0.986	0.969-1.000

Parsim-info: number of parsimony informative sites, Parsim%: rate of parsimony informative sites, Allele type: number of alleles for the gene fragments and the concatenated sequence (sequence type); Simpson's ID: index of diversity, CI: 95% confidence interval.

Isolates collected from the same farm but in different years were examined through three examples: Bácsalmás, Jánoshalma and Hajdúszoboszló. In the case of Bácsalmás 11 isolates between 2014 and 2019 were collected and analysed, while two isolates were analysed from Jánoshalma from 2016 and 2017 and four isolates were evaluated from Hajdúszoboszló between 2014 and 2019. Most of the isolates from Bácsalmás formed a sub-clade, representing two STs, ST2 and ST3. However, the latest two isolates (MycSu141, MycSu152 from herd A) formed a different branch representing ST4 and ST5 (STs highlighted purple in Figure 12). The number of different nucleotides between STs are listed in Table 15 (highlighted purple).

Table 15: Number of different nucleotides between sequence types (ST) within farms.

Origin	Bácsalmás				Jánoshalma		Hajdúszoboszló		
ST	ST2	ST3	ST4	ST5	ST8	ST13	ST22	ST30	ST38
ST2									
ST3	1(1)								
ST4	10(5)	9(5)							
ST5	6(3)	5(3)	4(2)						
ST8									
ST13					12(6)				
ST22									
ST30							8(4)		
ST38							2(2)	8(4)	

Background information of the sequence types and corresponding isolates are detailed in Supplementary table 4. The number of genes on which the polymorphisms were detected are given in parenthesis.

From Hajdúszoboszló two isolates (MycSu24 and MycSu27) represented the same ST (ST22) and the other two (MycSu76 and MycSu160 isolated in 2016 and 2019) represented two other STs, ST30 and ST38 respectively (green highlights on Figure 12) the number of differences between STs are listed in Table 15. The two isolates from Jánoshalma (MycSu86, MycSu103; yellow highlight on Figure 12) represented two distinct STs, ST13 and ST8, respectively and SNPs (12 in total) were found in every studied gene fragments (Table 15). No further correlations were identified between integration, year of isolation, sample type and ST. The three isolates originating from different tissue types (i-synovial fluid, t-tonsil, s-serosa) of the same animal (MycSu32) shared the same ST (ST3; Figure 12).

By the developed MLVA the tested isolates represented 38 genotypes with Simpson's index of diversity of 0.999 (CI 0.986-1.000). Based on the analysis of 41 isolates three alleles (Mhr438, Mhr441, Mhr444) are highly diverse; the number of polymorphisms in these alleles ranged between 14 and 16. The other three alleles (Mhr205, Mhr396, Mhr442) are less diverse with three different numbers of repeat units in the examined isolates. The repeat numbers in each allele and genotypes of each isolate are listed in Supplementary table 4. No correlation between the sample source or integration and the genotype was found with the MLVA method. Based on the developed MLVA two isolates from Bácsalmás showed identical genotype (Supplementary figure 1), however, by combining the developed MLST and MLVA methods, all isolates were discriminated except for the isolates from different tissues of the same animal (MycSu32i, MycSu32t, MycSu32s; Figure 12).

5.3.3 Comparison of the developed and previous genotyping assays

Comparison of the two previously published (MLSTp, Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016; and MLSTs, Clavijo *et al.*, 2019) and the here developed MLST (MLSTn) schemes was based on the analyses of the same 45 isolates (Supplementary table 2 and 4), which were used during the validation of MLSTn, except for MycSu32i and MycSu32s (Figure 13). The isolates showed high diversity with both MLSTp (31 STs) and MLSTs (32 STs). Several novel allele types were identified in both systems. In details, with MLSTp one new allele type on alleles *rpoB*, *gyrB* and *gmk*, three new types on allele *gltX* and four new types in allele *adk* were described, while 25 novel STs were found among the examined isolates (Supplementary table 4, Supplementary figure 2). The new allele types, STs and isolate data are available in the PubMLST database (https://publmst.org/mhyorhinis/). In the case of the MLSTs assay five new allele types on *pdhB*, three on *p95* and 14 on *mtlD* were identified, and our isolates represented 28 novel STs (Supplementary table 4, Supplementary figure 3). Sequences of the gene fragments from the MLSTs scheme are available in the GenBank database (*mtlD*: MT569073-MT569072; *p95*: MT568999-MT569035; *pdhB*: MT569073-MT569109; *ung*:

MT569110-MT569146). No correlations were found between background information and STs of the MLSTp and MLSTs systems examining all the isolates from the public databases and from this study (Supplementary figure 2 and 3).

To compare the three MLST systems, parsimony informative sites and Simpson's diversity of the gene fragments separately and of the concatenated sequences were calculated (Table 16). The rate of parsimony informative sites for the concatenated sequences was the highest for the MLSTs system (8.12%) mainly due to the high variance on the surface protein coding gene *mtlD* (20.30%). The Simpson's diversity indexes of the concatenated sequences were the highest for MLSTn (0.986). By using different gene fragments from *gltX* and *rpoB* in the MLSTn and MLSTp systems the rate of parsimony informative sites increased from 0.81% and 0.40% (MLSTp) to 1.46% and 1.48% (MLSTn), respectively based on the same sample set (Suppelementary table 5).

Table 16: Parsimony informative sites (number and percent), number of allele or sequence types and Simpson index of diversity of the three compared multi-locus sequence typing (MLST) methods.

MLST system	Parsim-info	Parsim%	Allele type	Simpson's ID	CI (95%)
MLSTn	38/3063	1.24	38	0.986	0.969-1.000
MLSTp	15/2304	0.65	31	0.976	0.958-0.994
MLSTs	117/1441	8.12	32	0.964	0.928-1.000

Parsim-info: number of parsimony informative sites, Parsim%: rate of parsimony informative sites, Allele type: number of alleles for the gene fragments and the concatenated sequence (sequence type); Simpson's ID: index of diversity, CI: 95% confidence interval.

The robustness of the constructed phylogenetic trees was compared based on the number of branches with bootstrap values of over 70%. On the phylogenetic tree of MLSTn system, five branches had bootstraps over 70%, while in the case of MLSTp there were none and for MLSTs the number of these branches was 12 (Figure 13).

To compare the results of the phylogenetic analysis, isolates that were grouped together by one of the MLST schemes or originated from the same farm were analysed. The number of differing nucleotides for MLSTp and MLSTs systmes between isolates from the same farm are summarized in Table 17 and Table 18 respectively.

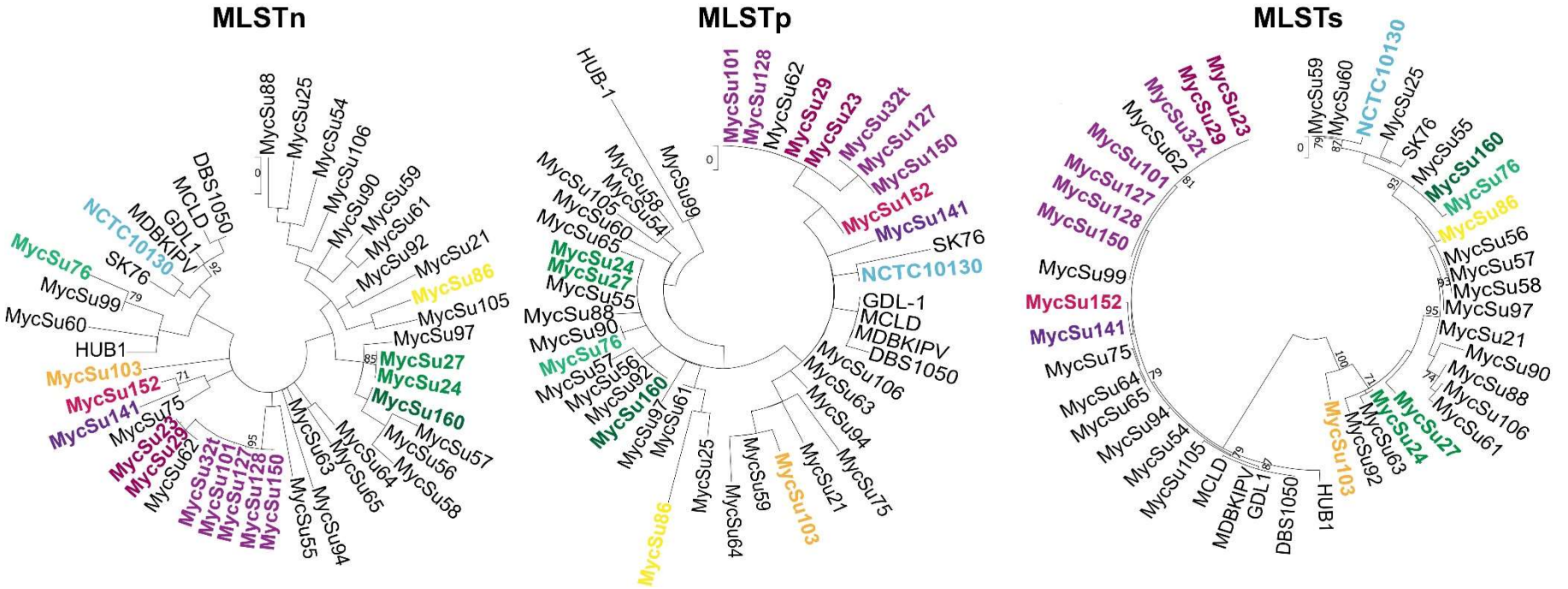

Figure 13: Molecular typing of *Mycoplasma hyorhinis* isolates from this study by previously published multi-locus sequence typing (MLST) based methods.

MLSTn: according to the here developed MLST scheme, **MLSTp:** according to Tocqueville *et al.* (2014) and Trüeb *et al.* (2016). **MLSTs:** MLSTc according to Clavijo *et al.* (2019). The phylogenetic trees were constructed by the Maximum Likelihood method with 1000 bootstraps (only bootstrap values >70% are presented). *M. hyorhinis* type strain is highlighted blue, the *M. hyorhinis* isolates from the same farm are coloured according to their sequence types in the MLSTn scheme (Figure 12).

Table 17: Number of different nucleotides between sequence types (ST) within farms in the MLSTp scheme.

Origin	Bácsalmás				Jánoshalma		Hajdúszoboszló		
ST	ST105	ST107	ST114	ST115	ST124	ST125	ST40	ST88	ST108
ST105									
ST107	1(1)								
ST114	2(2)	3(3)							
ST115	1(1)	3(2)	3(2)						
ST124									
ST125					6(4)				
ST40									
ST88							1(1)		
ST108							1(1)	1(1)	

MLSTp (Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016). Background information of the sequence types and corresponding isolates are detailed in Supplementary table 4. The number of genes on which the polymorphisms were detected are given in parenthesis.

Table 18: Number of different nucleotides between sequence types (ST) within farms in the MLSTs scheme.

Origin	Bácsalmás			Jánoshalma		Hajdúszoboszló			
ST	ST14	ST41	ST49	ST57	ST62	ST52	ST53	ST64	ST68
ST14									
ST41	1(1)								
ST49	4(2)	3(1)							
ST57									
ST62				31(3)					
ST52									
ST53						14(1)			
ST64						34(2)	30(2)		
ST68						31(1)	27(1)	3(1)	

MLSTs (Clavijo *et al.*, 2019). Background information of the sequence types and corresponding isolates are detailed in Supplementary table 4. The number of genes on which the polymorphisms were detected are given in parenthesis.

The isolates from Bácsalmás represented four STs in the MLSTp scheme similar to MLSTn (purple highlights on Figure 13). The two latest isolates from herd A represented ST114 (MycSu141) and ST115 (MycSu152), while isolates from the previous years formed ST105 (MycSu23, MycSu29, MycSu101, MycSu128) and ST107 (MycSu32t, MycSu127, MycSu150). The number of differing nucleotides are detailed in Table 17. In the case of the MLSTs system, these isolates formed three STs, all isolates except for the two latest formed ST49, while MycSu141 were characterized as ST14 and MycSu152 as ST41 (purple highlights on Figure 13). No differences were found between the isolates from Bácsalmás in the house-keeping

genes, SNPs were only detected either on both or one of the surface protein coding genes *p95* and *mtlD*. The number of differences between the STs are detailed in Table 18. The two isolates from Jánoshalma showed similar results in all MLST systems (yellow highlights on Figure 13 and Table 15, 17 and 18), the two isolates represented ST124 and ST127 and differed in six SNPs in MLSTp. While in MLSTs, the main differences were found on surface protein coding genes but one SNP was detected in a house-keeping gene fragment also. The four isolates from Hajdúszoboszló formed three STs in the MLSTp scheme. Similarly to the here developed system MycSu24 and MycSu27 were similar (ST40), MycSu76 formed ST88 and MycSu160 represented ST108 (highlighted green on Figure 13, Table 17). In contrast with the MLSTn results, the isolates from Hajdúszoboszló were grouped with isolates from other farms by MLSTp. MycSu55 also belongs to ST40, MycSu90 to ST88 and MycSu56 and MycSu92 to ST108 (Figure 13). In the MLSTs system, the four isolates showed four STs (ST52, ST53, ST64 and ST68; green highlight on Figure 13 and Table 18). Only MycSu76 differed from MycSu24, MycSu27 and MycSu160 on a house-keeping gene fragment, all other isolates from Hajdúszoboszló only differed on surface protein coding genes (Table 18).

There were two pairs of isolates (MycSu63-MycSu106 and MycSu56-MycSu92) which shared the same ST in the MLSTp system, but represented four distinct STs in the MLSTs and MLSTn systems, in congruence with their diverse origin. In three cases, isolates sharing the same ST in the MLSTs system belonged to different STs in the other two assays. In details, MycSu64 and MycSu65 shared ST40 with MLSTs, while they represented two different STs (ST9, ST10, respectively) within the same branch with the MLSTn system, and were located on different branches of the phylogenetic tree based on the MLSTp. MycSu56, MycSu57 and MycSu58 belonged to ST54 with the MLSTs, while represented three different STs with the other two MLST schemes. Finally, MycSu59 and MycSu60 were grouped in ST67 with MLSTs and in two distinct STs with the previous and the here developed MLST schemes. Interestingly, in both MLSTp and MLSTs MycSu29 and MycSu101 isolate, which showed identical genotypes in the MLVA system (Supplementary figure 1) were grouped together, in contrast, these isolates were differentiated with the MLSTn system in accordance with the epidemiologic background of the isolates.

The adjusted Rand coefficients defining the proportion of congruence between the genotyping systems were similar for the MLST assays. The MLVA system showed low congruence with all MLST schemes (Table 19).

Table 19: Adjusted Rand coefficients of the genotyping systems.

	MLSTn	MLSTp	MLSTs	MLVA
MLSTn				
MLSTp	0.445			
MLSTs	0.541	0.463		
MLVA	0	0.089	0.056	

5.4 Challenge model

5.4.1 Clinical observations

No clinical alterations were detected in the Control Group throughout the study and no body temperature higher than 40.3°C was recorded in any of the groups. One pig in Group IV-IP had body temperatures higher than 40°C on three consecutive days (D4-6, Supplementary table 6). No respiratory signs were recorded in the challenge groups.

Swollen joints were detected as early as D6 in Group IV-IP and D8 in Group IV-IV. Typically, the first swollen joint was one of the tarsal joints. By D15 all pigs in Group IV-IP had at least one swollen joint, 3/6 pigs had two swollen tarsi and in one animal joints of the front legs were also affected. In Group IV-IV swollen tarsal joint was observed in 4/6 pigs (one side only) and in one animal both tarsi were affected, while no swollen joints were detected in one pig. The appearance of swollen joints in the challenge groups is presented in Figure 14.

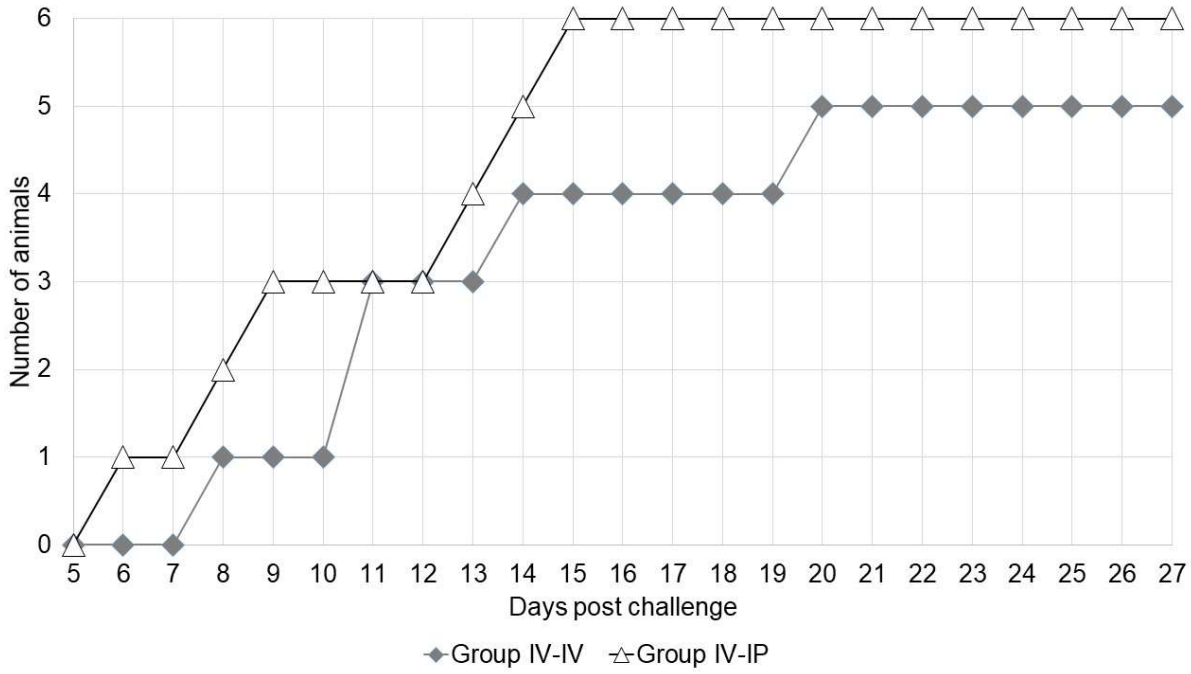

Figure 14: Appearance of at least one swollen joint in the challenge groups.

The weight gain dynamics of the different groups are shown in Figure 15. The average starting weight of the groups were 10.5 kg (SD 1.2), 10.3 kg (SD 1.2) and 10.3 kg (SD 1.1) in Group IV-IV, IV-IP and Control while at the end of the study average body weight of the groups were 18.0 kg (SD 4.3), 16.0 kg (SD 2.5) and 22.0 kg (SD 4.1), respectively. Mean ADWG was 223 g, 170 g and 350 g in Group IV-IV, IV-IP and Control Group. Significant differences in ADWG were detected between Groups Control and IV-IV ($p=0.05$) and Groups Control and IV-IP ($p<0.01$; Supplementary data 2a).

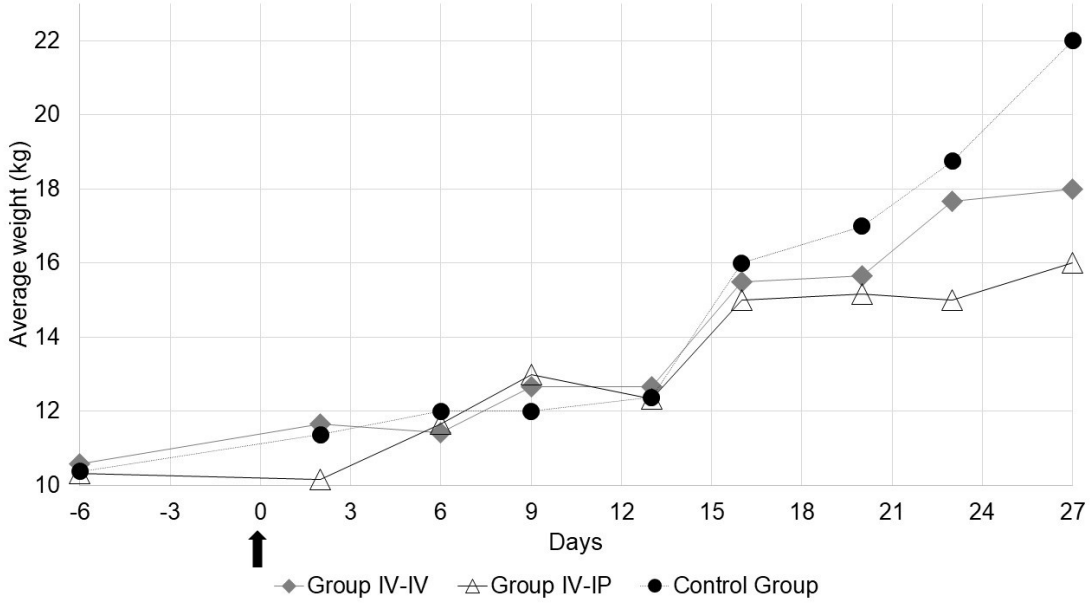

Figure 15: Weight gain dynamics and average weight of the study groups at each sampling point.
The arrow marks the first day of the challenge.

5.4.2 *Mycoplasma* detection and bacteriology

Nasal swabs of all animals were negative for *M. hyorhinis* by PCR and isolation at the beginning of the study (D-2). After the inoculation of the pigs, one sample from each challenged group was positive by isolation, which was positive also by PCR either at the same time or at different sampling times. These animals remained PCR positive for two-four consecutive sampling points. Further two animals in Group IV-IV and one animal in Group IV-IP were PCR positive as well at one sampling point. All nasal samples of the Control Group were negative by PCR and isolation for *M. hyorhinis* throughout the study (Supplementary table 7).

The number of PCR-positive samples is presented in Table 20. During the challenge study two nasal isolates and isolates from six necropsy samples (tarsal, carpal, elbow and stifle joints) were collected, details of the re-isolates are listed in Supplementary table 1. All samples from the Control Group were negative for *M. hyorhinis*. *M. hyopneumoniae* or *M. hyosynoviae* were not detected in any samples collected during the necropsy.

Table 20: Number of *Mycoplasma hyorhinis* specific PCR positive necropsy samples in each challenge group.

	Group IV-IV	Group IV-IP
Pericardium	1/6	0/6
Pleura	1/6	0/6
Peritoneum	1/6	0/6
Stifle	2/6	2/6
Elbow	4/6	4/6
Tarsus	5/6	4/6
Carpus	4/6	1/6
Conjunctiva	0/6	0/6
Meninx	0/6	0/6
Lung	1/6	0/6

The re-isolates were first genotyped using MLVA. Two re-isolates in Group IV-IP differed from the challenge strain on one allele (MHR444; Supplementary table 4, Figure 16). The sequence types of these two isolates, two other isolates from the same animals and one isolate from Group IV-IV were also determined by MLST. All the re-isolated strains showed the same MLST ST as the challenge strain (Figure 16, Supplementary table 4).

None of the cultures of the necropsy samples showed growth of pathogenic bacteria that could also be associated with the lesions, other than *M. hyorhinis*.

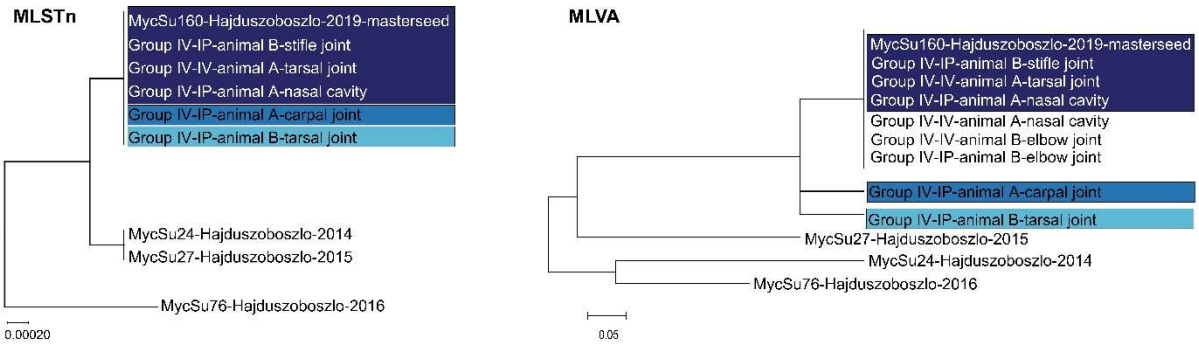

Figure 16: Multi-locus sequence typing and multiple-locus variable-number tandem-repeat analysis of the isolates from Hajdúszoboszló and the challenge strain (MycSu160) re-isolates.

The MLST tree was constructed using the Maximum Likelihood method using gene fragments of *lepA*, *rpoB*, *rpoC*, *gltX*, *uvrA* and *valS* with 1000 bootstraps (only bootstraps>70% are presented). The resolution by MLVA is based on the alleles: Mhr205, Mhr396, Mhr438, Mhr441, Mhr442 and Mhr 444, the trees were constructed using the Neighbour-Joining method. Re-isolates with the same genotype as the challenge strain are highlighted dark blue and the ones which differ are light blue.

5.4.3 Gross pathological examination

Arthritis of at least one joint was observed in all pigs in the challenge groups. Mild to severe arthritis was found in more than one joints of two animals in Group IV-IV, while in one animal three joints, and in another all joints were affected. A single joint was involved in the remaining two animals in this group (Supplementary table 8). Mild to severe arthritis was found in three, two or one joints of two-two pigs in Group IV-IP (Supplementary table 8). Arthritis manifested as serous or fibrinopurulent inflammation and was detected most often in the tarsus (8/12) followed by the elbow (6/12), stifle (5/12) and carpus (4/12) on one or both sides (Figure 2, 17 and 18).

Diffuse, severe, chronic pericarditis resulting in diffuse adhesions was detected in two animals in both groups (Supplementary table 8; Figure 3, 17 and 18). Additionally, mild or moderate chronic pleuritis with filamentous adhesions (Figure 3 and 17) were detected in two animals and mild chronic peritonitis presenting filaments of connective tissues occurred in one other animal in Group IV-IV (Figure 18; Supplementary table 8). Total macroscopic scores and number of affected animals are detailed in Table 21. Macroscopic scores of lesions in the affected organs are demonstrated in Figure 18 and detailed in Supplementary table 8.

No gross pathological alterations were found in the remaining organs examined. No gross pathological lesions were detected in any examined organs in the Control Group. Body condition in all groups was normal (Table 8). Significant differences were detected when pathological scores of joint lesions and total scores of the different groups were compared: pathological scores in both challenge groups were significantly higher than the Control Group ($p=0.03$ and $p=0.02$ regarding joint lesions, $p=0.02$ and $p=0.03$ regarding total scores for Groups IV-IV – Control and Groups IV-IP – Control, respectively), but the challenge groups did not show difference. No significant difference was found when scores of serosal lesions were compared (Supplementary data 2b-d).

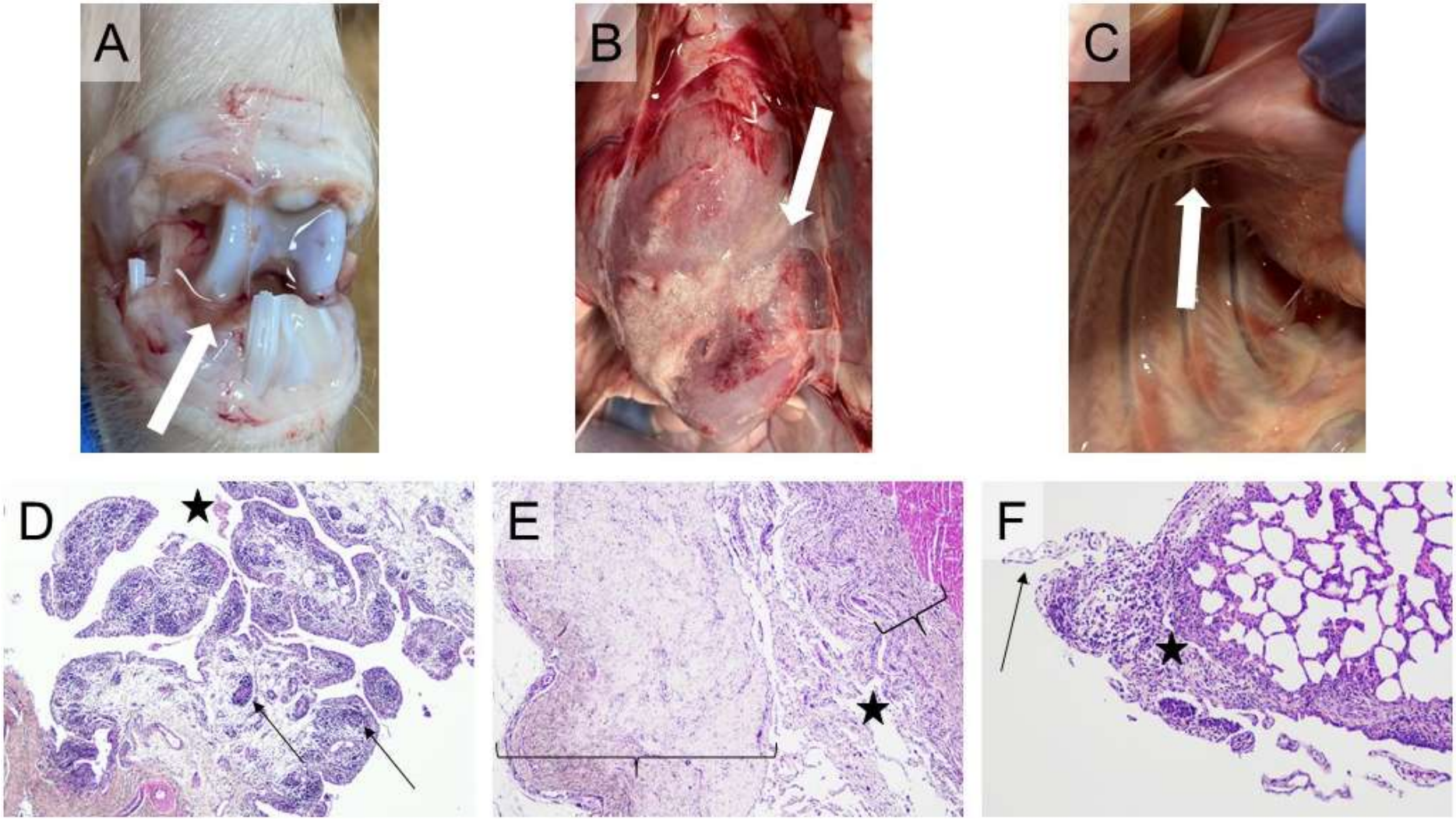

Figure 17: The main macroscopic and histopathological lesions of *Mycoplasma hyorhinis* infection.

Histopathologic lesions (D-F) are from the presented macroscopic lesions (A-C). A: acute fibrinopurulent arthritis in tarsus, B: diffuse severe chronic pericarditis with diffuse adhesion, C: Pleural adhesions, D: severe arthritis with the formation of perivascular lymphoid follicles (arrows) and fibrin exudates in the joint cavity (star; H&E, 40×), E: Large number of filamentous projections consisting of connective tissue (star), and pronounced thickening of the pericardium and epicardium by proliferating connective tissue (braces; H&E, 40×), F: Filamentous projections consisting of connective tissue (arrow), and focal, moderate thickening of the pleura by proliferating connective tissue with infiltration by mixed inflammatory cells (star; H&E,100×).

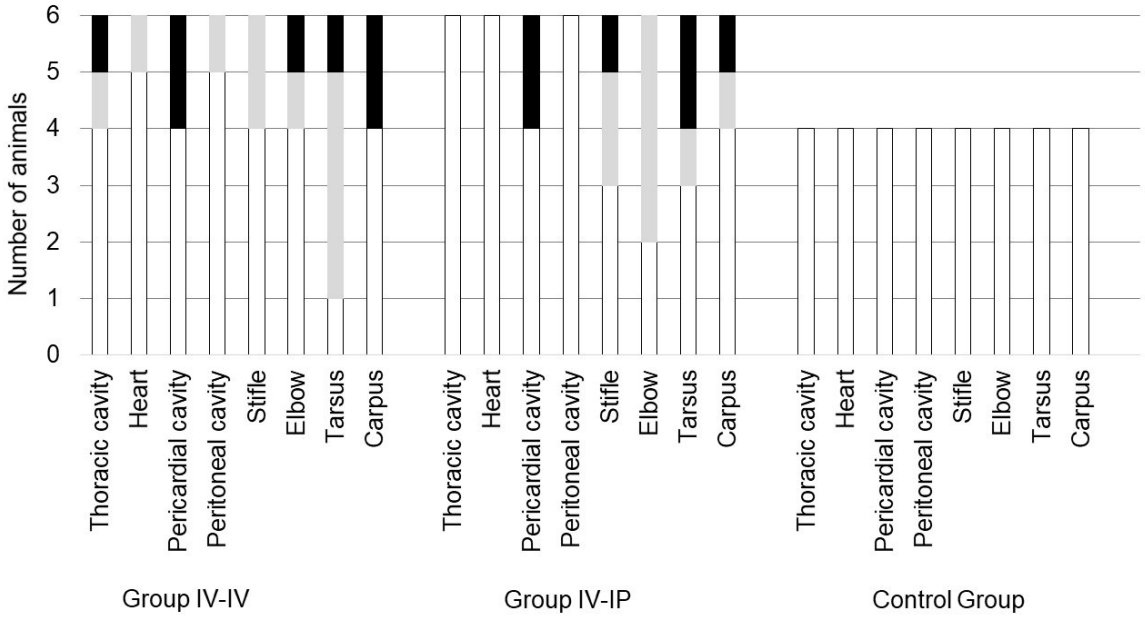

Figure 18: Macroscopic lesion scores of the affected organs of the study groups.
Organs were scored between 0-2 based on the severity of the lesion, except for the heart where score 1 was the maximum (Table 7). On the charts white colour indicates the number of animals with score 0; light grey is score 1 and black is score 2. The number of animals in each group is indicated on the Y-axis, there were six animals in the challenge groups and four in the Control group.

Table 21: Total macroscopic and histopathological scores of the challenge groups.

Group	Group IV-IV		Group IV-IP		Control Group	
Organ	Macro. score	Histo. score	Macro. score	Histo. score	Macro. score	Histo. score
Pericardium	4 (2/6)	7 (3/6)	4 (2/6)	6 (2/6)	0	0
Pleura	3 (2/6)	9 (3/6)	0	6 (4/6)	0	0
Peritoneum	1 (1/6)	2 (1/6)	0	1 (1/6)	0	0
Stifle	2 (2/6)	3 (1/6)	4 (3/6)	12 (4/6)	0	0
Elbow	3 (2/6)	5 (2/6)	4 (4/6)	7 (3/6)	0	0
Tarsus	6 (5/6)	11 (5/6)	5 (3/6)	7 (3/6)	0	0
Carpus	4 (2/6)	9 (3/6)	3 (2/6)	4 (2/6)	0	0
Conjunctiva	0	2 (1/6)	0	1 (1/6)	0	0
Heart	1 (1/6)	0	0	0	0	0
Lung	0	0	0	0	0	0
Nasal cavity/ mucosal membrane	0	1 (1/6)	0	0	0	0
Other organs	0	0	0	0	0	0

Number of annimals affected by the lesion is indicated in parenthesis. Scores are detailed in Supplementary table 8. Abbreviations: Macro. score: macroscopic score, Histo. score: histopathological score

5.4.4 Histological examination

The most important lesions were detected in the joints and in the serosa of parenchymal organs in the thoracic and abdominal cavities. Joint lesions were evident in several cases only with histological examination. A total of 24 joints presented histological lesions (score 1 to 3; Figure 6) and most of these lesions (16 cases) were given score 3 (Figure 20, Supplementary table 8). In the joints, inflammation was characterized by infiltration of mononuclear cells and in more severe cases hyperplasia of the synoviocytes (Table 9, Figure 6). Lymphoid follicles formed around the blood vessels (Figure 4) and fibrin exudates in the joint cavity were observed in several infected animals (Figure 17, Supplementary table 8). One animal in the Group IV-IP presented acute-subacute erosive synovitis associated with acute haemorrhages, frequent occurrence of fibrin exudates, blood cells and neutrophyl granulocytes in the joint cavity (Figure 19). Lesions of the serosal membranes (pleura, pericardium and peritoneum) were characterized by the presence of filamentous projections consisiting of connective tissue and by different severity of serosal thickening caused by proliferating connective tissue (Table 9, Figure 7, 8, 9 and 17, Supplementary table 8). Multinucleated giant cells in the joints in two cases and in the pleura in one of those cases were detected in the animals from Group IV-IV (Supplementary table 8). In the nasal cavity mild to moderate, acute rhinitis in one case and acute ulcerative conjunctivitis were detected in an other case from Group IV-IV (Figure 19, Supplementary table 8).

All detected lesions and histopathological scores are detailed in Supplementary table 8. Total scores and number of affected animals per group are shown in Table 21 and Figure 20. Based on the statistical analysis, scores of joints and total scores differed significantly between groups. In both cases, significant differences were detected between Group IV-IV and Control ($p=0.04$ regarding joint lesions, $p=0.04$ regarding total score; Supplementary data 2e-g).

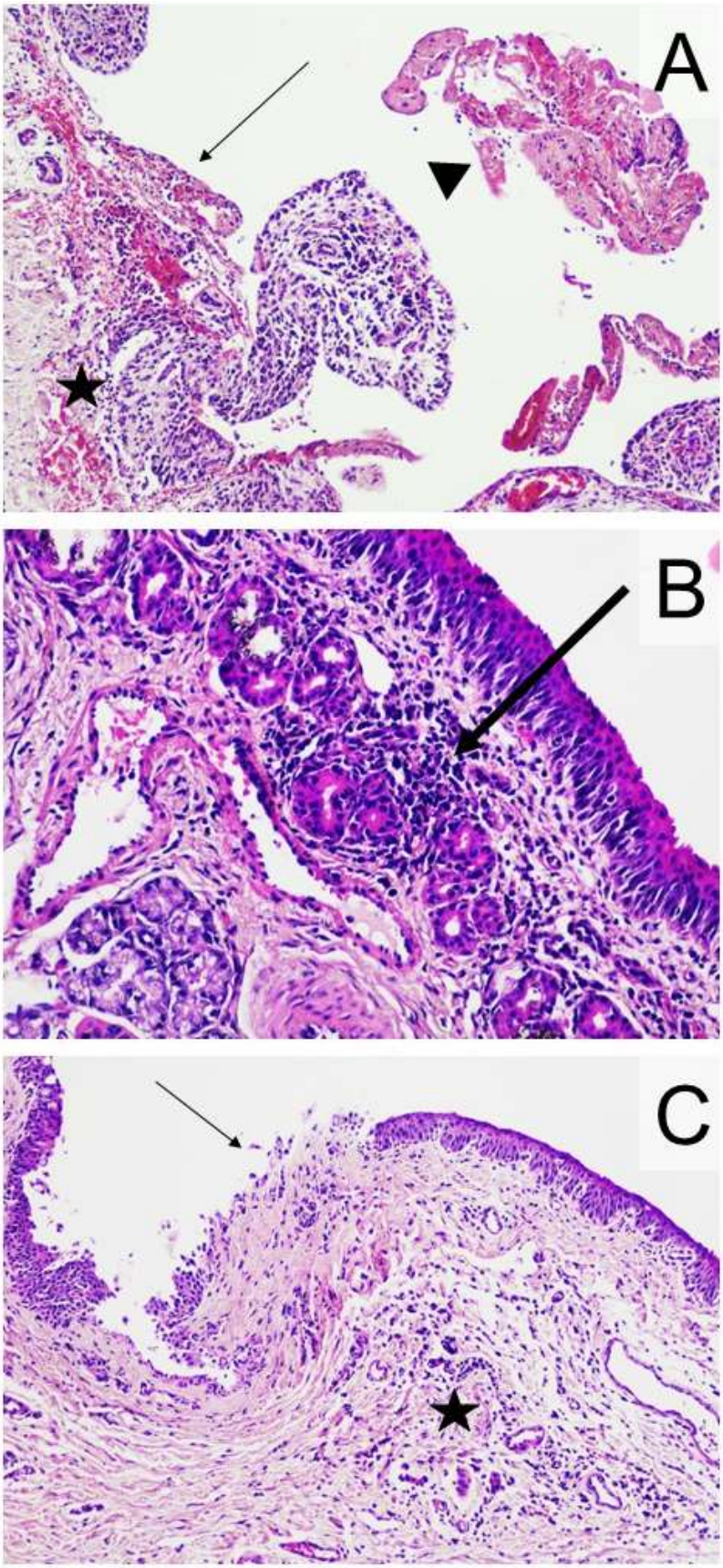

Figure 19: Histopathological lesions of *Mycoplasma hyorhinis* infection.

A: erosive synovitis (arrow) associated with acute haemorrhages (star), frequent occurrence of fibrin exudates, blood cells and neutrophyl granulocytes in the joint cavity (arrowhead; 100×; H&E stain), B: Moderate mixed inflammatory cell infiltration around the mucosal glands (arrow) in the nasal mucosal membrane (200×; H&E stain), C: Acute, ulcerative (arrow) inflammation (star) of the conjunctiva (100×; H&E stain).

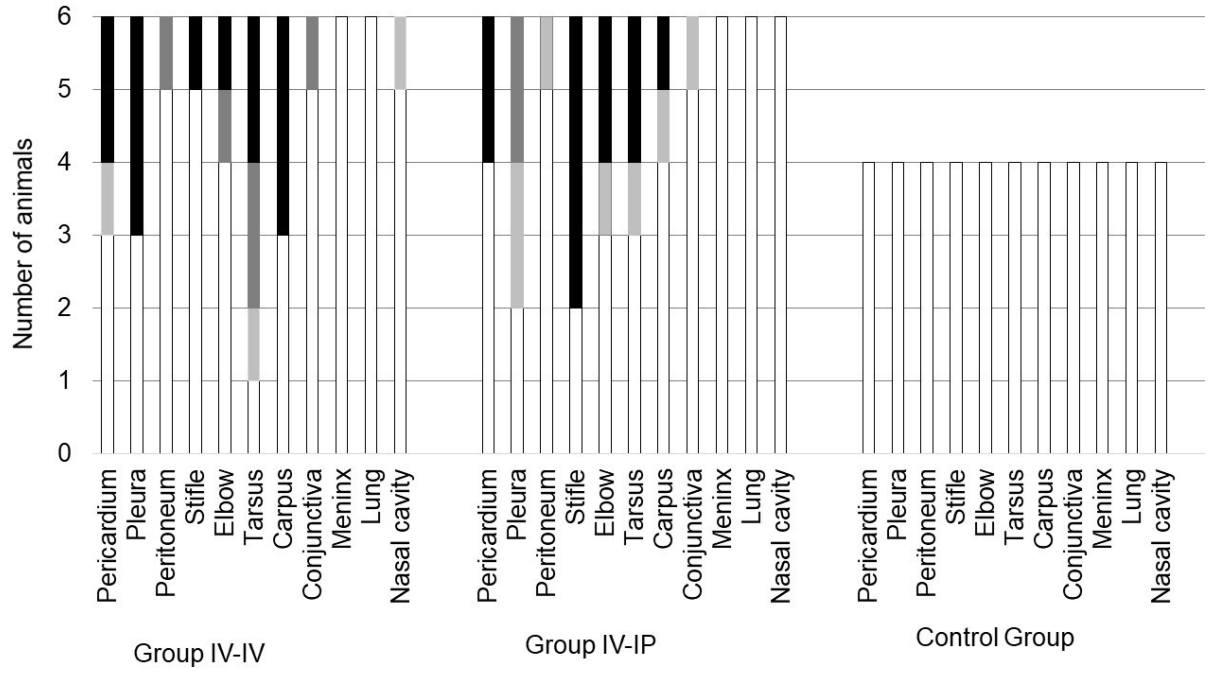

Figure 20: Histopathological lesion scores of the affected organs in the study groups. Organs were scored between 0-3 based on the severity of the lesion. In the charts white indicates the number of animals with score 0 (no lesion), light grey indicates score 1 (mild lesions), dark grey indicates score 2 (moderate lesions) and black indicates score 3 (severe lesions). The number of animals in each group is indicated on the Y-axis, there were six animals in the challenge groups and four in the Control group.

5.4.5 Serology

All animals were serologically negative to *M. hyorhinis* at the beginning of the study. The positive serological response appeared in both challenge groups on D5, S/P% of 1/6 pigs in Group IV-IV and 2/6 pigs in Group IV-IP was higher than 40%. By D28 all challenged animals were ELISA positive. However, one animal in Group IV-IP, presented a positive serological response just at the last sampling point. The mean S/P% of the groups throughout the study are demonstrated in Figure 21. Significant differences in S/P% on D28 were detected between Control and Group IV-IV ($p<0.01$) and Control and Group IV-IP ($p<0.01$; Supplementary data 2h). Animals from the Control Group remained negative throughout the study.

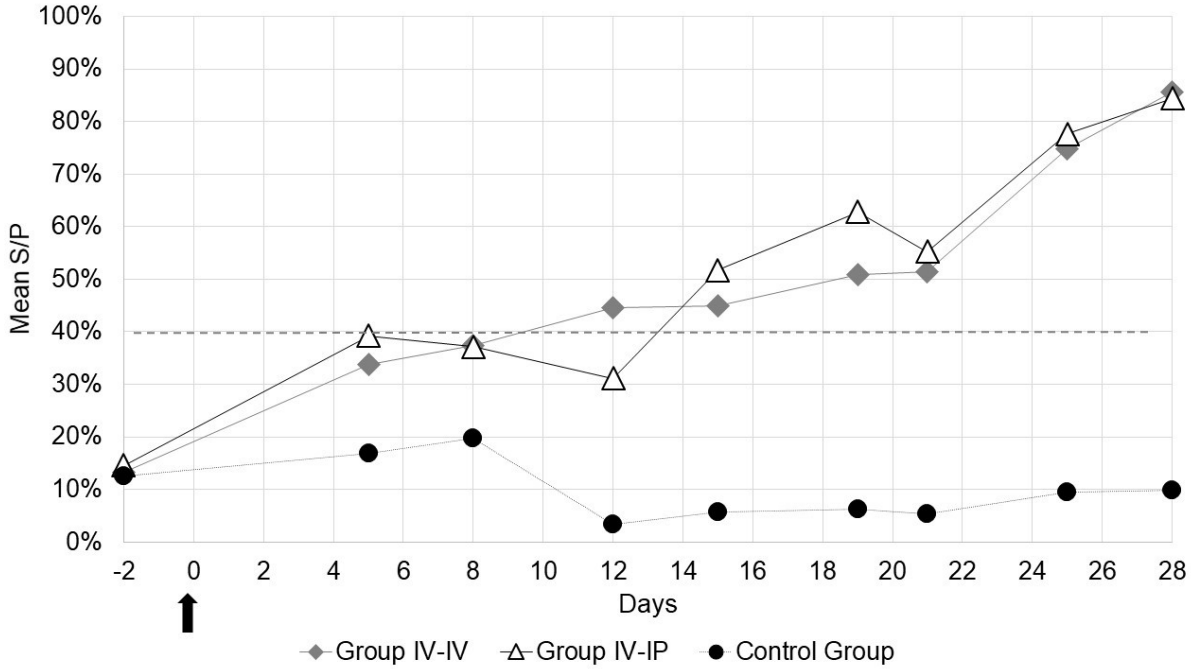

Figure 21: Mean sample-to-positive ratios (S/P%) of the blood samples taken during the study.

The arrow marks the first day of the challenge. Above the dotted line (40% mean S/P) samples were considered positive.

6. Discussion

6.1 Antibiotic susceptibility testing

Antibiotic susceptibility data of *M. hyorhinis* is available since 1989 (Hannan *et al.*, 1989), yet (similarly to other veterinary mycoplasmas) there is no standardised method for either broth or agar microdilution testing. In case of human mycoplasmas standardised susceptibility testing is only available since 2011 (Waites *et al.*, 2011). The fastidious nature of these bacteria regarding the time of growth and complex media requirement makes the standardisation difficult. To institutionalize the susceptibility testing the following conditions should be optimized: (1) the media used (2) length of incubation (3) quality control strains and (4) MIC reference ranges. Regarding the media ideally commercially available broth and agar plates would be used to unify the recipe among laboratories (Waites *et al.*, 2012). In case of *M. hyorhinis* either there are no data about the used media or at least three kinds of media were used in the published antibiotic susceptibility studies (Hannan *et al.*, 1989, 1997; Ter Laak *et al.*, 1991; Kobayashi *et al.*, 1996b, 1996a, 2005; Hannan, 2000; Wu *et al.*, 2000; Jin *et al.*, 2014; Jang *et al.*, 2016; Bekő *et al.*, 2019a; Rosales *et al.*, 2020). Also, cell-wall synthesis inhibiting antimicrobials added to the *Mycoplasma* media to support selective growth can interfere with the antibioitic susceptibility test, which needs to be considered (Käbisch *et al.* 2021). As of the quality control strain for *M. hyorhinis,* most studies used the type strain (NCTC 10130; Ter Laak *et al.*, 1991; Hannan *et al.*, 1997; Kobayashi *et al.*, 1996a, 2005; Wu *et al.*, 2000; Jang *et al.*, 2016; Bekő *et al.*, 2019a; Rosales *et al.*, 2020; present study), however, the use of surrogate quality controls with standardised MIC ranges were also suggested (Käbisch *et al.* 2021). Besides the difficulities of the standardisation antibiotic susceptibility testing of veterinary mycoplasmas is time consuming and requires special expertise. Therefore, in most cases the targeted antibiotic treatment is inaccessible and the selection of antibiotics for therapeutic use is often empirical. For this reason, recent and comparable MIC data is essential, however, susceptibility data of recent European isolates are scarce in the literature (Ter Laak *et al.*, 1991; Bekő *et al.*, 2019a; Rosales *et al.*, 2020).

Accordingly, antibiotic susceptibility of 76 European (Belgian, German, Hungarian, Italian and Polish) *M. hyorhinis* isolates from 2019 to 2021 was examined. These isolates were inhibited by low concentrations of tiamulin, tetracyclines (doxycycline and oxytetracycline) and florfenicol, while moderate concentrations of enrofloxacin were also effective against them. On the other hand, in the case of the tested macrolides and lincomycin a bimodal MIC distribution was observed, with 42-56% of the isolates showing decreased susceptibility to these drugs.

The MIC values of European *M. hyorhinis* isolates over the years were compared. Consistent low MIC values for tiamulin and tetracyclines were detected and MIC values remained in the same range over the observed period (around 35 years; Table 11). The described results indicate the effectiveness of these antimicrobials against *M. hyorhinis* isolates.

The pharmacokinetic characteristics of the used antimicrobial agents is also crucial for their provided clinical efficacy. In the present study, the tested ten antimicrobials (doxycycline, oxytetracycline, florfenicol, enrofloxacin, tylosin, tilmicosin, tylvalosin, tulathromycin, lincomycin and tiamulin) are characterised by excellent distribution and are able to achieve high concentration in tissues (Nielsen and Gyrd-Hansen, 1998; El Korchi *et al.*, 2001; Prats *et al.*, 2005; Giguére, 2013; Bladek *et al.*, 2015; Pallarés *et al.*, 2015; Wang *et al.*, 2016; Huang *et al.*, 2018; Lei *et al.*, 2018; Xiong *et al.*, 2018). In addition, it has been described that florfenicol is able to achieve therapeutic concentrations in the joint synovial fluid of pigs (Somogyi *et al.*, 2022). These properties of the antimicrobials are crucial for the therapy against a pathogen like *M. hyorhinis*, which causes systemic lesions and polyarthritis in infected animals. These factors influence the *in vivo* efficacy of the antibiotics, which should be considered during the evaluation of the results of *in vitro* antibiotic susceptibility tests.

During the Pan-European study slight differences in the MIC_{50} values of macrolides and lincomycin between countries were detected. The Hungarian and Polish isolates showed MIC_{50} values below the here defined breakpoints, while the isolates from Germany and Italy (except for tylvalosin) had MIC_{50} values above the breakpoint. These minimal differences can be explained by the hypothesised differences between countries in the route of administration (parenteral or feed/water) and frequency of use. In a study including four European countries (Belgium, France, Germany and Sweden) marked differences in the recommended dosages were found, for example recommended dosages in Germany were lower than in the other countries (Postma *et al.*, 2015). The detected bimodal susceptibility patterns for macrolides and lincomycin and the comparison of the susceptibility profiles of European *M. hyorhinis* isolates over the years from literature data emphasize the importance of regular susceptibility monitoring.

6.2 Mismatch amplification mutation assay

Macrolide and lincosamide antibiotics are chemically distinct, but share a similar mode of action as their binding sites overlap (also with streptogamins, ketolides and oxazolidinones). Both inhibit the bacterial protein synthesis by reversly binding to the 50S subunit of the ribosome. Resistance to macrolides and lincomycin can evolve through three methods (1) target site modification through methylation or mutation which prevents binding of the antibiotic, (2) active efflux mechanism, (3) drug inactivation (Leclercq, 2002). Ribosomal methylation is the most widespread mechanism of resistance among bacteria, ribosomal methylation is the result of the activity of the *erm* (erythromycin ribosome methylase) genes. Erm methylases are expressed in a wide range of microorganism (Leclercq, 2002). The presence of *erm* genes have been described in human mycoplasmas and ureaplasmas (Varghese and Kerkar, 2020; Ma *et al.*, 2023) but not yet in veterinary species. Nonetheless, target site modification through mutations in the 23S rRNA gene has been described in several veterinary species and mutations in the L4 and L22 ribosomal proteins in some avian and ruminant mycoplasmas has also been identified (Gautier-Bouchardon, 2018; Bekő *et al.*, 2020, Grózner *et al.*, 2022). Active efflux mechanism in tilmicosin resistance was also described recently (Xia *et al.*, 2022).

A point mutation in *M. hyorhinis* isolates in the 23S rRNA gene V domain A2059G (according to *E. coli* numbering) was described earlier in connection with decreased susceptibility to macrolides (Kobayashi *et al.*, 2005). In this study, a transition substitution in the same region of the 23S rRNA gene at position A2066G (according to the numbering of 23S rRNA gene in the *M. hyorhinis* type strain; A2058G according to *E. coli* numbering) was identified in isolates with decreased susceptibility to macrolides and lincomycin.

A MAMA was designed to detect the identified SNP and provide a cost effective tool for rapid determination of macrolide and lincomycin susceptibility. Results of the developed assay were in line with the *in vitro* MIC values. In the few cases when the MIC values for macrolides and lincomycin were not uniform, the results of the molecular tests were in accordance with the isolates' susceptibility profile for the majority of the tested macrolides and showed false results for the antibiotic with the outlier MIC values (Supplementary table 3). In most cases (MycSu94 being the exception) the isolates were susceptible to one macrolide and high MICs were detected for the others, accordingly, the use of macrolides and lincomycin is not advised in these cases and the result of the MAMA supports that (genotype H was detected). In the case of MycSu94, an evolving resistance is assumed indicated by the elevated MIC for tilmicosin, which did not show up in nucleotide change at the examined position. Similar event was observed in case of three recent isolates showing high MIC values of all tested macrolides and lincomycin (>64 µg/ml for all except tylvalosin where MICs of 5->10 µg/ml was detected)

but revealing genotype L (Ge-13, Po-4 and Po-9). Sequence analysis of the partial 23S rRNA gene sequence in these isolates confirmed the presence of the nucleotide associated with high susceptibility to macrolides (adenine in isolates Po-4 and Po-9), or its substitution to an atypical variant (thymine in Ge-13). Considering these results, presence of other resistance mechanisms can be suspected also. Besides the above mentioned mechanism, biofilm formation was also connected with elevated MIC values in the case of *M. hyopneumoniae* (Tassew *et al.*, 2017). The resistance mechanisms in the here examined four isolates warrant further investigation, yet the low number of samples limited these studies.

During the validation of the assay on DNA samples both genotypes were detected in a clinical specimen (Su21) by the agarose-MAMA, while genotype H was detected in the corresponding isolate (MycSu101; Supplementary table 3) in line with the isolate's antibiotic susceptibility profile determined by the broth microdilution assay. This can indicate the presence of more than one variations of the pathogen in a clinical sample with different antibiotic susceptibility profiles. Therefore, the use of the designed molecular assay, which is suitable for the examination of DNA extracted directly from clinical specimens, can prevent false result due to the selection of the filter cloning step. However, to avoid misinterpretation of the results, presence of *M. flocculare* needs to be excluded first and in case of low DNA concentration (below 10^3 copies/reaction) isolation prior to testing might be inevitable.

Considering the described incongruencies of the assay, detection rate of the developed MAMA was 97.5% in the case of tylosin, tulathromycin and lincomycin, 96.7% for tilmicosin and gamithromycin and 90.2% for tylvalosin based on the analysed samples. As there are no official breakpoints for mycoplasmas, the described false detection rates for the different macrolides may be altered when standard breakpoints will be determined. This could be expected especially in the case of tylvalosin, as MIC values of isolates with false genotypes were only one or two dilution steps below the here defined breakpoint. Considering all the above, the assay is reliable to guide antibiotic therapy when the susceptibility data for macrolides and lincomycin are required rapidly.

6.3 Genotyping assays

Efficient genotyping tools are the key to better understand and monitor *M. hyorhinis* infections and to conduct epidemiological investigations. The first methods to identify relationships of *M. hyorhinis* isolates were restriction endonuclease analysis (Darai *et al.*, 1982) and pulse-filed gel electrophoresis (Barlev and Borchsenius, 1991). Then sequencing based methods became widespread. First, sequences of the *p37* gene were compared among *M. hyorhinis* isolates, but the discrimination index for the examined population was low (0.934; Tocqueville *et al.*, 2014). Next, a six-gene based MLST was developed, the discriminatory

power of the assay was 0.989, analysing the same population which was examined by *p37* sequencing. The MLST assay detected high variability of sequence types, with identical sequence types within farms and individual animals (MLSTp; Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016), still the reproducibility of the assay remained low. Therefore a modified MLST scheme including surface protein coding genes was applied (MLSTs; Clavijo *et al.*, 2019), however, the use of surface protein genes limits the reliability of the assay as conserved genes are more reliable in indicating relationships (Estoup *et al.*, 2002; Urwin and Maiden, 2003). Ultimately, a core genome MLST scheme has been established, after the publication of the here developed MLST system, providing the highest resolution of pure isolates (Bünger *et al.*, 2021). Beside the different MLST schemes a two allele based MLVA was also described for the characterization of *M. hyorhinis* isolates (Dos Santos *et al.*, 2015).

In the present study, high resolution MLST (Simpson's ID 0.986) and MLVA (Simpson's ID 0.999) were developed. Isolates from routine diagnostic sampling and from slaughter houses over a short time period (2014-2019) mainly from Hungary were used for the validation of the developed assays. Accordingly, no complex epidemiological analysis could be performed. Nevertheless, the examined isolates revealed high variability and certain correlations were observed among isolates from the same farm or the same animal. The same sample set was submitted for analyses with the previously published conventional MLST assays (MLSTp, Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016; MLSTs, Clavijo *et al.*, 2019) and the observed high diversity among the tested isolates was in line with the results of the here developed assays.

The limitation of the conventional MLST systems is that by using a limited number of house-keeping gene fragments differentiation of closely related isolates can not be achieved (Urwin and Maiden, 2003). Accordingly, differentiation among isolates originating from the same farm could not be achieved in most of the cases neither with MLSTp (Trüeb *et al.*, 2016) or MLSTs (Clavijo *et al.*, 2019). In the present study limited number of isolates from the same integration but different farms were available (i.e. integration A besides Bácsalmás three other farms: Beremend, Sárbogárd and Alsómocsolád; Supplementary table 1). While the isolate from Beremend shared an ST with isolates from Bácsalmás in all MLST schemes, the other two isolates from Sárbogárd and Alsómocsolád represented different STs. Similarly, the MLSTs system discriminated several STs within integrations (Clavijo *et al.*, 2019). The present study examined isolates from the same farms collected in variable timeframes (collections six months to six years apart). Typically, isolates from the same farm shared the same or closely related STs. This was observed in the case of Bácsalmás, where most of the isolates were typed into two, closely related STs according to the year of isolation (ST2: 2014-2015, ST3:2016-2019). The small difference between these two STs could be the result of an evolutional event, which is demonstrated by the single nucleotide change in the *rpoC* gene. The two latest isolates from this farm (MycSu141 and MycSu152) differ greatly from the

previous isolates (5-10 SNPs) and from each other (4 SNPs), thus the introduction of new strains to the herd is presumed in these cases. The two latest isolates from Bácsalmás were only isolated six months apart, yet there are more differences between them than isolates from different years from the same farm. Hence, the presence of more than one strain at the same time in the same herd is a possibility. Similarly, isolates with relatively distant STs were detected in the case of Jánoshalma (ST8 and ST13) and Hajdúszoboszló (ST22, ST30 and ST38), confirmed by the previously published MLST systems also (Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016; Clavijo *et al.*, 2019), assuming the spread of new *M. hyorhinis* variants with the sows. Similar hypothesis was established in the MLSTs study based on the finding that isolates from five different integrations shared the same ST (Clavijo *et al.*, 2019). Coinciding observations among the Austrian *M. hyorhinis* isolates were made in the cgMLST study, where large allele differences were observed between isolates from the same farm and isolates from the same integration also showed closer relationships than epidemiologically not related isolates (Bünger *et al.*, 2021). Presence of more than one bacterial population in a farm was also observed among *M. hyopneumoniae* isolates (Vranckx *et al.*, 2011).

The isolates from the same animal (MycSu32i,s,t) were grouped to the same MLST ST and MLVA genotype. The observation that the same isolate can be found in different organs indicates that the same strain can cause macroscopic lesions in every affected tissue. On the other hand, different allele types from the same animal were identified by the cgMLST, without indication to affinity of certain allele types to any organ (Bünger *et al.*, 2021).

The MLSTn was compared with the previously published MLST schemes based on the same 45 whole genome sequences. By using the selected six highly diverse gene fragments, the number of parsimony informative sites almost doubled compared to MLSTp; parsimony% of the MLSTn was 1.24% (38/3063 nucleotides) while it was 0.65% (15/2304) for MLSTp (Table 16; Supplementary table 5). MLSTs still has higher number of parsimony informative sites (8.12%; 117/1441) mainly due to the surface protein coding gene *mtlD* (109/537; Supplementary table 5). Nevertheless, the here described system had the highest resolution among the examined three MLST assays (MLSTn 0.986, MLSTp 0.976 and MLSTs 0.964). The explanation for the highest resolution despite the lower number of parsimony informative sites can be the stochastic nature of mutations. The surface protein coding genes have higher mutation rates, indicating a higher chance for homoplastic alleles to appear (Estoup *et al.*, 2002; Urwin and Maiden, 2003). The most robust phylogenetic tree was made based on the MLSTs scheme, still the here developed system resulted in a more robust phylogenetic analysis than the MLSTp. High variability based on a limited number of parsimony informative sites might limit the robustness of the MLST (Urwin and Maiden, 2003). Using surface protein coding genes, especially *mltD* in the case of *M. hyorhinis* increased the robustness of the phylogenetic analysis, on the other hand resulted in less reliable detection of relationships

between isolates based on the background information of the analysed isolates and their relations in the two conventional MLST systems (MLSTn, MLSTp; Figure 13). As house-keeping genes are under stabilizing selection for the conservation of functions the indicated relationships based on these genes are more reliable than using highly variable genes, like surface protein coding genes (Estoup *et al.*, 2002; Urwin and Maiden, 2003).

During the development of the recent cgMLST scheme Bünger and his colleagues (2021) made a comparison between the cgMLST, MLSTn and MLSTp based on 73 Austrian and German *M. hyorhinis* isolates. Naturally, the cgMLST has higher discriminatory power than any conventional MLST, due to the high number of used house-keeping genes. When comparing the three systems based on the same isolates they found that the results of the MLSTn were more congruent with the cgMLST (Rand coefficient 0.496) compared to MLSTp (Rand coefficient 0.179). Moreover, similarly to the observations in the present study, certain distantly related isolates were grouped together by MLSTp, contradicting with the results of the cgMLST (Bünger *et al.*, 2021).

MLVA is a rapid and cost effective tool for fine-scale typing of isolates. As tandem-repeat regions have high mutation rates, their examination enabled the discrimination of short term evolutional events. With the developed MLVA differentiation of isolates within farms and the same MLST STs were possible. The proportion of genotype/number of isolates in the MLVA was 92.6% (38 genotype/41 isolates), higher than in the previous MLVA system (9.69%, 16 genotype/165 isolates) designed by Dos Santos *et al.* (2015). The use of higher number of VNTR regions in the MLVA supports higher robustness and decrease the chance of homoplastic profiles (Vergnaud and Pourcel, 2006), as demonstrated in the present study also (using six tandem-repeat regions in the here described assay instead of the two alleles in the previous system). Still, with the use of six alleles, homoplastic profiles may occur. Two isolates from Bácsalmás (MycSu29 and MycSu101) showed the same genotype by MLVA, but were collected from different epidemics and represented different STs by the MLSTn. In this case, we suppose that the stochastic nature of mutations is responsible for the detection of the same genotype in two unrelated isolates (Estoup *et al.*, 2002), therefore the developed MLVA is not suitable alone to conduct phylogenetic analysis.

The MLSTn and MLVA represent convenient, well reproducible and high resolution molecular tools for the typing of *M. hyorhinis* isolates. To explore relationships of closely related isolates in phylogenetic studies the combined MLSTn and MLVA method is recommended, but the MLVA alone is suitable for epidemiologic studies with basic laboratory equipment and techniques.

6.4 Challenge model

Even though the natural course of *M. hyorhinis* starts with the colonization of the nasal cavity and upper respiratory tract (Kobisch and Friis, 1996), intranasal inoculation to induce clinical signs is usually not sufficient. Based on the available literature data, a single dose intranasal or intratracheal challenge can only induce one aspect of the infection [mainly polyserositis or lung lesions; (Lin *et al.*, 2006; Gomes Neto *et al.*, 2014; Lee *et al.*, 2018; Fourour *et al.*, 2019; Wei *et al.*, 2020)] or may not generate any clinical signs or macroscopic lesions at all (Switzer, 1955). Seroconversion tested after the above mentioned inoculation routes was either low or not detectable (Gomes Neto *et al.*, 2014; Fourour *et al.*, 2019). Even intranasal challenge combined with tonsillar swabbing resulted in low serological conversion with no clinical signs or macroscopic lesions (Merodio *et al.*, 2021). All piglets were challenged within the susceptible period, either at six weeks of age (Lin *et al.*, 2006; Neto *et al.*, 2014; Lee *et al.*, 2018; Fourour *et al.*, 2019; Merodio *et al.*, 2021) or ten weeks of age (Wei *et al.*, 2020).

Our study plan was based on the work of Martinson and co-workers (2018a) where one-dose intranasal, intravenous and intraperitoneal inoculations were compared to two or three dose inoculations with combined challenge routes in seven-week-old animals. The results of the study of Martinson *et al.* (2018a) also confirmed that a single dose challenge is not sufficient to induce all typical lesions, with the mildest clinical signs observed in the intranasally infected group. On the other hand, in the intravenously infected group, the rate of pigs with pericarditis and pleuritis was similar to or higher than in the groups with combined challenge routes. The authors suggested the combination of intravenous, intraperitoneal and intranasal routes on three consecutive days to induce both polyserositis and polyarthritis (Martinson *et al.*, 2018a; Wang *et al.*, 2022a).

In the present study, two challenge routes were compared by using the same virulent clinical isolate. The double dose IV challenge (which was not mentioned in previous publications) produced equal involvement of joints as the mix of IV-IP route (arthritis of at least one joint was detected in 6/6 animals in both groups), which exceeded the rate of animals affected with arthritis in the previous study (single dose IV challenge resulted arthritis in only 1/10 animal; Martinson et al 2018a). The combination of the used infection routes (Group IV-IP) resulted in the earlier appearance of more pronounced clinical signs of arthritis like swollen joints and lameness. In the majority of animals affected with arthritis the lesions indicated a subacute state of inflammation. Only a single animal in IV-IP Group presented an acute inflammation in two joints. On the other hand, in the group which was challenged by the IV route on two consecutive days (Group IV-IV) the thoracic and peritoneal cavity were more commonly affected by serositis than in Group IV-IP, with equal envolvement of the pericardial cavity in both groups (Figure 18). Most of the rest of the lesions appeared to be chronic in both infected

groups. Chronic inflammation of the serosa presents as filamentous projections, serosal thickening and adhesions between the serosal surfaces, which was detected both in the pericardium, peritoneum and in the pleura of the affected animals (Figure 7-9 and 17; Ennis *et al.*, 1971). The low rate of re-isolation compared to PCR positive samples also indicate the late phase of infection. Therefore, the reduction of the length of the study is suggested. Based on field observations and data of other challenge studies the clinical signs gradually start to alleviate two weeks after the first clinical signs (Barden and Decker, 1971; Wang *et al.*, 2022a). Consequently, the length of the study should be determined based on the appearance of the first clinical signs (five days post challenge here) and should be termined 14 days after (around 19 days post challenge in the present case).

Genotyping of the re-isolates from the challenge revealed that the challenge strain was isolated from the affected organs. The re-isolates shared the same ST with the here developed MLST and the same genotype with two exceptions (Group IV-IP A-c isolated from the carpus and Group IV-IP B-t from the tarsus, Figure 16), where only one allele differed. These are presumed to be microvariants due to within-host evolution. This finding further confirms our previous conclusion during the validation of the genotyping systems that the same isolate can induce clinical signs and pathological lesions in all organs affected by *M. hyorhinis* infection.

Although the natural route of infection is not yet fully understood, the results of the presented and previous challenge models using the IV route indicate that the circulatory system has important role in the systemic spread of *M. hyorhinis* (Martinson *et al.*, 2018a). In the present study, both of the applied challenge routes included intravenous infection, and accordingly systemic spread of *M. hyorhinis* was obtained in both cases. Furthermore, despite inoculating directly the peritoneum in Group IV-IP, peritonitis was only detected in one animal by histopathology without macroscopic lesions, while in Group IV-IV both macroscopic and more pronounced histopathologic lesions of peritonitis were detected. Lesions of the pleura and pericardium were also more severe in Group IV-IV. Nevertheless, as with both challenge methods the main lesions of *M. hyorhinis* infection were induced, both models can be recommended for the future studying of *M. hyorhinis* infection or vaccine efficacy studies. Considering the hypothesis of the natural spread of the pathogen via the circulatory or lymphatic system and the more severe pathological lesions in Group IV-IV, a two-dose intravenous challenge is recommended by the authors.

7. Overview of the new scientific results

Ad. 1.: The performed antibiotic susceptibility tests with 76 *M. hyorhinis* isolates from five European countries provide essential information regarding minimal inhibitory concentration values of ten frequently used antimicrobials in the swine industry. The presented data are useful for current targeted antibiotic treatments and in future analyses of trends in changes of antibiotic susceptibility.

Ad. 2.: A previously described molecular marker of decreased susceptibility to macrolides and lincomycin was identified among the examined *M. hyorhinis* isolates, and a rapid and reliable molecular assay (MAMA) was developed for the detection of this point mutation. The developed assay is a feasible tool to guide targeted antibiotic treatment.

Ad. 3.: Novel, PCR-based, genotyping assays (MLST and MLVA) were developed using the latest technology for the phylogenetic and epidemiologic analyses of *M. hyorhinis* isolates and clinical specimens. The comparison of the developed MLST with the previously published MLST schemes revealed that our MLST scheme has the highest discriminatory power and reflects best the genome-wide relationships among the conventional MLST methods.

Ad. 4.: Genotyping and phylogenetic analyses was performed with the developed MLST and MLVA systems on 41 *M. hyorhinis* isolates. The developed MLST method differentiated *M. hyorhinis* isolates with high discriminatory power. In combination with the developed MLVA, differentiation between closely related isolates could be achieved.

Ad. 5.: Two *M. hyorhinis* challenge models were established in five-week-old piglets. Both the double dose intravenous and the combined intravenous and intraperitoneal challenge could induce typical lesions. The challenge models are suitable to be used in further vaccine efficacy studies.

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9. Scientific publications

Publications in the topic of the dissertation:

In peer-reviewed journals

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10. Supplements

Supplementary table 1: Background information of all isolates and DNA samples used in this study.

Original ID	Sample*	Sample type	Country of origin	Herd	Isolation year	Integration
DBS1050	CP006849.1	cell culture	USA	-	2012	-
GDL-1	CP003231.1	cell culture	USA	-		-
HUB-1	CP002170.1	respiratory tract	China	-		-
MCLD	CP002669.1	cell culture	Israel	-	2004	-
MDBK-IPV	CP016817.1	cell culture	Brazil	-	2014	-
SK76	CP003914.1	synovial fluid	USA	-	1973	-
NCTC 10130 (*Mycoplasma hyorhinis* type strain)	culture	nasal cavity	USA	-	1955	-
MycSu21	culture	lung	Hungary	Heves	2014	-
MycSu23	culture	lung	Hungary	Bácsalmás	2014	A
MycSu24	culture	lung	Hungary	Hajdúszoboszló	2014	B
MycSu25	culture	lung	Hungary	Hajdúdorog	2014	-
MycSu27	culture	lung	Hungary	Hajdúszoboszló	2015	B
MycSu29	culture	lung	Hungary	Bácsalmás	2015	A
MycSu32i	culture	synovial fluid	Hungary	Bácsalmás	2016	A
MycSu32s	culture	serosa	Hungary	Bácsalmás	2016	A
MycSu32t	culture	tonsil	Hungary	Bácsalmás	2016	A
MycSu54	culture	lung	Hungary	Lovasberény	2016	-
MycSu55	culture	lung	Hungary	Felsőbabád	2016	-
MycSu56	culture	lung	Hungary	Nemesnádudvar	2016	-
MycSu57	culture	lung	Hungary	Osli	2016	-
MycSu58	culture	lung	Hungary	Somogycsicsó	2016	-
MycSu59	culture	lung	Hungary	Felsőszentiván	2016	-
MycSu60	culture	lung	Slovakia	not available	2016	-
MycSu61	culture	lung	Hungary	Pápa	2016	-
MycSu62	culture	lung	Hungary	Beremend	2016	A

Supplementary table 1 continued.

Original ID	Sample	Sample type	Country of origin	Herd	Isolation year	Integration
MycSu63	culture	pleural exudate	Hungary	Sárbogárd	2016	A
MycSu64	culture	pleural exudate	Hungary	Csongrád	2016	-
MycSu65	culture	nasal swab	Hungary	Borota	2016	-
MycSu74	culture	pericardium	Hungary	Mohács	2016	
MycSu75	culture	synovial fluid	Hungary	Mohács	2016	-
MycSu76	culture	pleural exudate	Hungary	Hajdúszoboszló	2016	B
MycSu78	culture	synovial fluid	Hungary	Hajdúszoboszló	2016	B
MycSu86	culture	lung	Hungary	Jánoshalma	2016	-
MycSu88	culture	synovial fluid	Hungary	Derecske	2016	-
MycSu90	culture	lung	Hungary	Orosháza	2016	-
MycSu92	culture	lung	Hungary	Baracska	2016	-
MycSu94	culture	nasal swab	Hungary	Fadd	2017	-
MycSu97	culture	nasal swab	Hungary	Alsómocsolád	2017	A
MycSu99	culture	nasal swab	Hungary	Szalánta	2017	-
MycSu101	culture	pericardial matter	Hungary	Bácsalmás, Herd A	2017	A
MycSu103	culture	lung	Hungary	Jánoshalma	2017	-
MycSu105	culture	meninx	Hungary	Tiszainoka	2017	-
MycSu106	culture	bursa	Hungary	Létavértes	2017	-
MycSu107	culture	pericardial matter	Hungary	Nagymágocs	2017	-
MycSu109	culture	meninx	Hungary	Bábolna	2017	-
MycSu110	culture	pericardial matter	Hungary	Városföld	2017	-
MycSu111	culture	lung (wild boar)	Hungary	Óbánya	2017	-
MycSu112	culture	conjunctiva swab	Hungary	Vásárosnamény	2017	-
MycSu113	culture	pleura	Hungary	Hajdúnánás-Tedej	2017	-
MycSu114	culture	synovial fluid	Hungary	Szőny	2017	-
MycSu115	culture	pleural exudate	Hungary	Mohács	2019	-
MycSu116	culture	pericardium	Hungary	Gerde	2019	-
MycSu117	culture	pleural exudate	Hungary	Veszprémvasvári	2019	-

Supplementary table 1 continued.

Original ID	Sample	Sample type	Country of origin	Herd	Isolation year	Integration
MycSu127	culture	serosa	Hungary	Bácsalmás, Herd B	2018	A
MycSu128	culture	serosa	Hungary	Bácsalmás, Herd A	2018	A
MycSu134	culture	serosa	Hungary	Szeged	2019	-
MycSu141	culture	serosa	Hungary	Bácsalmás Herd A	2019	A
MycSu150	culture	lung	Hungary	Bácsalmás Herd B	2019	A
MycSu152	culture	serosa	Hungary	Bácsalmás, Herd A	2019	A
MycSu158	culture	serosa	Hungary	Hajdúszovát	2019	-
MycSu160	culture	pericardium	Hungary	Hajdúszoboszló	2019	B
MycSu162	culture	serosa	Hungary	Városföld	2019	-
MycSu166	culture	serosa	Hungary	Bácsalmás, Herd A	2019	A
MycSu167	culture	serosa	Hungary	Baracska	2019	-
MycSu171	culture	lung	Hungary	Szentes	2019	-
MycSu181	culture	serosa	Hungary	Bácsalmás, Herd A	2020	A
MycSu189	culture	lung	Hungary	Kisújszállás	2020	-
MycSu199	culture	synovial fluid	Hungary	Lajoskomárom	2020	-
MycSu204	culture	pleural exudate	Hungary	Szarvas	2020	-
MycSu206	culture	serosa	Hungary	not available	2020	-
MycSu207	culture	serosa	Hungary	Eger	2020	-
MycSu210	culture	lung	Hungary	Nádudvar	2020	-
MycSu212	culture	lung, serosa	Hungary	Bácsalmás, Herd A	2020	A
MycSu213	culture	nasal swab	Hungary	Komárom	2020	-
MycSu223	culture	serosa	Hungary	Zámoly	2021	-
MycSu230	culture	pericardium	Hungary	Palé	2021	-
Ge-1	culture	serosa	Germany		2020	-
Ge-2	culture	serosa	Germany		2020	-
Ge-3	culture	joint	Germany		2019	-
Ge-4	culture	joint	Germany		2019	-
Ge-5	culture	joint	Germany		2019	-

Supplementary table 1 continued.

Original ID	Sample	Sample type	Country of origin	Herd	Isolation year	Integration
Ge-6	culture	serosa	Germany		2019	-
Ge-7	culture	lung	Germany		2021	-
Ge-8	culture	serosa	Germany		2021	-
Ge-9	culture	lung	Germany		2021	-
Ge-10	culture	lung	Germany		2020	-
Ge-11	culture	lung	Germany		2019	-
Ge-12	culture	lung	Germany		2021	-
Ge-13	culture	lung	Germany		2021	-
Ge-14	culture	lung	Germany		2021	-
Ge-15	culture	joint	Germany		2021	-
Bl-1	culture	joint	Belgium		2021	-
Bl-2	culture	joint	Belgium		2021	-
It-1	culture	joint	Italy		2020	-
It-2	culture	lung	Italy		2020	-
It-3	culture	lung	Italy		2020	-
It-4	culture	lung	Italy		2020	-
It-5	culture	pericardium	Italy		2019	-
It-6	culture	pericardium	Italy		2020	-
It-7	culture	lung	Italy		2020	-
It-8	culture	lung	Italy		2020	-
It-9	culture	lung	Italy		2020	-
It-10	culture	nasal swab	Italy		2020	-
It-11	culture	lung	Italy		2020	-
It-12	culture	lung	Italy		2019	-
It-13	culture	lung	Italy		2019	-
It-14	culture	lung	Italy		2019	-
It-15	culture	pericardium	Italy		2020	-
It-16	culture	pericardium	Italy		2021	-

Supplementary table 1 continued.

Original ID	Sample	Sample type	Country of origin	Herd	Isolation year	Integration
It-17	culture	pericardium	Italy		2020	-
It-18	culture	lung	Italy		2019	-
It-19	culture	lung	Italy		2019	-
It-20	culture	lung	Italy		2020	-
Po-1	culture	nasal swab	Poland		2020	-
Po-2	culture	nasal swab	Poland		2020	-
Po-3	culture	nasal swab	Poland		2020	-
Po-4	culture	lung	Poland		2021	-
Po-5	culture	serosa	Poland		2021	-
Po-6	culture	abdominal exudate	Poland		2021	-
Po-7	culture	lung	Poland		2020	-
Po-8	culture	lung	Poland		2019	-
Po-9	culture	lung	Poland		2019	-
Po-10	culture	lung	Poland		2019	-
Po-11	culture	lung	Poland		2020	-
Po-12	culture	lung	Poland		2020	-
Po-13	culture	pericardium	Poland		2020	-
Po-14	culture	lung	Poland		2020	-
Po-15	culture	pericardium	Poland		2020	-
Po-16	culture	lung	Poland		2020	-
Po-17	culture	pericardium	Poland		2021	-
Po-18	culture	lung	Poland		2021	-
Po-19	culture	lung	Poland		2021	-
Group IV-IV A-n	culture	nasal swab	MycSu160 re-isolate			-
Group IV-IV A-t	culture	tarsus swab	MycSu160 re-isolate			-
Group IV-IV B-e	culture	elbow swab	MycSu160 re-isolate			-
Group IV-IP A-c	culture	carpus swab	MycSu160 re-isolate			-
Group IV-IP A-n	culture	nasal swab	MycSu160 re-isolate			-

Supplementary table 1 continued.

Original ID	Sample	Sample type	Country of origin	Herd	Isolation year	Integration
Group IV-IP B-e	culture	elbow swab	MycSu160 re-isolate			-
Group IV-IP B-t	culture	tarsus swab	MycSu160 re-isolate			-
Group IV-IP B-s	culture	stifle swab	MycSu160 re-isolate			-
S556/1 (MycSu76)	DNA	pleural exudate	Hungary	Hajdúszoboszló	2016	B
Su2 (MycSu88)	DNA	synovial fluid	Hungary	Derecske	2016	-
Su21 (MycSu101)	DNA	pericardial matter	Hungary	Bácsalmás, Herd A	2017	A
Su28 (MycSu105)	DNA	meninx	Hungary	Tiszainoka	2017	-
Su29 (MycSu106)	DNA	bursa	Hungary	Létavértes	2017	-
Su31 (MycSu107)	DNA	pericardial matter	Hungary	Nagymágocs	2017	-
Su38 (MycSu110)	DNA	pericardial matter	Hungary	Városföld	2017	-
Su41 (MycSu112)	DNA	conjunctiva swab	Hungary	Vásárosnamény	2017	-
Su47 (MycSu114)	DNA	synovial fluid	Hungary	Szőny	2017	-
Su117 (MycSu161)	DNA	pericardial matter	Hungary	Hajdúszoboszló	2019	B
Su124 (MycSu166)	DNA	serosa	Hungary	Bácsalmás, Herd A	2019	A
Su131 (MycSu171)	DNA	lung	Hungary	Szentes	2019	-
20206/4 (MycSu206)	DNA	serosa	Hungary	not available	2020	-
20249/1 (MycSu212)	DNA	lung, serosa	Hungary	Bácsalmás, Herd A	2020	A
20251/A (MycSu213)	DNA	nasal swab	Hungary	Komárom	2020	-

* samples for which only whole genome sequences were used are indicated with the GenBank accession numbers.

Supplementary table 2: Summary of the tests which were performed on the samples in this study.

ID	AB	MAMA	WGS	MLSTn	MLSTp	MLSTs	MLVA	ELISA
DBS1050				+	+	+		
GDL-1				+	+	+		
HUB-1				+	+	+		
MCLD				+	+	+		
MDBK-IPV				+	+	+		
SK76				+	+	+		
NCTC 10130	X*	+		+	+	+	+	
MycSu21		+	+	+	+	+	+	
MycSu23		+	+	+	+	+	+	
MycSu24		+	+	+	+	+	+	
MycSu25		+	+	+	+	+	+	
MycSu27		+	+	+	+	+	+	
MycSu29		+	+	+	+	+	+	
MycSu32i				+			+	
MycSu32s				+			+	
MycSu32t		+	+	+	+	+	+	
MycSu54		+	+	+	+	+	+	
MycSu55		+	+	+	+	+	+	
MycSu56		+	+	+	+	+	+	
MycSu57		+	+	+	+	+	+	
MycSu58		+	+	+	+	+	+	
MycSu59		+	+	+	+	+	+	
MycSu60	X	+	+	+	+	+	+	
MycSu61		+	+	+	+	+	+	
MycSu62		+	+	+	+	+	+	
MycSu63		+	+	+	+	+	+	
MycSu64		+	+	+	+	+	+	
MycSu65		+	+	+	+	+	+	
MycSu74								+
MycSu75		+	+	+	+	+	+	

Supplementary table 2 continued.

ID	AB	MAMA	WGS	MLSTn	MLSTp	MLSTs	MLVA	ELISA
MycSu76		+	+	+	+	+	+	
MycSu78								+
MycSu86		+	+	+	+	+	+	
MycSu88		+	+	+	+	+	+	
MycSu90		+	+	+	+	+	+	
MycSu92		+	+	+	+	+	+	
MycSu94		+	+	+	+	+	+	
MycSu97		+	+	+	+	+	+	
MycSu99		+	+	+	+	+	+	
MycSu101		+	+	+	+	+	+	
MycSu103		+	+	+	+	+	+	
MycSu105		+	+	+	+	+	+	+
MycSu106		+	+	+	+	+	+	
MycSu107		+						
MycSu109		+						
MycSu110		+						
MycSu111		+						
MycSu112		+						
MycSu113		+						
MycSu114		+						
MycSu115	X*	+						
MycSu116	X*	+						
MycSu117	X*	+						
MycSu118	X*	+						
MycSu127	X	+	+	+	+	+	+	
MycSu128	X	+	+	+	+	+	+	
MycSu134	X*	+						
MycSu141	X*	+	+	+	+	+	+	+
MycSu150	X*	+	+	+	+	+	+	
MycSu152	X	+	+	+	+	+	+	

Supplementary table 2 continued.

ID	AB	MAMA	WGS	MLSTn	MLSTp	MLSTs	MLVA	ELISA
MycSu158	X*	+						
MycSu160	X*	+	+	+	+	+	+	
MycSu162	X*	+						
MycSu166	X	+						
MycSu167	X*	+						
MycSu171	X*	+						
MycSu181	X	+						
MycSu189	X*	+						
MycSu199	X*	+						+
MycSu204	X*	+						
MycSu206	X	+						+
MycSu207	X*	+						
MycSu210	X*	+						
MycSu212	X	+						
MycSu213	X*	+						
MycSu223	X*	+						
MycSu230	X*	+						
Ge-1	X*	+						
Ge-2	X*	+						
Ge-3	X*	+						
Ge-4	X*	+						
Ge-5	X*	+						
Ge-6	X*	+						
Ge-7	X*	+						
Ge-8	X*	+						
Ge-9	X*	+						
Ge-10	X*	+						
Ge-11	X*	+						
Ge-12	X*	+						
Ge-13	X*	+						

Supplementary table 2 continued.

ID	AB	MAMA	WGS	MLSTn	MLSTp	MLSTs	MLVA	ELISA
Ge-14	X*	+						
Ge-15	X*	+						
Bl-1	X*	+						
Bl-2	X*	+						
It-1	X*	+						
It-2	X*	+						
It-3	X*	+						
It-4	X*	+						
It-5	X*	+						
It-6	X*	+						
It-7	X*	+						
It-8	X*	+						
It-9	X*	+						
It-10	X*	+						
It-11	X*	+						
It-12	X*	+						
It-13	X*	+						
It-14	X*	+						
It-15	X*	+						
It-16	X*	+						
It-17	X*	+						
It-18	X*	+						
It-19	X*	+						
It-20	X*	+						
Po-1	X*	+						
Po-2	X*	+						
Po-3	X*	+						
Po-4	X*	+						
Po-5	X*	+						
Po-6	X*	+						

Supplementary table 2 continued.

ID	AB	MAMA	WGS	MLSTn	MLSTp	MLSTs	MLVA	ELISA
Po-7	X*	+						
Po-8	X*	+						
Po-9	X*	+						
Po-10	X*	+						
Po-11	X*	+						
Po-12	X*	+						
Po-13	X*	+						
Po-14	X*	+						
Po-15	X*	+						
Po-16	X*	+						
Po-17	X*	+						
Po-18	X*	+						
Po-19	X*	+						
Group IV-IV A-n							+	
Group IV-IV A-t				+			+	
Group IV-IV B-e							+	
Group IV-IP A-c				+			+	
Group IV-IP A-n				+			+	
Group IV-IP B-e							+	
Group IV-IP B-t				+			+	
Group IV-IP B-s				+			+	
S556/1 (MycSu76)		+						
Su2 (MycSu88)		+						
Su21 (MycSu101)		+						
Su28 (MycSu105)		+						
Su29 (MycSu106)		+						
Su31 (MycSu107)		+						
Su38 (MycSu110)		+						
Su41 (MycSu112)		+						
Su47 (MycSu114)		+						

Supplementary table 2 continued.

ID	AB	MAMA	WGS	MLSTn	MLSTp	MLSTs	MLVA	ELISA
Su117 (MycSu161)		+						
Su124 (MycSu166)		+						
Su131 (MycSu171)		+						
20206/4 (MycSu206)		+						
20249/1 (MycSu212)		+						
20251/A (MycSu213)		+						
Total	**85**	**138**	**38**	**52**	**45**	**45**	**49**	**6**

Markings: X: antibiotic susceptibility by broth microdilution for one or more macrolide and/or lincomycin X*: antibiotic susceptibility testing of recent *M. hyorhinis* isolates from Europe, +: mismatch amplification mutation assay, whole genome sequencing, genotyping or used for development of enzyme-linked immunosorbent assay (ELISA) in this study. Abbreviations: AB: antibiotic susceptibility testing by broth microdilution, MAMA: mismatch amplification mutation assay, WGS: whole genome sequencing, MLST: multi-locus sequence typing MLSTn: novel MLST, this study, MLSTp: previously published MLST (Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016), MLSTs: previously published MLST with surface protein genes (Clavijo *et al.*, 2019), MLVA: multiple-locus variable-number tandem-repeat analysis. For the DNA samples, the corresponding isolates are indicated in brackets.

102

Supplementary table 3: Antibiotic susceptibility test results by broth microdilution and molecular biological mismatch amplification mutation assay (MAMA) methods.

ID	Minimal Inhibitory Concentrations (µg/ml)											Melt-MAMA		Agarose-MAMA	
	Tia	Dox	Oxy*	Flo	Enr	Tyl	Til	Tyv*	Tul	Gam	Lin	Melt (°C)	GT	Size (bp)	GT
NCTC 10130	0.078	≤0.039	≤0.125	2	0.625	≤0.25	2	≤0.039	4	0.5	0.5	78.6	L	119	L
MycSu21						≤0.25	2	≤0.25	2	0.5	0.5	78.6	L	119	L
MycSu23						0.5	1	≤0.25	4	0.5	0.5	78.6	L	119	L
MycSu24						64	>64	8	>64	>64	>64	81.6	H	139	H
MycSu25						0.5	2	≤0.25	4	2	1	78.6	L	119	L
MycSu27						>64	>64	8	>64	>64	>64	81.6	H	139	H
MycSu29						1	4	≤0.25	4	2	1	78.6	L	119	L
MycSu32t						>64	>64	8	>64	>64	>64	81.6	H	139	H
MycSu54						0.5	2	≤0.25	2	1	≤0.25	78.6	L	119	L
MycSu55						≤0.25	2	≤0.25	2	1	≤0.25	78.3	L	119	L
MycSu56						0.5	4	≤0.25	4	2	0.5	78.3	L	119	L
MycSu57						64	>64	4	>64	>64	>64	81.3	H	139	H
MycSu58						0.5	4	≤0.25	4	2	0.5	78.6	L	119	L
MycSu59						0.5	2	≤0.25	4	2	0.5	78.6	L	119	L
MycSu60						64	>64	4	>64	32	>64	81.6	H	139	H
MycSu61						0.5	2	≤0.25	4	1	0.5	78.6	L	119	L
MycSu62						>64	>64	8	>64	>64	>64	81.6	H	139	H
MycSu63						0.5	4	≤0.25	4	1	0.5	78.6	L	119	L
MycSu64						≤0.25	1	≤0.25	2	0.5	≤0.25	78.3	L	119	L
MycSu65						0.5	2	≤0.25	4	1	0.5	78.3	L	119	L
MycSu75						0.5	2	≤0.25	4	1	0.5	78.6	L	119	L
MycSu76						≤0.25	1	≤0.25	4	1	0.5	78.6	L	119	L
MycSu86						>64	>64	4	>64	>64	>64	81.6	H	139	H
MycSu88						≤0.25	1	≤0.25	4	1	≤0.25	78.6	L	119	L
MycSu90						>64	>64	4	>64	>64	>64	81.6	H	139	H
MycSu92						1	2	≤0.25	2	1	0.5	78.6	L	119	L

Supplementary table 3 continued.

ID	Minimal Inhibitory Concentrations (µg/ml)											Melt-MAMA		Agarose-MAMA	
	Tia	Dox	Oxy	Flo	Enr	Tyl	Til	Tyv	Tul	Gam	Lin	Melt (°C)	GT	Size (bp)	GT
MycSu94						1	16	≤0.25	4	2	≤0.25	78.6	L	119	L
MycSu97						≤0.25	2	≤0.25	4	1	0.5	78.6	L	119	L
MycSu99						0.5	2	≤0.25	2	0.5	0.5	78.6	L	119	L
MycSu101						>64	>64	4	>64	>64	>64	81.6	H	139	H
MycSu103						64	>64	4	>64	>64	>64	81.6	H	139	H
MycSu105						0.5	2	≤0.25	2	1	≤0.25	78.6	L	119	L
MycSu106						>64	>64	4	>64	>64	>64	81.6	H	139	H
MycSu107						≤0.25	1	≤0.25	4	2	1	78.6	L	119	L
MycSu109						≤0.25	1	≤0.25	4	2	1	78.9	L	119	L
MycSu110						≤0.25	1	≤0.25	4	1	0.5	78.6	L	119	L
MycSu111						≤0.25	1	≤0.25	4	1	0.5	78.6	L	119	L
MycSu112						≤0.25	1	≤0.25	4	1	0.5	78.6	L	119	L
MycSu113						>64	>64	4	>64	>64	>64	81.6	H	139	H
MycSu114						>64	>64	4	>64	>64	>64	81.6	H	139	H
MycSu115	0.078	0.078	0.25	1	0.625	32	>64	2.5	>64		>64	82.20	H		
MycSu116	0.078	≤0.039	≤0.125	0.5	0.312	≤0.25	2	≤0.039	4		0.5	78.90	L		
MycSu117	0.078	≤0.039	≤0.125	0.5	0.312	≤0.25	2	≤0.039	4		0.5	78.60	L		
MycSu118	0.078	0.156	0.5	4	0.625	32	>64	5	>64		>64	82.20	H		
MycSu127						0.5	2	<0.25	1	1	0.5	78.6	L	119	L
MycSu128						32	64	4	64	1	>64	81.6	H	139	H
MycSu134	0.078	≤0.039	≤0.125	0.25	0.312	0.5	1	≤0.039	4		0.5	78.60	L		
MycSu141	0.078	0.078	≤0.125	2	0.625	32	>64	5	>64	16	>64	81.60	H	139	H
MycSu150	≤0.039	0.156	0.5	1	0.625	≤0.25	2	≤0.039	4	2	0.5	78.60	L	119	L
MycSu152						64	>64	4	>64	8	>64	81.6	H	139	H
MycSu158	0.078	≤0.039	0.25	1	0.625	32	>64	5	>64		>64	81.60	H		
MycSu160	0.078	≤0.039	≤0.125	0.5	0.312	≤0.25	2	≤0.039	4		0.5	79.50	L		

Supplementary table 3 continued.

ID	Minimal Inhibitory Concentrations (µg/ml)											Melt-MAMA		Agarose-MAMA	
	Tia	Dox	Oxy	Flo	Enr	Tyl	Til	Tyv	Tul	Gam	Lin	Melt (°C)	GT	Size (bp)	GT
MycSu162	≤0.039	≤0.039	0.25	0.5	0.312	≤0.25	2	≤0.039	4		0.5	78.90	L		
MycSu166						>64	>64	4	>64	16	>64	81.6	H	139	H
MycSu167	0.156	≤0.039	≤0.125	2	0.625	32	>64	2.5	>64		>64	81.60	H		
MycSu171	≤0.039	0.078	≤0.125	1	0.625	≤0.25	2	≤0.039	4		0.5	78.60	L		
MycSu181									>64			81.9	H	139	H
MycSu189	0.156	≤0.039	≤0.125	1	0.625	64	>64	2.5	>64		>64	82.20	H		
MycSu199	0.078	≤0.039	≤0.125	2	0.312	≤0.25	2	≤0.039	8		0.5	78.60	L		
MycSu204	0.156	≤0.039	≤0.125	1	0.625	≤0.25	2	0.078	4		≤0.25	78.90	L		
MycSu206							2		2			78.9	L	119	L
MycSu207	0.156	≤0.039	≤0.125	2	0.625	≤0.25	2	≤0.039	4		0.5	78.90	L		
MycSu210	0.156	≤0.039	≤0.125	1	0.078	≤0.25	1	≤0.039	8		≤0.25	78.60	L		
MycSu212						64	>64	4	>64	16	>64	81.9	H	139	H
MycSu213	0.078	≤0.039	≤0.125	1	0.312	64	>64	1.25	>64		>64	82.20	H		
MycSu223	0.156	≤0.039	≤0.125	1	0.625	64	>64	2.5	>64		>64	82.20	H		
MycSu230	0.312	0.156	0.25	2	10	>64	>64	5	>64		>64	82.20	H		
Ge-1	0.312	0.156	≤0.125	1	0.625	>64	>64	5	>64		>64	81.60	H		
Ge-2	0.156	0.078	≤0.125	2	0.625	0.5	4	≤0.039	8		1	78.60	L		
Ge-3	0.156	0.078	≤0.125	2	0.625	0.5	4	≤0.039	8		1	78.90	L		
Ge-4	0.156	0.078	≤0.125	2	0.625	0.5	4	0.078	8		1	78.60	L		
Ge-5	0.312	≤0.039	≤0.125	2	0.312	>64	>64	2.5	>64		64	82.50	H		
Ge-6	0.156	0.078	0.25	2	0.625	64	>64	2.5	>64		>64	81.60	H		
Ge-7	0.312	0.078	≤0.125	2	0.625	64	>64	5	>64		>64	81.60	H		
Ge-8	0.312	≤0.039	≤0.125	1	1.25	>64	>64	5	>64		>64	82.20	H		
Ge-9	0.156	≤0.039	≤0.125	2	0.625	>64	>64	5	>64		>64	82.20	H		
Ge-10	0.078	≤0.039	≤0.125	1	0.312	0.5	1	≤0.039	2		≤0.25	78.90	L		
Ge-11	0.312	≤0.039	≤0.125	1	0.625	>64	>64	5	>64		>64	82.20	H		

Supplementary table 3 continued.

ID	Minimal Inhibitory Concentrations (µg/ml)											Melt-MAMA		Agarose-MAMA	
	Tia	Dox	Oxy	Flo	Enr	Tyl	Til	Tyv	Tul	Gam	Lin	Melt (°C)	GT	Size (bp)	GT
Ge-12	0.312	0.078	≤0.125	2	1.25	64	>64	10	>64		>64	81.90	H		
Ge-13	0.312	0.078	≤0.125	2	1.25	>64	>64	>10	>64		>64	78.90	L		
Ge-14	0.312	0.078	≤0.125	1	0.625	>64	>64	5	>64		>64	82.20	H		
Ge-15	0.312	0.078	≤0.125	1	0.625	>64	>64	5	>64		>64	81.90	H		
Bl-1	0.312	0.078	≤0.125	2	0.625	>64	>64	5	>64		>64	82.20	H		
Bl-2	0.312	0.078	≤0.125	1	0.625	>64	>64	5	>64		>64	82.20	H		
It-1	0.156	0.078	≤0.125	2	0.625	64	>64	5	>64		>64	82.20	H		
It-2	0.156	0.078	0.25	2	0.625	64	>64	2.5	>64		>64	81.90	H		
It-3	0.156	0.156	0.25	2	0.625	32	>64	2.5	>64		>64	82.20	H		
It-4	0.156	≤0.039	≤0.125	2	0.625	64	>64	2.5	>64		>64	82.20	H		
It-5	0.312	0.078	≤0.125	2	0.625	>64	>64	5	>64		>64	81.90	H		
It-6	0.312	0.625	≤0.125	0.5	1.25	>64	>64	5	>64		>64	82.20	H		
It-7	0.156	0.078	≤0.125	2	0.625	0.5	4	≤0.039	8		0.5	79.20	L		
It-8	0.156	0.078	≤0.125	2	0.625	0.5	2	≤0.039	8		0.5	78.90	L		
It-9	0.156	0.156	0.25	2	0.625	0.5	4	0.078	2		1	78.90	L		
It-10	0.156	0.078	≤0.125	2	0.625	0.5	4	0.078	2		1	78.90	L		
It-11	0.078	≤0.039	≤0.125	1	0.625	≤0.25	2	≤0.039	4		≤0.25	78.90	L		
It-12	0.312	≤0.039	≤0.125	2	0.625	64	>64	5	>64		>64	82.50	H		
It-13	0.156	0.078	≤0.125	2	0.625	0.5	4	0.078	4		0.5	78.90	L		
It-14	0.312	0.312	0.5	2	0.625	>64	>64	>10	>64		>64	81.90	H		
It-15	0.156	0.156	0.25	2	0.625	≤0.25	2	≤0.039	4		0.5	78.90	L		
It-16	0.312	0.156	0.5	2	0.625	64	>64	2.5	>64		>64	82.20	H		
It-17	0.156	≤0.039	≤0.125	1	0.625	64	>64	5	>64		>64	82.20	H		
It-18	0.312	0.156	0.25	4	0.625	64	>64	5	>64		>64	81.90	H		
It-19	0.312	0.156	0.25	≤0.125	0.625	>64	>64	5	>64		>64	82.20	H		
It-20	0.312	0.078	0.25	0.5	0.625	64	>64	5	>64		>64	82.20	H		

Supplementary table 3 continued.

ID	Minimal Inhibitory Concentrations (µg/ml)											Melt-MAMA		Agarose-MAMA	
	Tia	Dox	Oxy	Flo	Enr	Tyl	Til	Tyv	Tul	Gam	Lin	Melt (°C)	GT	Size (bp)	GT
Po-1	0.625	≤0.039	≤0.125	2	0.625	>64	>64	5	>64		>64	82.50	H		
Po-2	0.312	≤0.039	≤0.125	0.5	0.625	>64	>64	5	>64		>64	82.50	H		
Po-3	0.625	≤0.039	≤0.125	1	0.312	>64	>64	5	>64		>64	82.50	H		
Po-4	0.156	0.078	0.25	2	0.625	>64	>64	10	>64		>64	78.90	L		
Po-5	0.156	≤0.039	≤0.125	1	0.312	≤0.25	2	≤0.039	4		0.5	79.20	L		
Po-6	0.156	0.156	0.25	≤0.125	2.5	≤0.25	4	≤0.039	4		0.5	78.90	L		
Po-7	0.312	≤0.039	≤0.125	1	0.625	≤0.25	4	0.078	4		0.5	79.50	L		
Po-8	0.312	≤0.039	≤0.125	≤0.125	0.625	>64	>64	5	>64		>64	82.50	H		
Po-9	0.078	≤0.039	≤0.125	1	0.625	>64	>64	5	>64		>64	78.90	L		
Po-10	0.156	≤0.039	≤0.125	0.5	1.25	0.5	2	≤0.039	4		≤0.25	78.90	L		
Po-11	≤0.039	0.078	0.5	2	0.625	0.5	4	0.078	4		0.5	78.90	L		
Po-12	0.156	0.078	0.5	2	0.625	0.5	4	0.078	4		1	79.50	L		
Po-13	0.078	0.078	0.5	2	0.625	0.5	4	0.078	4		1	78.90	L		
Po-14	0.625	0.078	≤0.125	0.25	2.5	>64	>64	5	>64		>64	81.90	H		
Po-15	0.078	0.078	≤0.125	2	0.312	≤0.25	1	≤0.039	4		≤0.25	79.50	L		
Po-16	0.156	≤0.039	≤0.125	0.5	1.25	64	>64	5	>64		>64	81.90	H		
Po-17	0.078	0.078	0.5	2	0.625	0.5	4	0.078	4		0.5	79.50	L		
Po-18	0.156	0.078	≤0.125	0.5	0.625	≤0.25	1	0.078	4		≤0.25	78.90	L		
Po-19	0.156	0.078	0.5	1	0.625	1	2	0.078	4		1	78.90	L		
S556/1 (MycSu76)												78.6	L	nd	nd
Su2 (MycSu88)												78.6	L	119	L
Su21 (MycSu101)												78.6	L	119/139	H and L
Su28 (MycSu105)												nd	nd	139	H
Su29 (MycSu106)												81.9	H	139	H
Su31 (MycSu107)												78.6	L	119	L
Su38 (MycSu110)												78.6	L	119	L

Supplementary table 3 continued.

ID	Minimal Inhibitory Concentrations (µg/ml)											Melt-MAMA		Agarose-MAMA	
	Tia	Dox	Oxy	Flo	Enr	Tyl	Til	Tyv	Tul	Gam	Lin	Melt (°C)	GT	Size (bp)	GT
Su41 (MycSu112)												78.6	L	119	L
Su47 (MycSu114)												81.6	H	139	H
Su117 (MycSu161)												81.6	H	139	H
Su124 (MycSu166)												81.6	H	139	H
Su131 (MycSu171)												78.3	L	119	L
20206/4 (MycSu206)												78.6	L	119	L
20249/1 (MycSu212)												81.6	H	139	H
20251/A (MycSu213)												81.6	H	139	H

Genotype H samples are highlighted grey, inconsistent results are highlighted black.

Abbreviations: melt-MAMA: differentiating based on melting temperature of the product, agarose-MAMA: differentiating based on size of the product, bp: base pair, GT: genotype, L/H: genotype L or H based on the point mutation in the 23S rRNA, nd: not detected.

Abbreviations of the antibiotics: Tia: tiamulin, Dox: doxycycline, Oxy: oxytetracycline, Flo: florfenicol, Enr: enrofloxacin, Tyl: tylosin, Til: tilmicosin, Tyv: tylvalosin, Tul: tulathromycin, Gam: gamithromycin, Lin: lincomycin. * marks the antibiotics which were examined in different ranges in the Pan-European study from the ones used by Bekő and co-workers (2019a).

Susceptibility testing of isolates with only data for macrolides and lincomycin were published before in the work of Bekő and co-workers (2019a).

Supplementary table 4: Results of the genotyping assays.

ID	MLSTn							MLSTp							MLSTs					MLVA						
	lA	*rB*	*rC*	*gX*	*uA*	*vS*	ST	*dA*	*rB*	*gB*	*gX*	*ak*	*gk*	ST	*pB*	*p95*	*mD*	*ug*	ST	Mhr 205	Mhr 396	Mhr 438	Mhr 441	Mhr 442	Mhr 444	GT
NCTC 10130	1	1	1	1	1	1	1	1	3	1	1	1	3	16	2	1	1	2	26	3	2	14	13	2	12	1
MycSu23	7	2	9	1	10	2	2	13	1	11	1	1	3	105	1	12	10	2	49	1	2	10	13	2	4	9
MycSu29	7	2	9	1	10	2	2	13	1	11	1	1	3	105	1	12	10	2	49	1	2	11	13	2	21	10
MycSu62	7	2	9	1	10	2	2	13	1	11	1	1	3	105	1	12	10	2	49	1	2	16	13	2	24	7
MycSu32i	7	2	4	1	10	2	3													1	2	np	8	2	22	2
MycSu32s	7	2	4	1	10	2	3													1	2	np	8	2	22	2
MycSu32t	7	2	4	1	10	2	3	13	1	11	1	1	1	107	1	12	10	2	49	1	2	np	8	2	22	2
MycSu101	7	2	4	1	10	2	3	13	1	11	1	1	3	105	1	12	10	2	49	1	2	11	13	2	21	10
MycSu127	7	2	4	1	10	2	3	13	1	11	1	1	1	107	1	12	10	2	49	1	2	np	13	2	15	8
MycSu128	7	2	4	1	10	2	3	13	1	11	1	1	3	105	1	12	10	2	49	1	2	np	8	2	25	3
MycSu150	7	2	4	1	10	2	3	13	1	11	1	1	1	107	1	12	10	2	49	1	2	np	17	2	24	6
MycSu141	4	3	2	1	7	3	4	1	9	11	1	1	3	114	1	4	3	2	14	1	2	np	11	2	24	5
MycSu152	4	2	2	1	10	3	5	13	1	1	1	1	3	115	1	12	3	2	41	1	2	np	16	2	26	4
MycSu94	5	8	4	10	3	2	6	1	1	1	1	1	4	112	1	2	11	2	44	2	2	np	12	2	15	33
MycSu63	4	2	7	1	1	9	7	1	1	1	1	1	3	13	1	2	23	2	51	2	2	21	2	2	23	34
MycSu103	4	11	3	2	1	2	8	1	19	4	9	1	3	127	2	1	15	2	62	1	2	3	15	2	24	17
MycSu64	4	10	8	1	11	9	9	1	19	14	1	1	11	122	9	1	3	2	40	1	3	19	2	2	22	28
MycSu65	4	15	8	11	11	9	10	1	1	2	16	1	3	123	9	1	3	2	40	1	2	22	11	2	25	21
MycSu55	11	7	4	7	1	7	11	1	1	2	1	1	3	40	8	1	16	2	63	1	2	22	12	2	24	24
MycSu75	11	2	2	1	1	8	12	8	1	9	1	1	3	111	1	3	3	2	42	1	2	21	15	np	19	36
MycSu86	8	2	2	5	5	4	13	3	1	6	9	11	3	124	1	2	29	2	57	2	2	20	15	2	27	35
MycSu105	10	2	1	1	9	9	14	1	1	11	15	1	3	128	11	1	11	2	45	1	2	24	14.5	2	17	12
MycSu59	8	12	4	4	4	4	15	1	19	4	1	1	3	120	1	1	34	2	67	1	2	23	12	2	24	25
MycSu61	8	13	4	5	1	4	16	1	1	2	9	1	3	110	1	1	31	2	58	1	2	10	4	2	23	15
MycSu90	9	15	4	6	1	4	17	10	1	2	1	1	3	88	3	1	33	2	61	1	1	23	np	2	4	19
MycSu106	11	15	6	1	6	4	18	1	1	1	1	1	3	13	12	1	30	2	59	1	2	21	12	2	27	23
MycSu54	6	15	5	8	1	4	19	1	1	2	15	1	3	118	1	1	11	2	43	1	2	24	16	2	24	14

Supplementary table 4 continued.

ID	MLSTn							MLSTp							MLSTs					MLVA						
	lA	*rB*	*rC*	*gX*	*uA*	*vS*	ST	*dA*	*rB*	*gB*	*gX*	*ak*	*gk*	ST	*pB*	*p95*	*mD*	*ug*	ST	Mhr 205	Mhr 396	Mhr 438	Mhr 441	Mhr 442	Mhr 444	GT
MycSu88	6	14	4	4	4	5	20	1	1	2	1	10	3	125	1	1	32	2	60	1	2	23	14	2	28	27
MycSu25	6	15	10	4	8	4	21	1	1	8	9	1	4	106	3	1	35	2	66	1	2	24	6	2	23	13
MycSu24	11	6	3	2	1	4	22	1	1	2	1	1	3	40	1	2	22	2	52	3	2	23	7	2	23	30
MycSu27	11	6	3	2	1	4	22	1	1	2	1	1	3	40	1	2	24	2	53	1	2	16	12	2	25	22
MycSu97	4	2	3	3	1	4	23	1	1	8	17	1	3	126	1	1	26	2	55	1	2	17	15	2	23	16
MycSu92	8	2	4	2	1	6	24	3	1	2	1	1	3	108	1	2	21	2	50	2	2	np	15	np	18	37
MycSu57	8	5	11	1	1	4	25	3	1	2	1	1	4	109	1	2	28	2	54	1	2	24	5	2	28	11
MycSu56	8	2	11	2	1	4	26	3	1	2	1	1	3	108	1	2	28	2	54	2	2	np	11	2	27.5	32
MycSu58	8	2	11	8	1	4	27	1	1	8	15	1	3	119	1	2	28	2	54	1	1	5	15	2	24	18
MycSu21	8	2	4	8	8	9	28	1	1	14	4	1	3	117	1	1	27	2	56	1	2	23	10	2	25	26
MycSu60	2	9	4	8	1	9	29	1	19	2	4	12	3	121	1	1	34	2	67	1	2	22	16	2	15	20
MycSu76	3	2	2	9	1	4	30	10	1	2	1	1	3	88	10	2	16	2	64	3	2	np	14.5	2	22	31
MycSu99	1	2	2	8	1	4	31	1	1	6	4	1	3	113	1	11	10	2	48	1	2	19	14	np	22	29
GDL-1	1	2	16	1	1	10	32	1	1	1	1	1	1	1	2	8	11	1	46							
MCLD	1	2	15	1	1	10	33	1	1	1	1	1	1	1	2	10	11	1	47							
SK76	1	1	4	1	12	2	34	2	3	3	1	1	1	14	1	1	6	1	65							
HUB-1	1	16	1	12	13	9	35	6	5	6	4	2	5	27	7	9	20	6	39							
DBS1050	1	2	12	1	1	10	36	1	1	1	1	13	1	116	2	8	11	1	46							
MDBK-IPV	1	2	1	1	1	10	37	1	1	1	1	1	1	1	2	8	11	1	46							
MycSu160	11	2	17	2	1	4	38	3	1	2	1	1	3	108	1	2	16	2	68	1	2	np	15	np	46	38
Group IV-IV A-t	11	2	17	2	1	4	38													1	2	np	15	np	46	38
Group IV-IP A-c	11	2	17	2	1	4	38													1	2	np	15	np	42	39
Group IV-IP A-n	11	2	17	2	1	4	38													1	2	np	15	np	46	38
Group IV-IP B-t	11	2	17	2	1	4	38													1	2	np	15	np	48	40

Supplementary table 4 continued.

ID	MLSTn							MLSTp							MLSTs					MLVA						
	lA	***rB***	***rC***	***gX***	***uA***	***vS***	**ST**	***dA***	***rB***	***gB***	***gX***	***ak***	***gk***	**ST**	***pB***	***p95***	***mD***	***ug***	**ST**	**Mhr 205**	**Mhr 396**	**Mhr 438**	**Mhr 441**	**Mhr 442**	**Mhr 444**	**GT**
Group IV-IV A-n																				1	2	np	15	np	46	38
Group IV-IV B-e																				1	2	np	15	np	46	38

Abbreviations: MLST: multi-locus sequence typing; MLSTn: novel MLST, this study, MLSTp: previously published MLST (Tocqueville *et al.*, 2014; Trüeb *et al.*, 2016), MLSTs: previously published MLST with surface protein genes (Clavijo *et al.*, 2019), MLVA: multiple-locus variable-number tandem-repeat analysis, *lA: lepA, rB: rpoB, rC: rpoC, gX: gltX, uA: uvrA, vS: valS, dA: dnaA, gB: gyrB, ak:adk, gk:gmk, pB: pdhB, mD: mtlD, ug:ung*, ST: sequence type, GT: genotype, np: no product.
Isolates are in the order of their ST in the MLSTn system. Cells are crossed when the test was not performed with the isolate. New allele types and sequence types are highlighted for each previously developed system.

Supplementary table 5: Parsimony informative sites (number and percent), number of allele or sequence types and Simpson index of diversity of the three compared multi-locus sequence typing (MLST) methods and the multiple-locus variable-number tandem-repeat analysis (MLVA).

		Parsim-info	Parsim%	Allele type	Simpson's ID	CI (95%)
MLSTn	*lepA*	6/444	1.35	11	0.876	0.845-0.907
	rpoB	7/472	1.48	15	0.685	0.534-0.836
	rpoC	8/565	1.41	15	0.860	0.784-0.935
	gltX	6/410	1.46	12	0.737	0.613-0.861
	uvrA	6/518	1.16	12	0.705	0.578-0.832
	valS	5/657	0.76	10	0.795	0.723-0.867
	concatenated	38/3063	1.24	38	0.986	0.969-1.000
MLSTp	*dnaA*	3/435	0.69	7	0.624	0.490-0.758
	rpoB	2/496	0.40	5	0.320	0.147-0.494
	gyrB	5/291	1.72	9	0.818	0.759-0.877
	gltX	3/370	0.81	6	0.484	0.311-0.656
	adk	none		6	0.212	0.050-0.375
	gmk	2/435	0.46	5	0.468	0.309-0.627
	concatenated	15/2304	0.65	31	0.976	0.958-0.994
MLSTs	*pdhB*	3/415	0.72	9	0.543	0.376-0.711
	p95	4/289	1.38	9	0.768	0.695-0.840
	mtlD	109/537	20.30	22	0.925	0.883-0.967
	ung	1/200	0.50	3	0.241	0.085-0.398
	concatenated	117/1441	8.12	32	0.964	0.928-1.000
MLVA	Mhr205			3	0.355	0.176-0.534
	Mhr396			3	0.148	1.000-0.299
	Mhr438			14	0.891	0.828-0.954
	Mhr441			16	0.924	0.888-0.967
	Mhr442			2	0.189	0.034-0.343
	Mhr444			16	0.923	0.878-0.968
	All alleles			38	0.999	0.986-1.000

Parsim-info: number of parsimony informative sites, Parsim%: rate of parsimony informative sites, Allele type: number of alleles for the gene fragments and the concatenated sequence (sequence type); Simpson's ID: index of diversity, CI: 95% confidence interval.

Supplementary table 6: Rectal temperatures (°C) measured during the challenge study.

Animal ID	Group IV-IV						Group IV-IP						Control Group			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Days																
D-2	38.3	38.6	38.8	39.1	39.6	39.2	38.1	39.7	39.4	39.5	39.0	39.0	39.6	39.2	38.5	39.6
D-1	38.6	38.8	39.0	39.4	38.7	39.0	37.2	39.5	39.2	39.6	39.0	38.7	39.0	39.4	38.8	39.6
D0	39.5	39.1	39.4	39.1	39.6	39.6	38.2	39.1	39.3	39.7	38.5	39.1	39.7	39.0	38.5	39.7
D1	39.6	39.2	38.9	39.0	39.4	39.4	39.2	39.3	39.5	39.9	39.4	39.4	39.2	39.1	38.9	39.6
D2	39.4	39.4	39.1	39.4	39.1	39.4	39.3	38.8	39.5	39.4	39.2	39.3	39.8	39.6	39.0	40.0
D3	39.3	39.5	38.9	39.3	39.3	38.9	38.9	39.5	39.2	39.7	39.3	39.1	39.7	39.2	39.0	39.5
D4	39.5	39.3	39.1	39.9	39.6	39.5	39.0	39.2	39.6	**40.0**	38.8	39.3	39.5	39.0	39.1	39.6
D5	40.0	38.8	39.0	39.5	39.2	39.5	38.8	38.9	39.1	**40.0**	38.5	39.1	39.2	39.0	38.2	39.6
D6	39.2	39.4	38.8	39.4	39.0	39.8	39.4	39.5	38.8	**40.3**	39.2	39.5	39.2	39.0	38.4	39.4
D7	39.0	39.4	38.7	38.8	38.3	39.0	39.2	39.1	39.0	39.5	39.3	38.5	39.1	39.1	39.0	39.2
D8	39.4	39.8	38.5	39.6	39.8	39.8	38.8	39.1	39.2	39.8	38.8	38.4	38.9	39.0	39.1	39.4
D9	38.8	39.1	39.1	39.2	39.0	39.9	38.7	38.7	38.1	39.4	38.6	38.7	38.7	38.2	38.3	39.0
D10	38.5	38.7	38.6	39.0	38.8	39.0	38.1	38.9	39.0	39.6	39.3	39.0	39.2	38.7	38.5	38.8
D11	38.9	38.7	38.5	39.0	38.5	38.6	38.6	39.4	38.9	39.4	39.6	39.1	39.2	38.3	38.4	39.2
D12	38.2	39.0	38.9	33.9	39.2	39.2	38.5	38.9	38.6	39.4	39.0	39.1	38.8	39.3	38.7	39.2
D13	39.0	39.4	38.8	39.6	39.1	39.2	38.4	38.9	38.6	39.3	38.8	39.2	38.8	38.8	37.8	39.1
D14	39.3	39.1	38.8	39.4	39.2	39.0	38.8	39.5	39.0	39.3	39.1	39.3	38.8	38.7	38.0	39.0
D15	39.4	39.7	38.4	39.3	39.1	39.4	38.9	39.2	39.1	39.6	37.4	39.2	39.5	39.4	38.8	39.1
D16	39.0	38.6	38.2	38.5	39.1	38.8	38.7	39.7	39.3	39.4	39.2	39.5	39.7	39.0	38.6	39.6
D17	39.5	39.2	39.0	39.4	39.2	39.0	39.1	39.4	38.9	39.4	39.1	39.7	39.7	39.1	38.0	39.8
D18	39.2	39.2	38.8	39.1	39.2	39.2	40.0	39.5	39.6	39.4	39.6	39.6	39.4	38.8	37.8	39.2
D19	39.1	39.0	39.1	39.5	39.5	39.3	39.2	39.0	39.5	40.0	38.7	39.2	39.2	38.2	38.1	39.1
D20	39.0	38.2	38.8	39.0	39.0	38.7	38.4	39.0	39.1	39.0	39.4	39.0	39.3	39.2	38.4	39.3
D21	39.0	39.1	38.9	38.9	39.0	38.9	39.0	39.1	39.2	39.5	39.2	38.8	39.7	39.0	38.7	39.4
D22	39.3	39.4	39.5	39.5	39.6	38.7	39.4	39.7	39.7	39.5	39.1	39.6	39.5	39.4	38.9	39.6
D23	39.1	39.2	39.1	39.3	39.9	39.3	38.5	38.8	38.5	39.2	39.1	38.7	39.2	39.1	38.6	39.1
D24	39.5	39.4	39.5	39.5	39.7	39.5	40.0	39.4	39.2	39.7	39.7	40.3	39.6	39.5	38.6	39.6
D25	39.3	39.4	39.0	39.5	39.5	39.4	39.0	39.0	38.8	39.1	38.8	39.1	39.6	39.2	38.8	39.7
D26	39.1	39.6	39.2	39.7	39.5	39.6	39.0	39.5	39.2	39.6	39.4	39.7	39.7	39.5	39.2	39.8
D27	39.4	39.5	39.3	39.4	39.6	39.3	38.8	39.4	39.0	39.2	39.4	39.4	39.2	39.1	38.0	39.5

Supplementary table 7: Results of isolation and nasal swab PCR tests from the challenge study.

	Group IV-IV						Group IV-IP						Control Group			
Animal ID **Sampling time**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**
D-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D5	-	-	-	-	-	-	-	-	+	++	-	-	-	-	-	-
D8	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-
D12	-	-	-	(+)	-	-	-	-	-	+	-	-	-	-	-	-
D15	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
D19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D21	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
D26	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-

114 (+): positive *Mycoplasma hyorhinis* isolation with negative PCR, +: positive *M. hyorhinis* PCR with negative isolation, ++: positive *M. hyorhinis* PCR and isolation.

Supplementary table 8: Detailed gross pathological and histopathological scores with the additional histopathological findings of each animal from the challenge study.

Group	Animal ID	Organ	Macro. score	Histo. score	Additional histological lesions
Group IV-IV	1	Pericardium	0	0	
		Pleura	0	0	
		Peritoneum	0	0	
		Stifle	1	0	
		Elbow	1	0	
		Tarsus	1	1	Scant occurrence of fibrin exudates and neutrophyl granulocytes in the cavity in one joint
		Carpus	2	3	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**5**	**4**	
Group IV-IV	2	Pericardium	2	3	
		Pleura	1	3	Multifocal, mixed inflammatory cell infiltration in the pleura
		Peritoneum	1	2	
		Stifle	1	3	
		Elbow	2	3	
		Tarsus	2	3	Scant occurrence of neutrophyl granulocytes in the cavity of all joints
		Carpus	0	3	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity/mucosal membrane	0	1	Mild to moderate mixed inflammatory cell infiltration around the mucosal glands
		Heart	1	0	
		Other organs	0	0	
		Total	**10**	**21**	

Supplementary table 8 continued.

Group	Animal ID	Organ	Macro. score	Histo. score	Additional histological lesions
Group IV-IV	3	Pericardium	0	0	
		Pleura	0	0	
		Peritoneum	0	0	
		Stifle	0	0	
		Elbow	0	0	
		Tarsus	1	2	Scant occurrence of neutrophyl granulocytes in the cavity of one joint
		Carpus	0	0	
		Conjunctiva	0	2	Acute, ulcerative inflammation
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	Hyperplasia of lymphoid follicles
		Heart	0	0	
		Other organs	0	0	
		Total	**1**	**4**	
Group IV-IV	4	Pericardium	0	0	
		Pleura	2	3	Multifocal subacute pleuritis, scant multinucleated giant cells in the pleura
		Peritoneum	0	0	
		Stifle	0	0	
		Elbow	0	0	
		Tarsus	1	3	Frequent occurrence of fibrin exudates and neutrophyl granulocytes in the cavity of one joint, and scant multinucleated giant cells in the intima and subintima of this joint
		Carpus	0	0	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**3**	**6**	

Supplementary table 8 continued.

Group	Animal ID	Organ	Macro. score	Histo. score	Additional histological lesions
Group IV-IV	5	Pericardium	2	3	Chronic, serous inflammation with adhesion
		Pleura	0	0	
		Peritoneum	0	0	
		Stifle	0	0	
		Elbow	0	0	
		Tarsus	0	0	
		Carpus	2	3	Scant occurrence of fibrin exudates and neutrophyl granulocytes in the cavity of one joint, and scant multinucleated giant cells in the intima and subintima of this joint
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**4**	**6**	
Group IV-IV	6	Pericardium	0	1	
		Pleura	0	3	
		Peritoneum	0	0	
		Stifle	0	0	
		Elbow	0	2	
		Tarsus	1	2	Scant occurrence of neutrophyl granulocytes in the cavity of one joint
		Carpus	0	0	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**1**	**8**	

Supplementary table 8 continued.

Group	Animal ID	Organ	Macro. score	Histo. score	Additional histological lesions
Group IV-IP	1	Pericardium	0	0	
		Pleura	0	2	Multifocal, mild infiltration of the pleura by mixed inflammatory cells
		Peritoneum	0	0	
		Stifle	1	3	Scant occurrence of neutrophyl granulocytes in the cavity of one joint
		Elbow	1	1	
		Tarsus	1	1	
		Carpus	0	0	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs: Trachea	0	0	Focal, mild mixed inflammatory cell infiltration in the adventitia of trachea
		Total	**3**	**7**	
Group IV-IP	2	Pericardium	0	0	
		Pleura	0	1	
		Peritoneum	0	0	
		Stifle	1	3	
		Elbow	0	0	
		Tarsus	0	0	
		Carpus	0	0	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**1**	**4**	

Supplementary table 8 continued.

Group	Animal ID	Organ	Macro. score	Histo. score	Additional histological lesions
Group IV-IP	3	Pericardium	2	3	Chronic, serous inflammation with adhesion
		Pleura	0	2	Multifocal, mild infiltration of the pleura by mixed inflammatory cells
		Peritoneum	0	1	
		Stifle	0	3	
		Elbow	1	3	Acute-subacute erosive synovitis associated with acute haemorrhages, frequent occurrence of fibrin exudates, blood cells and neutrophyl granulocytes in the cavity of two joints
		Tarsus	2	3	
		Carpus	0	1	
		Conjunctiva	0	1	Mild, multifocal, serous inflammation
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**5**	**17**	
Group IV-IP	4	Pericardium	2	3	Chronic inflammation with adhesion
		Pleura	0	1	Multifocal, mild infiltration of the pleura by mixed inflammatory cells
		Peritoneum	0	0	
		Stifle	0	3	
		Elbow	1	3	Scant occurrence of neutrophyl granulocytes in the cavity of two joints
		Tarsus	2	3	
		Carpus	2	3	Scant occurrence of neutrophyl granulocytes in the cavity of one joint and scant multinucleated giant cells in the intima and subintima of this joint
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**7**	**16**	

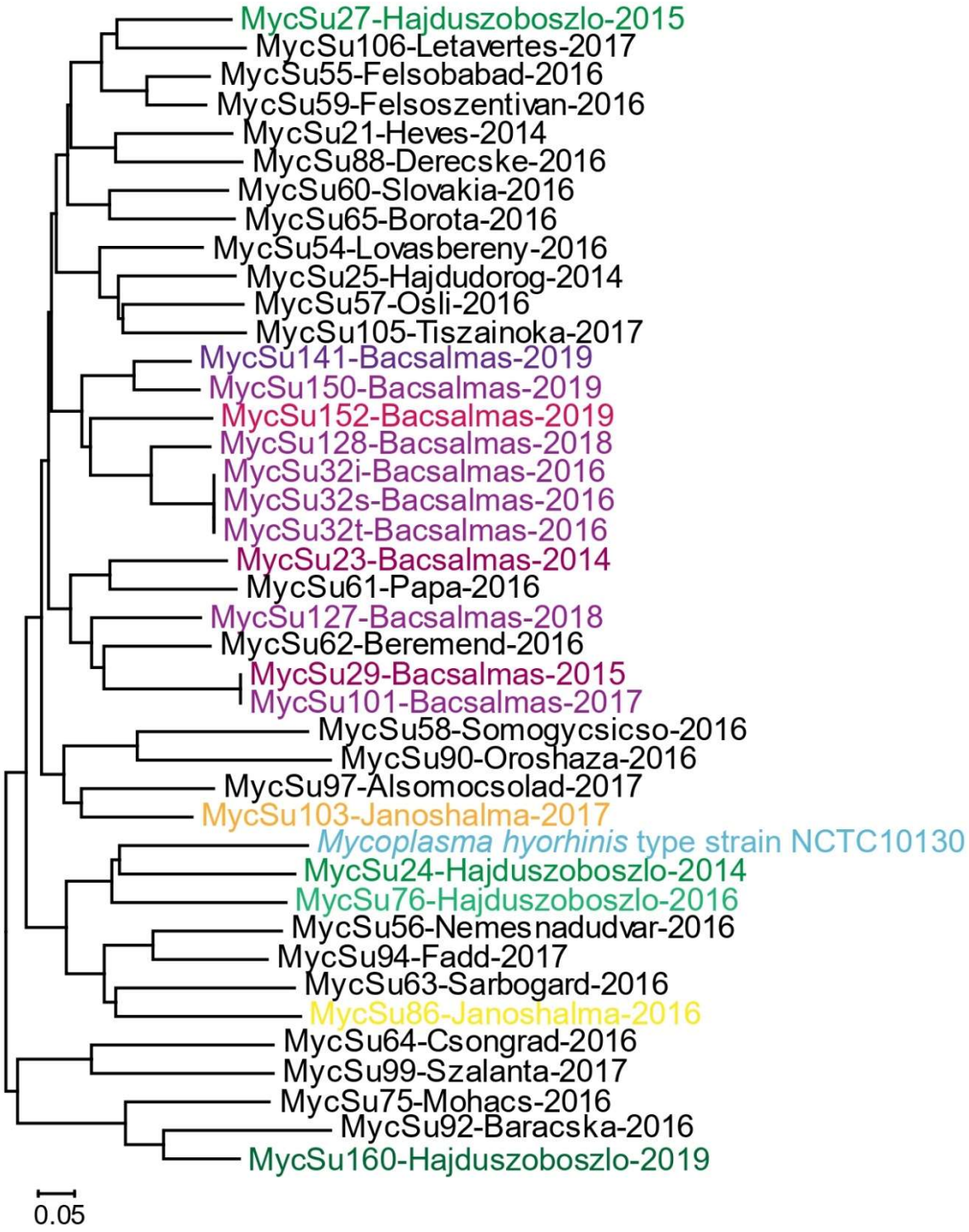
Supplementary table 8 continued.

Group	Animal ID	Organ	Macro. score	Histo. score	Additional histological lesions
Group IV-IP	5	Pericardium	0	0	
		Pleura	0	0	
		Peritoneum	0	0	
		Stifle	0	0	
		Elbow	1	0	
		Tarsus	0	0	
		Carpus	0	0	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**1**	**0**	
Group IV-IP	6	Pericardium	0	0	
		Pleura	0	0	
		Peritoneum	0	0	
		Stifle	2	0	
		Elbow	0	0	
		Tarsus	0	0	
		Carpus	1	0	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**3**	**0**	

Supplementary table 8 continued.

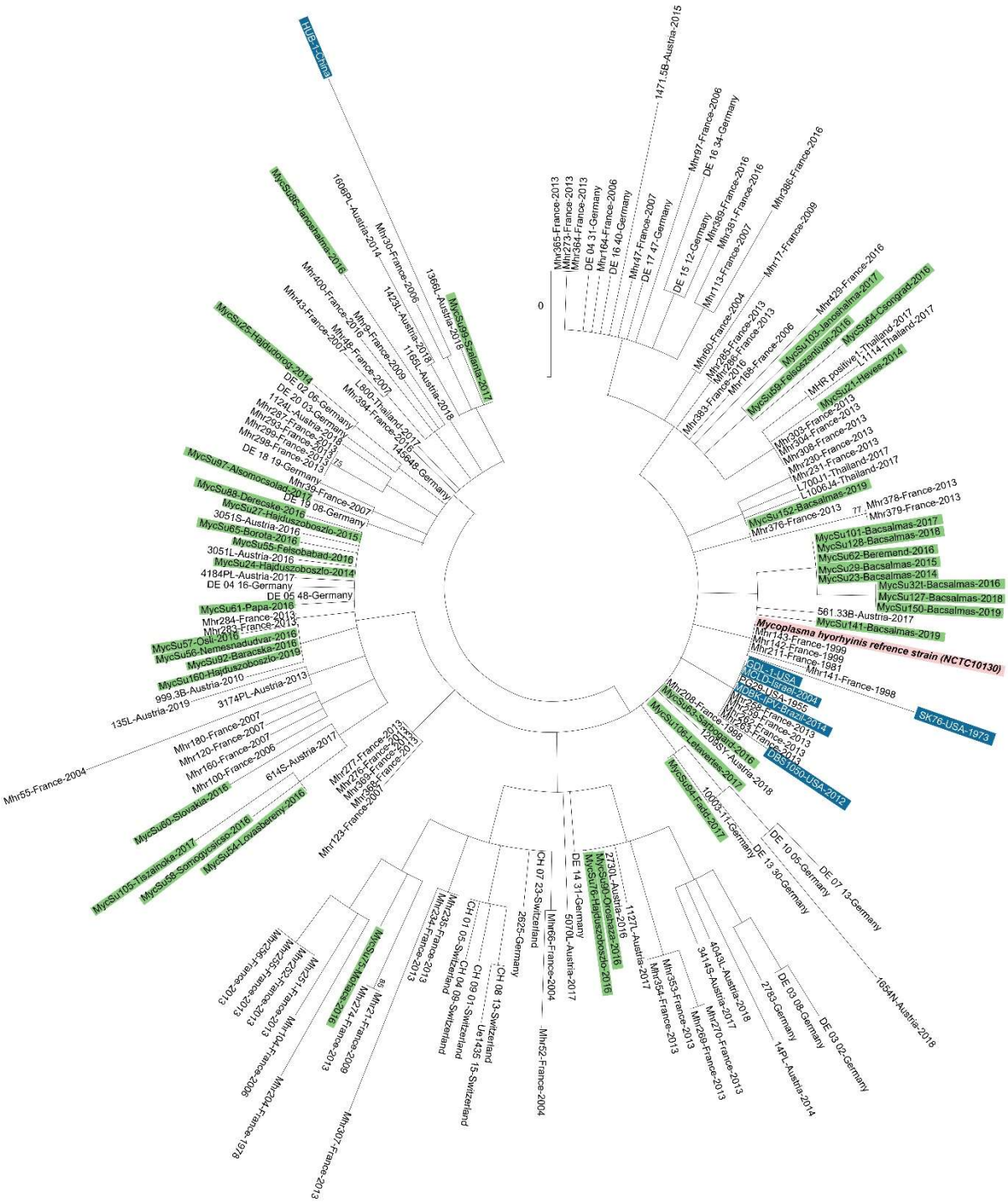
Group	Animal ID	Organ	Macro. score	Histo. score	Additional histological lesions
Control Group	1-4	Pericardium	0	0	
		Pleura	0	0	
		Peritoneum	0	0	
		Stifle	0	0	
		Elbow	0	0	
		Tarsus	0	0	
		Carpus	0	0	
		Conjunctiva	0	0	
		Brain	0	0	
		Lung	0	0	
		Nasal cavity /mucosal membrane	0	0	
		Heart	0	0	
		Other organs	0	0	
		Total	**0**	**0**	

Abbreviations: Macro. score: scores of macroscopic lesions; Histo. score: scores of histopathological lesions.



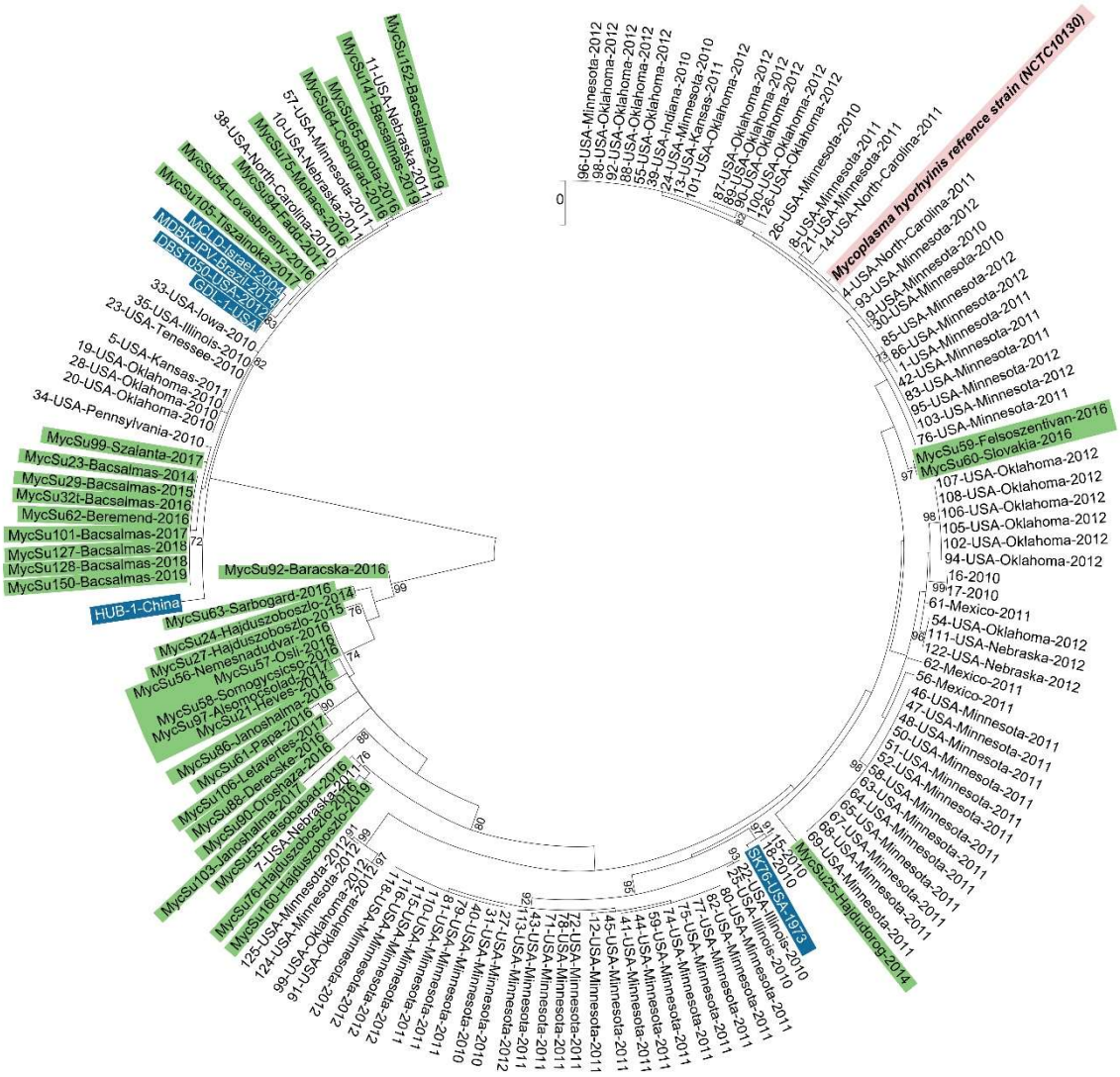
Supplementary figure 1: Molecular typing of *M. hyorhinis* isolates with the here developed multiple-locus variable-number tandem-repeat analysis.

The phylogenetic tree was constructed by the Neighbour joining method. *M. hyorhinis* type strain is highlighted blue, isolates from the same farm are highlighted with different tones of the same colour based on their sequence type with the here described multi-locus sequence typing method.



Supplementary figure 2: Molecular typing of *M. hyorhinis* isolates from PubMLST database and isolates described in this study (total of 173 isolates) by MLST according to Tocqueville *et al.* (2014) and Trüeb *et al.* (2016).

The phylogenetic tree was constructed by the Maximum Likelihood method with 1000 bootstraps (only bootstrap values >70% are presented). *M. hyorhinis* type strain is highlighted pink, the isolates described in this study are highlighted green, and the *M. hyorhinis* strains with available whole genomes are highlighted blue



Supplementary figure 3: Molecular typing of *M. hyorhinis* isolates from the study by Clavijo *et al.* (2019) and isolates described in this study (total of 148 isolates) by MLST according to Clavijo *et al.* (2019).

The phylogenetic tree was constructed by the Maximum Likelihood method with 1000 bootstraps (only bootstrap values >70% are presented). *M. hyorhinis* type strain is highlighted pink, the isolates described in this study are highlighted green, and the *M. hyorhinis* strains with available whole genomes are highlighted blue

Supplementary data

Supplementary data 1: Composition of polymerase chain reaction master mixes and thermocycling parameters

Supplementary data 1a: Reagents and thermocycling parameters used for the conventional species-specific polymerase chain reactions

Reagents		Volume (µl) for one reaction
Nuclease free water		12.05
5× Green GoTaq Flexi buffer (Promega)		5
$MgCl_2$ (25mM; Promega)		2
dNTP (10 mM; Qiagen)		0.75
Forward primer (10 µM)		2
Reverse primer (10 µM)		2
GoTaq Polymerase (5 U/µl; Promega)		0.2
template DNA		1
Total volume		25
Thermocycling parameters		
Initial denaturation		95°C 5 minutes
30 cycles	Denaturation	95°C 30 seconds
	Annelation	54°C 30 seconds
	Elongation	72°C 60 seconds
Final elongation		72°C 5 minutes

Primer sequences are listed in Table 1 for *M. hyorhinis* and Table 2 for contaminant mycoplasmas

Supplementary data 1b: Reagents and thermocycling parameters used for the species-specific real-time polymerase chain reactions

Reagent	Volume (µl) for one reaction
Nuclease free water	3
2× qPCRBIO Probe MIX No-ROX (PCR Biosystems Ltd., UK)	6
Forward primer (10 µM)	0.4
Reverse primer (10 µM)	0.4
Probe (10 µM)	0.2
template DNA	2
Total volume	12

Thermocycling parameters		
Initial denaturation		95°C 2 minutes
45 cycles	Denaturation	95°C 5 seconds
	Annelation	60°C 20 seconds

Primer sequences are listed in Table 1 for *M. hyorhinis* and Table 2 for contaminant mycoplasmas

Supplementary data 1c: Reagents and thermocycling parameters used for the universal *Mycoplasma* polymerase chain reactions

Reagent		Volume (µl) for one reaction
Nuclease free water		13.75
5× Green GoTaq Flexi buffer (Promega)		5
$MgCl_2$ (25mM; Promega)		2.5
dNTP (10 mM; Qiagen)		0.5
Forward primer (10 µM)		1
Reverse primer (10 µM)		1
GoTaq Polymerase (5 U/µl; Promega)		0.25
template DNA		1
Total volume		25
Thermocycling parameters		
Initial denaturation		95°C 2 minutes
40 cycles	Denaturation	95°C 30 seconds
	Annelation	55°C 30 seconds
	Elongation	72°C 60 seconds
Final elongation		72°C 5 minutes

Primer sequences are listed in Table 2

Supplementary data 1d: Reaction mixtures and thermocycling parameters for the conventional and real-time mismatch amplification mutation assays (MAMA)

agarose-MAMA			melt-MAMA		
Reagents		**Volume (µl) for one reaction**	**Reagents**		**Volume (µl) for one reaction**
Nuclease free water		9.8	Nuclease free water		1.6
5× Green GoTaq Flexi buffer (Promega)		5	2× HSTaq Mix (PCR Biosystems)		4.5
$MgCl_2$ (25mM; Promega)		2.5	EvaGreen (20×, Biotium)		0.5
dNTP (10 mM; Qiagen)		0.5			
23S-L primer(10 µM)		4	23S-L primer(10 µM)		1.6
23S-H primer(10 µM)		1	23S-H primer(10 µM)		0.4
23S-C primer(10 µM)		1	23S-C primer(10 µM)		0.4
GoTaq Polymerase (5 U/µl; Promega)		0.2			
template DNA		1	template DNA		1
Total volume		25	**Total volume**		10
Thermocycling parameters					
Initial denaturation		95°C 5 minutes	Initial denaturation		95°C 1 minute
35 cycles	Denaturation	95°C 30 seconds	35 cycles	Denaturation	95°C 15 seconds
	Annelation	60°C 30 seconds		Annelation	55°C 15 seconds
	Elongation	72°C 30 seconds		Elongation	72°C 5 seconds
Final elongation		72°C 5 minutes			
			Melt curve		55°C-95°C with 0.3°C increments

Primer sequences are listed in Table 3
agarose-MAMA: MAMA based on product size; melt-MAMA: MAMA based on melting temperature of the product. The primer named after the sensitivity specific point mutations in the 23S rRNA gene: L: genotype-L, low minimal inhibitory concentration (MIC) specific primer; H: genotype-H, high MIC specific primer; C: consensus primer.

Supplementary data 1e: Reagents and thermocycling parameters used for the 23S rRNA sequencing polymerase chain reactions

Reagent		Volume (µl) for one reaction
Nuclease free water		13
2× PCRBIO HS Taq Mix Red (PCR Biosystems)		25
Mhr-D5-1F (10 µM)		4
Mhr-D5-1R (10 µM)		4
template DNA		4
Total volume		50
Thermocycling parameters		
Initial denaturation		95°C 1 minutes
	Denaturation	95°C 15 seconds
40 cycles	Annelation	60°C 15 seconds
	Elongation	72°C 40 seconds

Previously published primers from Kobayashi and co-workers (2005) were used

Supplementary data 1f: Reagents and thermocycling parameters used for multi-locus sequence typing assay

Reagent		Volume (µl) for one reaction
Nuclease free water		10.75
5× Green GoTaq Flexi buffer (Promega)		5
$MgCl_2$ (25mM; Promega)		2.5
dNTP (10 mM; Qiagen)		0.5
Forward primer (10 µM)		2
Reverse primer (10 µM)		2
GoTaq Polymerase (5 U/µl; Promega)		0.25
template DNA		2
Total volume		25
Thermocycling parameters		
Initial denaturation		95°C 3 minutes
	Denaturation	95°C 30 seconds
40 cycles	Annelation	60°C 30 seconds
	Elongation	72°C 45 seconds
Final elongation		72°C 7 minutes

Primer sequences are listed in Table 4

Supplementary data 1g: Reagents and thermocycling parameters used for the multiple-locus variable-number tandem-repeat analysis

Reagent		Volume (µl) for one reaction
Nuclease free water		14.8
AmpliTaq™ Gold 10× PCR buffer (Applied Biosystems)		2.5
$MgCl_2$ (25 mM; Applied Biosystems)		2.5
dNTP (10 mM; Qiagen)		1
Forward primer (10 µM)		1
Reverse primer (10 µM)		1
AmpliTaq™ Gold Polymerase (5U/µl, Applied Biosystems)		0.2
template DNA		2
Total volume		25
Thermocycling parameters		
Initial denaturation		95°C 5 minutes
35 cycles	Denaturation	95°C 30 seconds
	Annelation	60°C 30 seconds
	Elongation	72°C 45 seconds
Final elongation		72°C 5 minutes

Primer sequences are listed in Table 5

Supplementary data 2: Details of the statistical analysis constructed for the challenge study

Supplementary data 2a: Statistical analysis of daily weight gain

	Number of animals	Mean	SD
Group IV-IV	6	223	86.90
Group IV-IP	6	170	41.00
Control	4	350	99.00

Shapiro-Wilk normality test

Null hypothesis: the distribution of the data is not significantly different from normal distribution.

W=0.89, p-value=0.06

one-way ANOVA					
	DF	Sum Sq	Mean Sq	F-value	Pr (>F)
Group	2	79067	39533	6.80	**<0.01**
Residuals	13	75533	5810		

Tukey multiple comparisons of means				
Comparison	diff	lwr	upr	adjusted p-value
Group IV-IP-Group IV-IV	-53.33	-169.54	-62.87	0.47
Control-Group IV-IV	126.67	-3.25	256.58	**0.05**
Control-Group IV-IP	180.00	50.08	309.92	**<0.01**

Supplementary data 2b: Statistical analysis of gross pathological scores of joint lesions

	Number of animals	Median	IQR
Group IV-IV	6	1.50	3.25
Group IV-IP	6	3.00	1.50
Control	4	0.00	0.00

Kruskal-Wallis test	
chi-squared	9.03
DF	2
p-value	**0.01**

Dunn-test			
Comparison	Z-value	unadjusted p-value	adjusted p-value
Group IV-IV-Group IV-IP	-0.25	0.80	1.00
Group IV-IV- Control	2.57	0.01	**0.03**
Group IV-IP- Control	2.79	0.01	**0.02**

Supplementary data 2c: Statistical analysis of gross pathological scores of serosa of the pericardium, pleura and peritoneum

	Number of animals	Median	IQR
Group IV-IV	6	0.50	1.75
Group IV-IP	6	0.00	1.50
Control	4	0.00	0.00

Kruskal-Wallis test	
chi-squared	2.54
DF	2
p-value	0.28

Supplementary data 2d: Statistical analysis of total gross pathological scores

	Number of animals	Median	IQR
Group IV-IV	6	3.00	3.50
Group IV-IP	6	3.00	3.00
Control	4	0.00	0.00

Kruskal-Wallis test	
chi-squared	8.76
DF	2
p-value	**0.01**

Dunn-test			
Comparison	Z-value	unadjusted p-value	adjusted p-value
Group IV-IV-Group IV-IP	0.06	0.95	1.00
Group IV-IV- Control	2.67	0.01	**0.02**
Group IV-IP- Control	2.62	0.01	**0.03**

Supplementary data 2e: Statistical analysis of joint histology scores

	Number of animals	Median	IQR
Group IV-IV	6	3.50	1.00
Group IV-IP	6	3.50	7.75
Control	4	0.00	0.00

Kruskal-Wallis test	
chi-squared	6.48
DF	2
p-value	**0.04**

Dunn-test			
Comparison	Z-value	unadjusted p-value	adjusted p-value
Group IV-IV-Group IV-IP	0.50	0.62	1.00
Group IV-IV- Control	2.46	0.01	**0.04**
Group IV-IP- Control	2.01	0.04	0.12

Supplementary data 2f: Statistical analysis of the histology scores of serosa of the pericardium, pleura and peritoneum

	Number of animals	Median	IQR
Group IV-IV	6	3.00	1.50
Group IV-IP	6	2.00	3.50
Control	4	0.00	0.00

Kruskal-Wallis test	
chi-squared	5.56
DF	2
p-value	0.06

Supplementary data 2g: Statistical analysis of total histology scores

	Number of animals	Median	IQR
Group IV-IV	6	6.00	3.00
Group IV-IP	6	5.50	12.80
Control	4	0.00	0.00

Kruskal-Wallis test	
chi-squared	6.64
DF	2
p-value	**0.04**

Dunn-test			
Comparison	Z-value	unadjusted p-value	adjusted p-value
Group IV-IV-Group IV-IP	0.62	0.53	1.00
Group IV-IV- Control	2.51	0.01	**0.04**
Group IV-IP- Control	1.95	0.51	0.15

Supplementary data 2h: Statistical analysis of ELISA S/P% from the last sampling point

	Number of animals	Mean	SD
Group IV-IV	6	85.70	24.40
Group IV-IP	6	84.40	29.40
Control	4	9.84	6.52

Shapiro-Wilk normality test

Null hypothesis: the distribution of the data is not significantly different from normal distribution.

W=0.94, p-value=0.38

one-way ANOVA					
	DF	Sum Sq	Mean Sq	F-value	Pr (>F)
Group	2	16974	8487	14.85	**<0.01**
Residuals	13	7428	571		

Tukey multiple comparisons of means				
Comparison	diff	lwr	upr	adjusted p-value
Group IV-IP-Group IV-IV	-1.25	-37.69	35.20	0.99
Control-Group IV-IV	-75.83	-116.57	-35.09	**<0.01**
Control-Group IV-IP	-74.59	-115.33	-33.84	**<0.01**

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