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Phenotypic and genotypic characterization of two novel *Mycoplasma* species isolated from the Eurasian badger (*Meles meles*)

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Abstract

Since 2003, several mycoplasma isolates that could not be identified to the species level have been recovered from the respiratory tract and in one case from the brain of Eurasian badgers (*Meles meles*) in Austria. Based on MALDI-ToF mass spectrometry these isolates could be divided into two groups (group 1 and 2) of which seven strains each were subjected to a comprehensive study to investigate their phenotypic and genotypic characteristics. The selected strains grew well in modified SP-4 medium presenting typical fried egg colony morphology on agar. Colonies from strains belonging to group 2 also produced "film and spots". Transmission electron microscopy revealed a cell morphology characteristic for members of genus *Mycoplasma* with spherically shaped cells for group 2 and ovoid to peanut shaped cells for group 1, bounded by a bi-layered cell membrane and lacking a cell wall. Both strain groups produced acid from glucose (glycolyse) but did not hydrolyze arginine or urea. Phylogenetic analyses of 16S rRNA sequences, the 16S-23S intergenic spacer region, and partial *rpoB* gene sequences revealed *M. mustelae* and *M. felis* to be the closest relatives to strains belonging to group 1, while strains of group 2 were most closely related to *M. molare* and *M. lagogenitalium*. Histopathological analyses revealed BALT hyperplasia with mononuclear cell infiltration in few lung tissue samples from which strains of group 1 had been isolated, suggesting a possible role of group 1 mycoplasmas as respiratory pathogens in the Eurasian badger.

Zusammenfassung

Seit dem Jahre 2003 wurden in Österreich Mykoplasmen aus dem Respirationstrakt und in einem Fall aus dem Gehirn von Eurasischen Dachsen (*Meles meles*) isoliert, die keiner Art zugeordnet werden konnten. Auf Grundlage der MALDI-ToF-Massenspektrometrie konnten diese Isolate in zwei Gruppen (Gruppe 1 und 2) eingeteilt werden, von denen jeweils sieben Stämme einer umfassenden Studie unterzogen wurden, um ihre phänotypischen und genotypischen Eigenschaften zu untersuchen. Die ausgewählten Stämme ließen sich gut in modifiziertem SP-4 Medium kultivieren und zeigten die typisch spiegeleiförmige Koloniemorphologie auf Agar-Medium. Die Kolonien der Stämme von Gruppe 2 wiesen außerdem "Film- und Spot"-Bildung" auf. Mithilfe der Transmissionselektronenmikroskopie konnte eine für die Gattung *Mycoplasma* charakteristische Zellmorphologie festgestellt werden, mit vorwiegend kugelförmigen Zellen für Gruppe 2- und ei- bis erdnussförmigen Zellen für Gruppe 1-Stämme, die von einer zweischichtigen Zellmembran begrenzt und

zellwandlos waren. Die Stämme beider Gruppen produzierten Säure aus Glukose (Glykolyse), konnten aber weder Arginin noch Harnstoff hydrolysieren. Phylogenetische Analysen der 16S rRNA-Gensequenzen, der intergenischen 16S-23S-Spacer-Region und von *rpoB*-Genfragmenten ergaben ein nahes Verwandtschaftsverhältnis mit *M. mustelae* und *M. felis* für Stämme der Gruppe 1, während die Stämme der Gruppe 2 einen engen Verwandtschaftsgrad mit *M. molare* und *M. lagogenitalium* aufwiesen. Bei der histopathologischen Untersuchung war in einigen Lungengewebeproben, aus denen zuvor Stämme der Gruppe 1 isoliert worden konnten, eine BALT-Hyperplasie mit mononukleärer Zellinfiltration feststellbar, was auf eine mögliche Rolle von Mykoplasmen der Gruppe 1 als Erreger von Atemwegserkrankungen beim Eurasischen Dachs hinweisen könnte.

Table of Contents

1. Introduction

When *Mycoplasma mycoides*, the etiologic agent of Contagious Bovine Pleuropneumonia, was isolated in the late 19th century, science discovered an entirely new class of bacteria even though it would only receive its current name in 1967: the class *Mollicutes* (1). Belonging to this class are members of genera *Mycoplasma*, *Ureaplasma*, and *Acholeplasma* among others which comprise bacteria sharing characteristics such as lack of a cell wall and reduced genome sizes (2, 3). With the advent of molecular technologies, such as polymerase chain reaction (PCR) and DNA sequencing in the late 20th century, the pace of bacterial discovery and identification drastically increased. This includes members of the genus *Mycoplasma* of which we count more than 150 recognized species nowadays. These bacteria usually occur within the respiratory and genital tracts of humans and animals but can also be isolated from joints, eyes, serous membranes, and mammary glands and can either be commensals or pathogens (4).

As many of these newly identified species of mycoplasmas have been isolated from wildlife, further research into this domain is warranted in order to assess the role of these microorganisms in their hosts including the potential of different wildlife species to serve as reservoirs for mycoplasma diseases in other animal species. One species of specific interest is the Eurasian badger (*Meles meles*). This medium-sized carnivore belonging to the family *Mustelidae* makes its habitat across the European mainland, the British Isles, and the Near East, spreading as far north as Lapland in Scandinavia, as far south as Israel, and as far east as Afghanistan (5–8). This not only represents an impressive capability to adapt to different biomes and environmental circumstances but also leads to common intersection between this species and the anthropogenic world.

The Eurasian badger already plays a relevant role in the epidemiology of bovine tuberculosis, serving as a prominent reservoir for *Mycobacterium bovis* within the British Isles which has led to significant economic losses over the years (9). According to more recent reports, this problematic seems to emerge currently across the Iberian Peninsula and southern France (10, 11). Additionally to this epidemiologic problematic nature in which Eurasian badgers are involved, this species has been described to live together in family groups within their burrows called “setts” which they share with a multitude of other wildlife such as crested porcupines (*Hystrix cristata*), red foxes (*Vulpes vulpes*), pine martens (*Martes martes*), wood mice (*Apodemus sp.*), brown rats (*Rattus norvegicus*), and nutria (*Myocastor coypus*) (12).

Altogether, this warrants a thorough surveillance of the potential pathogens that this species can harbor to avoid future epidemiologic outbreaks. This is also true in regard to mycoplasmas since hemoplasmas (hemotrophic mycoplasmas) have already been detected in the Japanese badger (*Meles meles anakuna*) and in Eurasian badgers in Spain displaying a potential for interspecies transmission (13, 14).

The aim of this thesis was to thoroughly characterize strains of two putative novel mycoplasma species which were isolated from Eurasian badgers in Austria between 2003 and 2020. These badgers were either found dead or shot and then send for necropsy to the Research Institute of Wildlife Ecology (FIWI), Vetmeduni Vienna. The mycoplasma strains examined in this study were all isolated from the respiratory tract except for one isolate which originated from the brain. This study sought to fulfill its goals by using a polyphasic taxonomic approach that followed the guidelines presented in the revised minimal standards for the description of new species in the class *Mollicutes* as well as recommendations published by the Subcommittee on the taxonomy of *Mollicutes* and included further analytical tests beyond these guidelines and recommendations (15, 16). In total, fourteen strains of which seven strains were selected to represent either group of mycoplasmas, were included in this study. These strains represented the overall diversity of either group including isolation source and year of isolation.

2. Materials and Methods

2.1 Mycoplasma strains

During this diploma thesis, 14 unidentifiable mycoplasma isolates were analyzed. Based on MALDI-ToF mass spectrometry (methodology see below) these 14 isolates could be divided into two groups of closer association. All but one strain originated from the respiratory tract of European badgers (*Meles meles*), while the remaining one was isolated from a badger's brain.

Table 1: Mycoplasma strains of group 1 included in this study (strain designation, isolation site/source, and year of isolation)

Strain designation	Isolation site / source	Year of isolation
6243	Lung	2018
4013	Lung	2015
4423	Lung	2016
5912	Lung	2017
AC1221	Trachea	2019
246B	Lung	2020
394	Brain	2020

Table 2: Mycoplasma strains of group 2 included in this study (strain designation, isolation site/source, and year of isolation)

Strain designation	Isolation site / source	Year of isolation
480	Lung	2003
1012	Lung	2011
5370	Lung	2017
CB776	Trachea	2018
613B	Trachea	2019
Z386	Trachea	2019
AC157	Trachea	2020

2.2 Cultivation of mycoplasmas

For cultivation, a small amount of each cryopreserved culture stock (approx. 10-50 µl) was taken and streaked onto a SP4 agar plate using a disposable loop. The inoculated agar plates were then incubated in a CO_2 incubator (7% CO_2 atmosphere) at 37°C for at least 72 hours. After colony formation, a single colony was picked from each agar culture using sterile glass pipettes under stereomicroscopic control and transferred into SP4 broth medium incubated aerobically at 37°C until a color change of the phenol red indicator from reddish to orange/yellow occurred (acidification due to glucose fermentation processes).

After aliquoting the mycoplasma cultures obtained from single colonies, they were stored at -80°C until further use.

The SP4 medium used is a complex and nutrient-rich culture medium, which was specially developed for the cultivation of mycoplasmas that are difficult to cultivate. The SP4 liquid medium was prepared as follows:

PPLO Broth (Difco™, Austria)	2,5 g
Tryptone (BD Difco™, Austria)	7 g
Peptone G (BD Difco™, Austria)	3,5 g
DNA (Sigma-Aldrich, Austria)	0,14 g
dH_2O	700 ml

After adjusting the pH to 7.5, the base medium was autoclaved at 121°C for 15 minutes. After cooling to 50°C, the following medium components were added:

Heat-inactivated horse serum (Biowest, France)	50 ml
Heat-inactivated pig serum (Biowest, France)	50 ml
Heat-inactivated fetal calf serum (Biowest, France)	20 ml
50% glucose solution (Roth, Germany)	7 ml
10 x CMLR 1066 (Thermo Scientific, GibcoTM, Austria)	35 ml
4 % yeast extract solution (Oxoid, Austria)	35 ml
4 % nicotinamide adenine dinucleotide solution (Roth, Germany)	11 ml
1 % L-cysteine solution (Sigma-Aldrich, Austria)	17 ml
Penicillin (Sandoz, Austria)	1.000.000 I.U.
10 % thallium acetate solution (Sigma-Aldrich, Austria)	1,4 ml
0,6 % phenol red solution	4 ml

The SP4 agar plates were prepared analogously to the SP4 liquid media, except for the addition of 8.5 g Agar Purified (Oxoid, Austria) after adjusting the pH to 7.5.

2.3 Genotypic characterization

Genotypic characterization was used to record the genetic or phylogenetic properties of the mycoplasma isolates to be characterized.

2.3.1 DNA Extraction

The GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) was used to extract DNA from the mycoplasma cultures obtained.

To prepare the strains for DNA extraction, 100 µl of the mycoplasma cultures cryopreserved at -80°C were transferred to SP4 medium and incubated aerobically at 37°C for 24 to 72 hours.

Subsequently, 1 ml of broth culture was centrifuged at 20,000 x g for 7 minutes, then the supernatant was pipetted off and the bacterial pellets obtained were stored temporarily at -24°C. To begin DNA extraction, the pellets were thawed at room temperature and the filter tubes included in the extraction kit were prepared by filling them with 500 µl of filter preparation solution and centrifuging them at 1200 x g for 1 minute. The thawed bacterial pellets were dissolved with 200 µl resuspension solution and finally 200 µl lysis solution C and 20 µl proteinase K solution was added.

The mixture was then incubated at 70°C for 10 minutes in a thermoblock (Eppendorf AG, Germany), after which 200 µl 95 % ethanol was added and vortexed for 5 seconds. The lysate was finally transferred to the prepared filter tube and centrifuged at 6,500 x g for 1 minute. The filtrate was discarded and the filter unit with the bound DNA was then rinsed during to washing steps with 500 µl wash solution each. The first wash was performed at 12,000 x g for 1 minute, the second at 14,000 x g for 3 minutes. In both washing steps, the filtrate was discarded. Finally, the filter tube was centrifuged again at 14,000 x g for 1 minute to completely remove residual wash solution. In the final step, the filter unit was transferred to a new tube and 200 µl of elution solution was added. After incubation at room temperature for 5 minutes, the tube was centrifuged at 12,000 x g for 1 minute and the extracted DNA was stored at -20°C until further use.

2.3.2 PCR

To prepare the master mix for PCR, OneTaq® Quick-Load® DNA polymerase (New England BioLabs®, USA) was mixed with the appropriate primers (Thermo Scientific, Austria) (see Tab. 3), transferred to the PCR tubes and the DNA templates were added. The PCR was performed in a Mastercycler® Nexus thermal cycler (Eppendorf, Germany).

Table 3: Primers (target sequence, primer ID, primer sequence, and reference)

Target sequence	Primer ID	Primer sequence	Reference
16S rRNA gene	27f	5'-AGA GTT TGA TCM TGG CTC AG-3'	Lane, 1991
	1492r	5'-TAC GGY TAC CTT GTT ACG ACT T-3'	
16S-23S intergenic spacer region	ITSavian-F	5'-CGT TCT CGG GTC TTG TAC AC-3'	Ramirez et al., 2008
	ITSavian-R	5'-CGC AGG TTT GCA CGT CCT TCA TCG-3'	

rpoB gene fragment	rpoB-F-MYC	5'-AGT TAT CAC AAT TTA TGG ATC AAA-3'	Volokhov et al., 2007
	rpoB-R-MYC	5'-GCT CAH ACT TCC ATT TCH CCA AA-3'	

2.3.3 Amplification of the 16S rRNA gene

For all isolates of both groups, the 16S rRNA gene was amplified for phylogenetic classification and the sequence was subsequently determined by Sanger sequencing (LGC, Berlin).

Table 4: Master mix formula for the amplification of the 16S rRNA gene (component and quantity)

Component	**Quantity/strain**
OneTaq	12,5 µl
Forward primer 27f	0,5 µl
Reverse primer 1492r	0,5 µl
DNA	2,5 µl
ddH_2O	9 µl
Total for one sample	25 µl

Table 5: Thermocyclic conditions for the amplification of the 16S rRNA gene (step, temperature, duration, and number of cycles)

Step	**Temperature (in °C)**	**Duration (in min.)**	**Cycles**
Initial denaturation	95	5	1
Denaturation	94	2	30
Annealing	50	1,5	
Elongation	72	5	
Final elongation	72	1	1

2.3.4 Amplification of the 16S-23S intergenic spacer region

For all isolates of both groups, the 16S-23S intergenic spacer region was amplified and the resulting amplicons were sequenced.

Table 6: Master mix formula for the amplification of the 16S-23S intergenic spacer region (component and quantity)

Component	Quantity/strain
OneTaq	12,5 µl
Forward primer ITSavianF	0,5 µl
Reverse primer ITSavianR	0,5 µl
DNA	2,5 µl
ddH_2O	9 µl
Total for one sample	25 µl

Table 7: Thermocyclic conditions for the amplification of the 16S-23S intergenic spacer region (step, temperature, duration, and number of cycles)

Step	Temperature (in °C)	Duration (in min.)	Cycles
Initial denaturation	94	0,25	1
Denaturation	95	0,25	35
Annealing	60	0,5	
Elongation	72	2	
Final elongation	72	5	1

2.3.5 Amplification of the *rpoB* gene fragment

To confirm the phylogenetic classification of all isolates of either group, a partial fragment of the *rpoB* gene was amplified and subjected to sequence analysis.

Table 8: Master mix formula for the amplification of the rpoB gene fragment (component and quantity)

Component	Quantity/strain
OneTaq	12,5 µl
Forward primer ITSavianF	0,5 µl
Reverse primer ITSavianR	0,5 µl
DNA	2,5 µl
ddH_2O	9 µl
Total for one sample	25 µl

Table 9: Thermocyclic conditions for the amplification of the rpoB gene fragment (step, temperature, duration, and number of cycles)

Step	Temperature (in °C)	Duration (in min.)	Cycles
Initial denaturation	95	6	1
Denaturation	95	1	45
Annealing	50	1	
Elongation	68	2	
Final elongation	68	5	1

2.3.6 Agarose gel electrophoresis

After performing PCR, the amplicons were visualised using agarose gel electrophoresis. For this purpose, a 2% agarose gel (6 g agarose (Sigma-Aldrich, Austria) in 300 ml TBE (TRIS-borate-EDTA) buffer solution (Roth, Germany)) was prepared. After heating, the agarose gel was poured into a gel chamber with a gel comb and allowed to harden at room temperature. Subsequently, 8 µl of each PCR product was pipetted into the middle gel wells, while 4.5 µl of a molecular weight marker (Gene Ruler 100 Base Pair plus DNA Ladder, Thermo Fisher Scientific, Germany) was added to the first and last gel lanes. The agarose gel was then run in a running chamber (Bio-Rad Laboratories GmbH, Austria) under voltage (220 volts) for approximately one hour. To visualise the amplification products, the gel was stained for 20 minutes in an ethidium bromide solution (2.5 mg/l, Bio-Rad Laboratories GmbH, Austria) and then excess ethidium bromide was removed by placing the gel in a water bath for 5 minutes. Finally, the PCR products were visualised using the transilluminator Gel Doc2000 (Bio-Rad Laboratories GmbH, Austria) and gel figures were stored in the system's internal data carrier.

2.3.7 Purification of PCR products

For subsequent sequencing, the PCR products were first purified. For each PCR product, 1.5 µl exonuclease I (Sigma-Aldrich, Austria) and 1.5 µl rSAP (shrimp alkaline phosphate) (Sigma-Aldrich, Austria) were mixed and 7 µl of the PCR product was added. The reaction mixture was then incubated in a Mastercycler® Nexus (Eppendorf, Germany) under specific temperature conditions.

Table 10: Temperature conditions for the purification of PCR products (step, temperature, and duration)

Step	**Temperature (in °C)**	**Duration (in min.)**
1	37	15
2	80	15

Subsequently, 10 µl dH_2O was added to the purified PCR product and 13 µl of this dilution was transferred to a new reaction tube and 2 µl of the corresponding primer (forward and reverse primer) was added.
Finally, sequencing of the amplicons was performed at LGC Genomics, Berlin.

2.3.8 Phylogenetic analysis

First, resulting sequences were compared with entries in the public GenBank database (www.ncbi.nlm.nih.gov) using the BLAST algorithm (www.blast.ncbi.nlm.nih.gov). Sequence similarity values were determined, and entries of the most closely related mycoplasma species were stored on a local data carrier. Subsequently, the sequences (16S rRNA gene, partial *rpoB* gene) were aligned using ClustalW (MegaX software package), and ambiguous nucleotides edited manually. To visualise phylogenetic relationships, phylogenetic trees were constructed using the Maximum Likelihood method with bootstrapping (1000 replications) and the Kimura-2 parameter substitution model (MegaX software package).

2.4 Phenotypic characterization

In the studies, the phenotypic properties of the mycoplasma isolates to be characterized were recorded.

2.4.1 Colony morphology

Using a stereomicroscope (Olympus, Germany) at 40-times magnification, the colony morphology of all 14 mycoplasma strains was determined. In preparation, 100 µl of the mycoplasma cultures cryopreserved at -80°C were spread onto SP4 agar plates, incubated at 37°C under 7 % CO_2 atmosphere for 3 to 4 days, before individual colonies were finally observed.

2.4.2 Growth temperature and oxygen requirements

Optimal growth temperature and oxygen requirements were determined for all investigated strains. For this purpose, 100 µl of each cryopreserved mycoplasma cultures were transferred into 3 ml of SP4 medium and incubated aerobically at 37°C for 24 to 72 hours, followed by plating 100 µl of broth culture onto SP4 agar. Optimal growth temperatures and oxygen requirements were determined by incubating these agar plates under aerobic, microaerobic (7 % CO_2 atmosphere), and anaerobic conditions (the latter using GasPak™ systems [BD, Austria] in culture jars) at different temperatures (4, 22, 28, 33, 37, and 42°C).

2.4.3 Digitonin sensitivity

Digitonin sensitivity was tested in one representative mycoplasma strain of each group (6243 and 480, respectively) for indirect detection of the dependence of growth on cholesterol and thus for the differentiation between the genera *Mycoplasma* and *Acholeplasma*. For this

purpose, 100 µl of broth cultures were spread onto SP4 agar plates and incubated for 5 minutes at room temperature. Then a filter disk containing 15 µl of a 1.5 % digitonin solution was placed in the centre of the inoculated SP4 agar plate and incubated under 7 % CO_2 atmosphere at 37°C for 4 days. Digitonin sensitivity and thus growth dependence on cholesterol was indicated by formation of a growth inhibition zone around the digitonin filter disk.

2.4.4 Enzymatic activity

The API-ZYM assay kit (Biomerieux, France) was used to determine the enzymatic activity of the mycoplasma isolates. This assay was performed on a representative mycoplasma strain of each group (6243 and 480, respectively) as well as the type strains of their closest relatives including *Mycoplasma* (*M.*) *mustelae* MX9^T, *M. felis* CO^T, *M. molare* H 542^T, and *M. lagogenitalium* 12MS^T. The following 19 enzymes were assayed: alkaline phosphatase, esterase (C4), esterase-lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, Naphthol AS-BI phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase.

For this purpose, 2 ml of the broth culture of each strain were centrifuged at 20.000 x g for 7 minutes, the supernatant discarded, and the bacterial pellet obtained resuspended with 100 µl of water (HPLC grade, Sigma-Aldrich, Austria), centrifuged again, and the supernatant discarded. The bacterial pellet was then resuspended again with 1 ml of water (HPLC grade, Sigma-Aldrich, Austria). The API-ZYM test kit was prepared by filling an incubation tray with 5 ml of distilled water to create a wet chamber. The chamber was then fitted with an enzyme test strip and 65 µl of the bacterial suspension were pipetted into each well of the enzyme test strip. The enzyme test strips were subsequently incubated aerobically at 37°C for 4.5 hours. After incubation, one drop of ZYM-A reagent and one drop of ZYM-B reagent were added to each individual well, incubated for 5 minutes at room temperature. A manufacturer's color scale was used to analyse the results.

2.4.5 Glucose-fermentation, arginine and urea hydrolysis

A representative mycoplasma strain for each group (6243 and 480, respectively) was tested for their capacity to ferment glucose or to hydrolyze arginine or urea. *M. bovirhinis* PG43^T, *M. arginini* G230^T, and *Ureaplasma* (*U.*) *diversum* A417^T were used as control strains for glucose fermentation, arginine and urea hydrolysis, respectively. For this purpose, 100 µl of cryopreserved mycoplasma cultures (6243, 480, *M. bovirhinis* PG43^T, *M. arginini* G230^T) were

transferred into 3 ml of SP4 medium (containing 50% glucose solution) and into 3 ml of modified SP4 medium (containing 50% L-arginine solution instead of the 50% glucose solution). In addition, 100 µl of cryopreserved mycoplasma cultures (6243, 480, *U. diversum* A417^T) were transferred into 3 ml of modified SP4 medium containing 50% urea solution instead of the 50% glucose solution. All of these culture preparations were incubated aerobically at 37°C for 24 to 48 hours. Thereafter, 100 µl of each liquid culture were plated onto SP4 or modified SP4 agar plates (containing 50% L-arginine or 50% urea solution instead of 50% glucose solution), incubated for 24 to 48 hours under 7 % CO_2 atmosphere at 37°C. The ability to ferment glucose was indicated by a color change of the SP4 broth and the SP4 agar medium from red to yellow (acidification). A positive reaction and thus the capacity to metabolize arginine was indicated by a color change of the modified SP4 broth and SP4 agar medium from red to pink or magenta (alkalization). Finally, the ability to hydrolyze urea was indicated by a color change of the modified SP4 broth and agar medium from yellow to pink or magenta (alkalinization).

2.4.6 Reduction of tetrazolium chloride and potassium tellurite

A representative mycoplasma strain for each group (6243 and 480, respectively) was tested for their ability to reduce tetrazolium chloride and potassium tellurite. Testing was performed on PPLO agar plates prepared as follows:

PPLO Broth (BD Difco, Austria)	21 g
dH_2O	700 ml
Agar Noble (BD Difco, Austria)	9,8 g

Following adjustment of the pH to 7,5, the base medium was autoclaved at 121°C for 15 minutes. After cooling to 50°C, the following medium components were added:

25% yeast extract solution (Merck, Germany)	85 ml
Heat-inactivated horse serum (Biowest, France)	170 ml
2% 2,3,5-triphenyltetrazolium chloride solution (Merck, Austria)	10 ml
OR	
11% potassium tellurite solution (Sigma-Aldrich, Austria)	5 ml
Penicillin (Sandoz, Austria)	1.000.000 I.U.

First, 100 µl of each of the mycoplasma cultures cryopreserved at -80°C was transferred into 3 ml of SP4 medium and incubated aerobically at 37°C for 24 to 72 hours. Subsequently, 100 µl of the liquid culture was plated onto PPLO agar supplemented with tetrazolium chloride or

potassium tellurite and incubated for up to 10 days under microaerobic (7 % CO_2) and anaerobic culture conditions (Gaspak^{TM}, BD, Austria) at 37°C. The ability to reduce tetrazolium chloride was indicated by red to pink-colored areas around colonies, while the ability to reduction of potassium tellurite was indicated by the production of black-colored colonies.

2.4.7 Hemolysis

A representative mycoplasma strain of each group (6243 and 480, respectively) was tested for their capacity to induce hemolysis on Columbia agar plates containing 5 % sheep blood (Becton Dickinson, Austria). For this purpose, 100 µl of each of these mycoplasma cultures cryopreserved at -80°C was transferred into 3 ml of SP4 medium and incubated aerobically at 37°C for 24 to 72 hours, followed by plating 100 µl of the broth culture onto Columbia agar. The plates were then incubated under 7 % CO2 atmosphere at 37°C for 4 days. The capacity to induce hemolysis was indicated by the appearance of transparent zones surrounding the mycoplasma colonies.

2.4.8 Cell morphology

To analyze the cell morphology, a representative mycoplasma strain of each group (6243 and 480, respectively) was examined by transmission electron microscopy at the Institute of Pathology at the Vetmeduni Vienna. For this purpose, 100 µl of each of these mycoplasma cultures cryopreserved at -80°C were transferred into 3 ml of SP4 medium and incubated aerobically at 37°C for 24 to 72 hours. Subsequently, 1 ml of the liquid culture was centrifuged at 12.000 x g for 15 minutes. The supernatant was then discarded and the pellet resuspended with 50 µl PBS (phosphate-buffered saline solution) and transferred to the Institute of Pathology.

2.4.9 MALDI-TOF protein mass fingerprinting

MALDI-TOF (matrix-assisted laser desorption ionization – time of flight) mass spectrometry was performed on all 14 mycoplasma strains. This served to group the strains tested and to determine relationships by comparing the mass spectra generated.

One ml of each broth culture in the late logarithmic growth phase was centrifuged at 20.000 x g for 7 minutes, the supernatant discarded, and the bacterial pellet washed with 100 µl water (HPLC grade, Sigma-Aldrich, Austria), and centrifuged again at 20.000 x g for 5 minutes. For protein extraction, the obtained bacterial pellet was then dissolved with 15 µl 70 % formic acid

(Sigma-Aldrich, Austria) and 15 µl 100 % acetonitrile (Sigma-Aldrich, Austria) and centrifuged at 20.000 x g for 2 minutes. Next, 1 µl of each protein extract was spotted onto a 96-target polished steel plate (Bruker Daltonik, Germany), air-dried, and overlaid with 1 µl α-cyano-4-hydroxycinnamic acid matrix solution (Sigma-Aldrich, Austria). Following a short period of air-drying, MALDI-TOF mass spectra were generated using the Microflex LT Biotyper (Bruker Daltonics, Germany) and the FlexControl 3.4 software. Subsequently, the generated spectra of the studied mycoplasma strains were compared with an in-house mycoplasma reference spectra database (containing a reference spectrum for strain 6243 and 480) to confirm grouping and species membership of the analyzed strains (score value ≥2.00). Using the FlexAnalysis 3.4 software, peak differences between the analyzed strains and the type strains of their closest relatives were visualized by overlaying the generated spectra.

2.4.10 Histopathology

To gain insights into their possible role as pathogens, tissue sections of animals from which mycoplasmas of group 1 (hosts of all seven analyzed strains and further seven hosts) and group 2 (hosts of three analyzed strains along with further four hosts) had previously been isolated, were histologically examined for alterations associated with mycoplasma infection. The histopathological examinations were performed at the FIWI, Vetmeduni, Vienna.

Table 11: Histopathological examination of tissue sections from which mycoplasmas of group 1 have been isolated (animal number, tissue, and year of mycoplasma isolation)

Animal number (identical to strain designation)	Tissue sections examined	Year of mycoplasma isolation
6243	Lung	2018
4013	Lung	2015
4423	Lung	2016
5912	Lung	2017
AC1221	Lung, trachea	2019
246B	Lung	2020
394	Lung, trachea, brain	2020
AC4	Lung, trachea	2019
Z388	Lung, trachea	2019
Z432	Lung, trachea	2019
Z981	Lung, trachea	2019
CB1022	Lung, trachea	2019
AC1244	Lung, trachea	2019
AC496	Lung, trachea	2020

Table 12: Histopathological examination of tissue sections from which mycoplasmas of group 2 have been isolated (animal number, tissue, and year of mycoplasma isolation)

Animal number (identical to strain designation)	Tissue sections examined	Year of mycoplasma isolation
5370	Lung	2017
613B	Lung, trachea	2019
Z386	Lung, trachea	2019
Z230	Lung, trachea	2019
Z388	Lung trachea	2019
AC154	Lung, trachea	2020
Z220A	Lung, trachea	2020

3. Results

3.1 Phylogenetic analysis

The phylogenetic relatedness of mycoplasma isolates and closely related mycoplasma species was determined by assessing sequence similarity values and by constructing phylogenetic trees.

For the seven mycoplasma isolates of group 1, 16S rRNA gene sequencing revealed highest sequence similarity values with *M. mustelae* MX9^T (95.49-95.78 %) (next related species <95 %). For the seven isolates of group 2, analysis of the 16S rRNA gene showed highest sequence similarity with *M. molare* H 542^T (98.14-98.28 %) and *M. lagogenitalium* 12MS^T (97.58-97.72 %) (next related species <95 %). A close relationship of these mycoplasma species to the examined isolates from both groups could be confirmed based on the constructed phylogenetic trees (Fig. 1).

By analyzing a fragment of the *rpoB* gene highest sequence similarity values with *M. mustelae* MX9^T (81.78-82.05%) for group 1 (next related species <80 %) and with *M. molare* H 542^T (83.63-83.99 %) and *M. lagogenitalium* 12MS^T (82.85-83.15 %) for group 2 strains (next related species <80 %) were obtained. While a close relationship of *M. mustelae* MX9^T with the examined isolates of group 1 was confirmed by the phylogenetic tree, the isolates of group 2 were shown to have a closer relationship with *M. lagogenitalium* 12MS^T than with *M. molare* H 542^T in the constructed phylogenetic tree (Fig. 2).

The 16S-23S intergenic spacer region of the seven mycoplasma isolates of group 1 showed highest sequence similarity values with *M. felis* CO^T (88.78-90.54 %) and only <88 % with *M. mustelae* MX9^T (next related species <83 %), whereas for group 2 strains highest sequence similarity values were obtained with *M. lagogenitalium* 12MS^T (89.98-90.11 %) and *M. molare* H 542^T (89.05-89.75 %) (next related species <83 %).

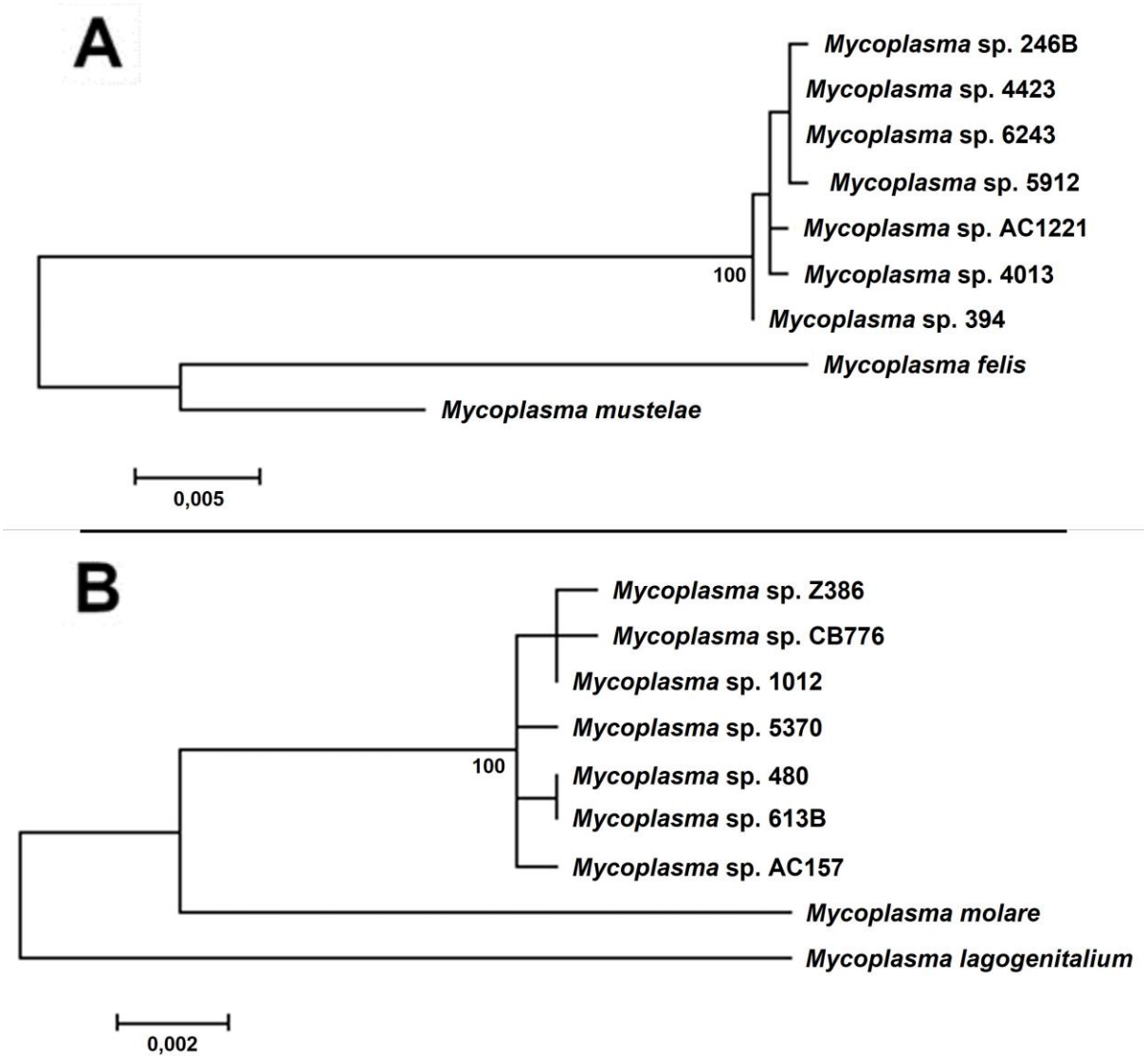

Fig. 1 Phylogenetic position based on 16S rRNA gene sequences of selected mycoplasma isolates from (A) group 1 and (B) group 2. The consensus tree was constructed from 1000 bootstrap replicates (only values above 70 are indicated). The scale bar conveys the distance between mycoplasma strains (substitution/position). The calculation was performed using MegaX.

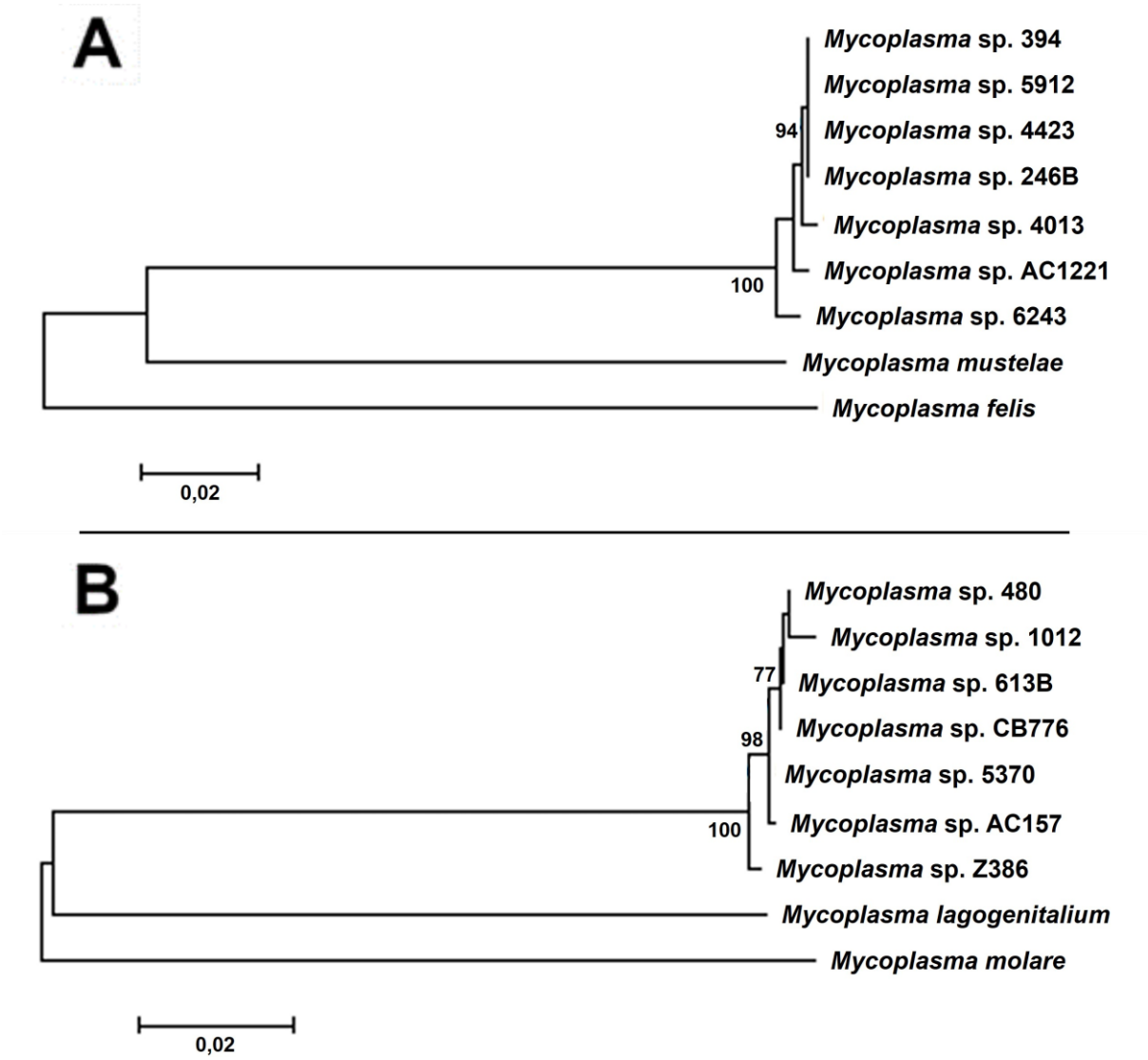

Fig. 2 Phylogenetic position based on partial *rpoB* gene sequences of selected mycoplasma isolates from (A) group 1 and (B) group 2. The consensus tree was constructed from 1000 bootstrap replicates (only values above 70 are indicated). The scale bar conveys the distance between mycoplasma strains (substitution/position). The calculation was performed using MegaX.

3.2 Phenotypic characterization

3.2.1 Colony morphology

All seven mycoplasma isolates of group 1 produced colonies with a typical fried egg shape which differed in opacity. While colonies of strains 4423, 5912, AC1221, 246B, and 394 presented a translucent morphotype, colonies of strains 6243 and 4013 were opaque (Fig. 3).

For all seven mycoplasma isolates of group 2, colonies with fried egg morphology were observed, although this typical shape was less clear in strains 5370, CB776, Z386, and AC157. These were also the strains demonstrating colonies with an opaque morphotype, while colonies of the strains 480, 1012 and 613B presented a translucent morphotype (Fig. 3). All strains of this group also produced 'film and spots' after 5 to 8 days of incubation (18).

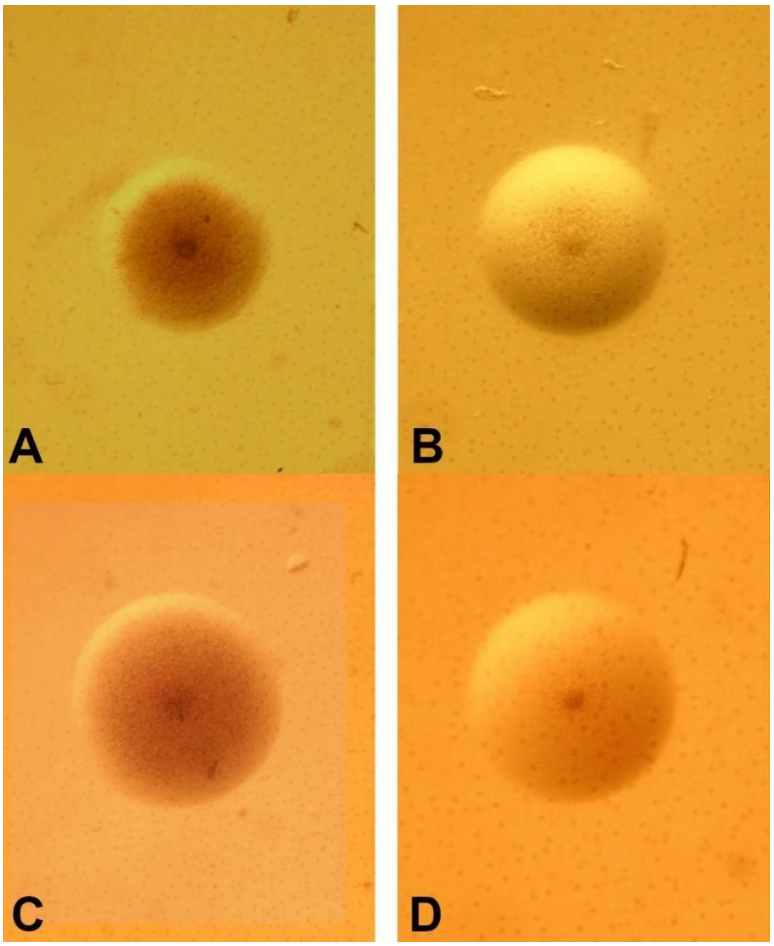

Fig. 3 A representative colony of (A) 6243 and (B) 5912 of group 1 and of (C) 5370 and (D) 480 of group 2 after 4 days of incubation at 40x magnification. 6243 and 5370 (A and C, respectively) represent the opaque morphotype, while 5912 and 480 (B and D, respectively) represent the translucent morphotype.

3.2.2 Growth characteristics

On SP4 agar plates, all strains grew rapidly at 37°C and more slowly at 28°C. Strains belonging to group 2 also showed rapid growth at 33°C, while those within group 1 only grew slowly. All strains did not grow at 4, 22, and 42°C but were shown to be facultative anaerobic at 37°C.

3.2.3 Digitonin sensitivity

Both strain 6243 (group 1) and 480 (group 2) were tested for digitonin sensitivity. They both displayed a clear growth inhibition zone around the digitonin filter disk after 48-72 hours of incubation. Since only members of genus *Mycoplasma* require cholesterol for growth, both strains could be assigned to genus *Mycoplasma* based on their digitonin sensitivity (19).

3.2.4 Enzymatic activity

The API-ZYM test kit (Biomerieux, France) was used to determine the enzymatic activity of a representative mycoplasma isolate of either group (6243 and 480, respectively). In both strains strong activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase and a weaker activity of naphthol-AS-BI-phosphohydrolase and α-glucosidase was observed. The activity of leucine arylamidase and N-acetyl-β-glucosaminidase was only detected in strain 6243.

3.2.5 Glucose fermentation and arginine and urea hydrolysis

All isolates of both groups were tested for their ability to ferment glucose or hydrolyze arginine or urea. While all mycoplasma isolates tested had the capacity to ferment glucose, none were able to metabolize arginine. Furthermore, none of the isolates proved to possess the characteristic capability of genus *Ureaplasma* to metabolize urea.

3.2.6 Reduction of tetrazolium chloride and potassium tellurite

The ability to reduce tetrazolium chloride and potassium tellurite under microaerobic and anaerobic growth conditions was tested in all mycoplasma strains. All strains demonstrated the capability to reduce tetrazolium chloride under anaerobic growth conditions, proven by a reddish color zone around colonies. In addition, all strains were able to reduce potassium tellurite under anaerobic and microaerobic growth conditions, indicated by the formation of black-colored colonies (20).

3.2.7 Hemolysis

The ability to induce hemolysis was tested on a representative mycoplasma isolate of either group (6243 and 480, respectively). For both strains the characteristic formation of transparent zones in the blood agar surrounding mycoplasma colonies (Fig. 4) could be observed, marking their capacity to cause hemolysis.

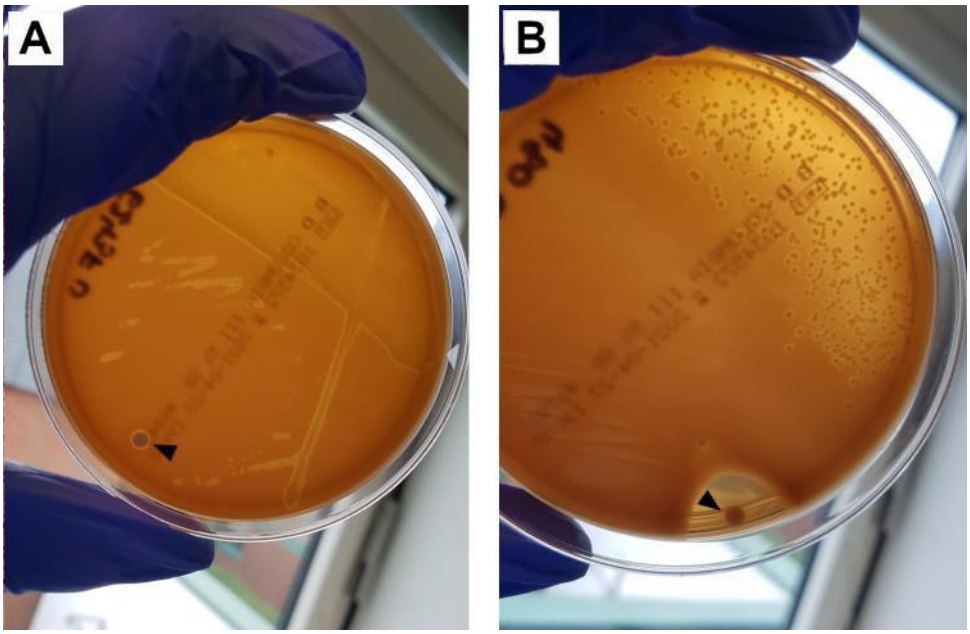

Fig. 4 Hemolysis surrounding the mycoplasma colonies of (A) 6243 and (B) 480 on a Columbia agar plate. Both plates were contaminated with another kind of hemolytic bacteria (arrows) which could be easily distinguished from mycoplasmas based on their much larger colony size.

3.2.8 Cell morphology

To illustrate the cell morphology, ultrathin sections of representative mycoplasma strains of both groups (6243 and 480, respectively) were examined by transmission electron microscopy. Cells lacked a cell wall and were only bounded by a bi-layered cell membrane that was fuzz-like coated possibly representing a glycocalyx. While cells of strain 480 were spherical with diameters ranging from 480 to 630nm, cells of 6243 were mostly ovoid with some being peanut-shaped (Fig. 5).

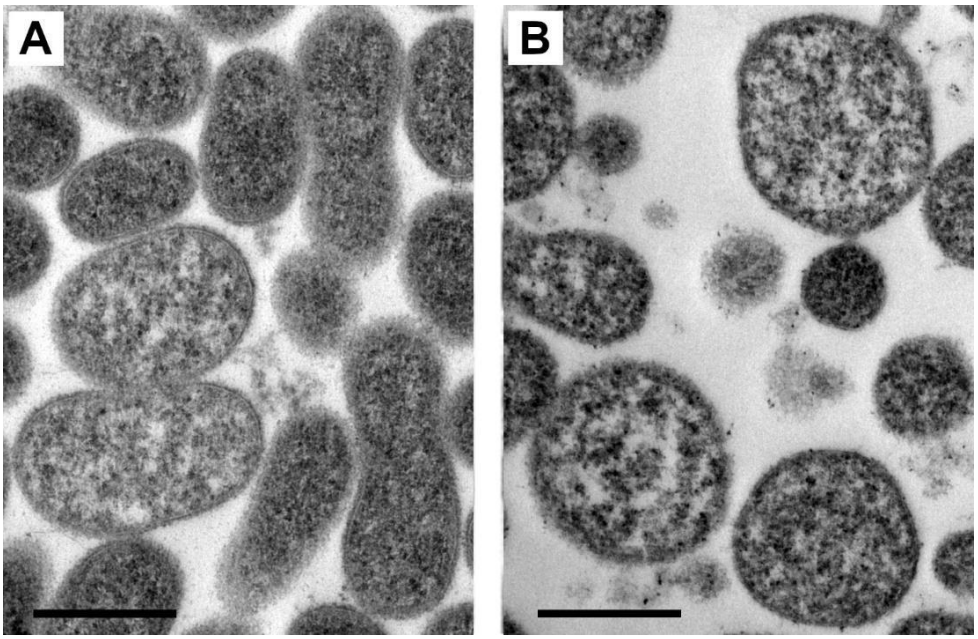

Fig. 5 Transmission electron micrographs of (A) 6243 and (B) 480. Note a fuzz-like coating on the surface of several 480 cells (B) which may possibly represent a glycocalyx. Bar, 500 nm.

3.2.9 MALDI-TOF protein mass fingerprinting

Comparison of generated spectra with reference spectra of an in-house mycoplasma database confirmed grouping of the analyzed strains by providing score value ≥2.00 for corresponding reference spectra of strain 6243 and 480. Moreover, spectrum overlays demonstrated a high level of congruency among isolates belonging to group 1, which was also observed when superimposing spectra of group 2 strains. This led to the confirmation that strains of group 1 belong to the same species of mycoplasma, while strains of group 2 are members of a distinct mycoplasma species.

When spectra of either group were superimposed with spectra generated for next related type strains, substantial peak differences were observed between *M. mustelae* or *M. felis* and spectra of group 1 strains, with the same being observed between *M. molare* or *M. lagogenitalium* and spectra of group 2 organisms (Fig. 6).

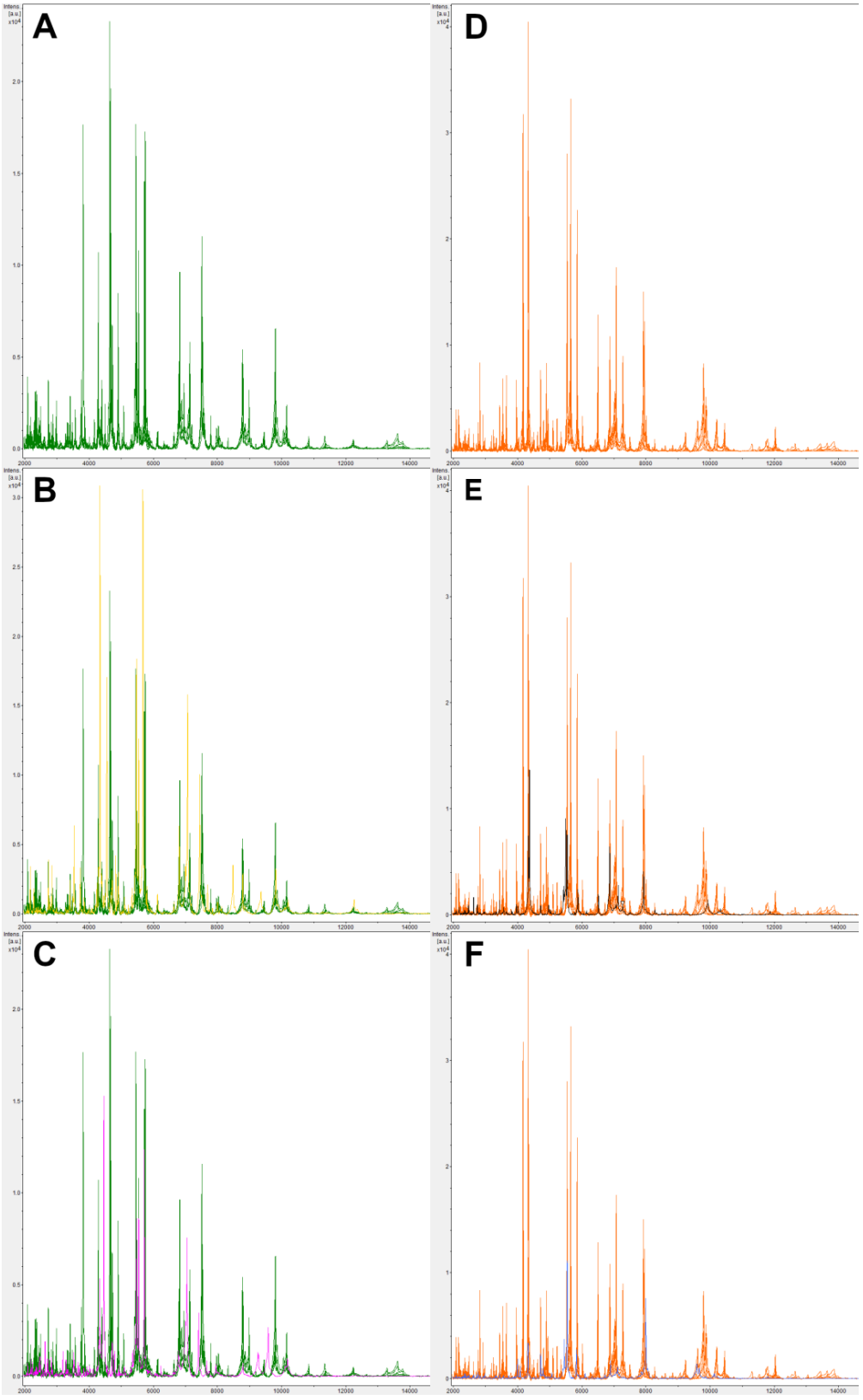

Fig. 6 Overlays of MALDI-ToF mass spectra generated from isolates belonging to (A) group 1 (in green) and (D) group 2 (in orange) displaying a high level of similarity but substantial differences to mass spectra of (B) *M. mustelae* (in yellow) and (C) *M. felis* (in pink) for group 1, as well as (E) *M. molare* (in black) and (F) *M. lagogenitalium* (in blue) for group 2. Y-axis - a.u. = arbitrary unit, x-axis - m/z = mass-to-charge ratio.

3.2.10 Histopathology

Tissue sections of animals from which group 1 and group 2 mycoplasmas had been isolated were screened for alterations characteristic for mycoplasma infection like BALT (bronchus-associated lymphoid tissue) hyperplasia with infiltration of mononuclear cells such as lymphocytes and macrophages. These criteria were clearly met without being associated with other factors in lung tissues originating from badgers from which strains 6243, 394, AC4, and AC496 (all group 1 strains) had been isolated (Fig. 7), while lung tissues of animals from which strains 246B (group 1) and 5370 (group 2) had been recovered were deemed questionable.

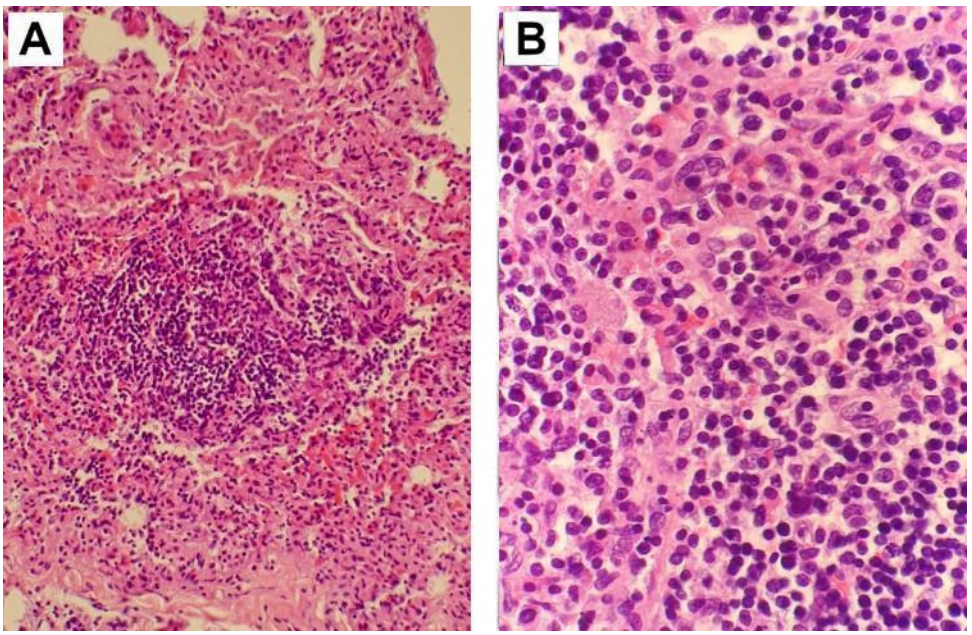

Fig. 7 Lung tissue section of animal 6243 (from which strain 6243 had been isolated) presenting BALT hyperplasia with infiltration of lymphocytes and macrophages indicating a non-pyogenic inflammation of the lung typical for mycoplasma infection (A) x100 and (B) x400 magnification.

4. Discussion

Even now with over 150 recognized species within the highly diverse genus *Mycoplasma*, we are only scratching the surface with most of these known *Mycoplasma* species having been isolated from humans and domesticated animals and a certain number of novel species being discovered in wildlife over the last decade. Therefore, the aim of this diploma thesis was the phenotypic and genotypic characterization of unidentifiable mycoplasma isolates recovered from the respiratory tract and the brain of Eurasian badgers (*Meles meles*). The investigated mycoplasma isolates represent two hitherto undescribed *Mycoplasma* species which was underlined through the results of the polyphasic taxonomic analyses. This study and the presented results thus build a solid foundation for further research such as whole genome sequencing of selected isolates. This will ultimately lead to the description of two novel species within genus *Mycoplasma*.

The taxonomic classification of unidentifiable mycoplasma isolates is based on phenetic, genetic, phylogenetic, and genomic characteristics. Traditionally, phenotypic characteristics are used to assign unknown mycoplasma isolates to higher taxonomic ranks such as the class *Mollicutes*, the order *Mycoplasmatales*, *Entomoplasmatales*, *Acholeplasmatales*, or *Anaeroplasmatales*, the families *Mycoplasmataceae*, *Entomoplasmataceae*, *Spiroplasmataceae*, *Acholeplasmataceae*, or *Anaeroplasmataceae*. This is effectuated through phenotypic tests that determine cell morphological characteristics such as the lack of a cell wall, and cell size and shape, as well as cultural and growth characteristics such as optimal growth temperature, oxygen sensitivity, colony morphology, sterol requirement for growth and cultivability (21). Mycoplasmas possess only limited biosynthetic and metabolic capacities, rendering biochemical analyses for taxonomic classification secondary. Biochemical tests only enable the assignment of a mycoplasma isolate to either the genus level (*Ureaplasma*) or a metabolic group such as glucose-, arginine- or organic acid utilizers (15, 22).

MALDI-TOF mass spectrometry, a method rapidly gaining popularity in taxonomic research, has been recognized as a reliable approach to distinguish and identify mycoplasma at the species level, displacing the traditional serological species identification method (23, 24). Importantly, this novel methodology has led to the detection of yet undescribed mycoplasma species in a variety of animal hosts, from pets and farm animals to wildlife (24). Moreover, MALDI-TOF has shown to be an effective tool for phenotypic profiling of novel species within genus *Mycoplasma* (25–27). Our research further underscores the credibility of MALDI-TOF

as a stand-alone phenotypic characterization technique, allowing us to classify all the studied mycoplasma isolates into two distinct species clearly separating them from other *Mycoplasma* species closely related with either group.

Taxonomic studies on mycoplasmas have long been informed by phylogenetic positioning based on 16S rRNA gene sequencing, a universal bacterial ribosomal RNA component recognized for its slow evolutionary pace (28, 29). Comparison of 16S rRNA gene sequences enables the assignment of a mycoplasma isolate to a genus or even a phylogenetic cluster within the genus category. It is widely accepted that if sequence similarities of <97 % are found, the mycoplasma isolate being studied can be considered a distinct species, while values >97 % imply close relationship (30). However, for certain closely related mycoplasma species, sequence similarity values between 98 and 99.5 % have been observed, making it difficult to delineate species only based on 16S rRNA gene sequencing. Hence, when 16S rRNA gene analysis reveals close phylogenetic relatedness (sequence similarity >97 %), additional phylogenetic markers such as the *rpoB* gene and the 16S-23S intergenic spacer region are used to define a species with arbitrary values of 90 % (*rpoB*) and 95 % (16S-23S intergenic spacer) proposed for species delineation (22). In our study, inconclusive results from 16S rRNA gene sequencing were only obtained for group 2 strains exhibiting highest sequence similarity values of 98.14-98.28 % with *M. molare* H 542^{T}. However, partial *rpoB* gene sequences and 16S-23S intergenic spacers showed highest similarity values to next related taxa (*rpoB* – 83-84 %, intergenic spacer – 89-90 %) far below the species delineation thresholds proposed (22), strongly suggesting that group 2 strains represent a novel species.

Histopathological examination of lung and trachea tissue sections revealed BALT hyperplasia with mononuclear cell infiltration in four lung samples from which mycoplasmas belonging to group 1 had been isolated previously. These pathohistological findings correlated with alterations associated with mycoplasma infection, indicating the group 1 mycoplasmas could possibly play a role as respiratory pathogens in the Eurasian badger (31).

Conclusively, the results of this study present ample proof that the seven unknown mycoplasma isolates of either group isolated from the Eurasian badger are members of two distinct novel species within genus *Mycoplasma*.

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List of tables and figures