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Genetic diversity and antibiotic resistance of *Mycoplasma bovis*

Ph.D. thesis

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2017

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Abbreviations

AFLP	amplified fragment length polymorphism
bp	base pair
CCU	colour changing unit
CI	confidence interval
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ELISA	enzyme-linked immunosorbent assay
gyrA gene	gene of DNA gyrase, A subunit
HRM	high resolution melt
MAMA	mismatch amplification mutation assay
MIC	minimum inhibitory concentration
MIC_{50}	minimum inhibitory concentration that inhibit 50% of the isolates
MIC_{90}	minimum inhibitory concentration that inhibit 90% of the isolates
MLST	multi-locus sequence typing
MLVA	multi-locus variable number of tandem repeats analysis
NCCLS	National Committee for Clinical Laboratory Standards
parC gene	gene of DNA topoisomerase IV, A subunit
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PG45	*Mycoplasma bovis* reference strain (NCTC10131)
QRDR	quinolone resistance-determining region
rrl1/*2* genes	23S ribosomal ribonucleic acid genes
rRNA	ribosomal ribonucleic acid
rrs1/*2* genes	16S ribosomal ribonucleic acid genes
RAPD	random amplified polymorphic deoxyribonucleic acid
S1-6	supplementary material 1-6
SE	standard error
SNP	single nucleotide polymorphism
ST	sequence type
subsp.	subspecies
T_{ann}	annealing temperature
Tet-1	primary tetracycline binding site
tet(M)	tetracycline-resistance determinant
T_m	melting temperature
TR	tandem repeat
uvrC gene	gene of excinuclease ABC, C subunit
VNTR	variable number tandem repeat
Vsp	variable surface protein

1. Summary

Mycoplasma bovis is a worldwide pathogen, the causative agent of respiratory disease, mastitis and arthritis of intensively farmed cattle, for which it had emerged as an economically significant infectious agent in North America and Europe. By the beginning of this century, the proportion of seropositive cattle herds in Hungary was almost two thirds, which value increased to 100% by 2008.

Efficient genotyping tools are essential for monitoring, control and epidemiological investigation of this agent. For the comparison and the genetic characterization of the Hungarian *M. bovis* population multi-locus sequence typing (MLST) and multi-locus variable-number tandem-repeat analysis (MLVA) were used. The thirty-one examined Hungarian *M. bovis* isolates were grouped into two clades and showed high genetic variation by both methods with little congruence between the results of the two typing systems. Therefore, the combined use of the two molecular epidemiological typing techniques are recommended for analysis of *M. bovis* isolates, as MLST provides information about mid-term evolutionary events while the short-term epidemiological relationships can be revealed by MLVA.

Since no effective vaccine is available against *M. bovis*, adequate housing and appropriate antibiotic treatment are promoted in the control of the diseases caused by this pathogen. In routine practice, the potential effectiveness of antimicrobials *in vivo* can be estimated by *in vitro* susceptibility testing with the determination of the minimum inhibitory concentration (MIC) values. Comprehensive study about the antibiotic susceptibility profiles of *M. bovis* strains isolated in Hungary has not been performed previously. Based on our *in vitro* examinations on 15 antibiotics of 8 antimicrobial families, fluoroquinolones are supposed to be the most effective agents in the therapy of *M. bovis* infections in Hungary. Our results confirmed the increasing resistance to antibiotics commonly used for the treatment of mycoplasma infections, primarily to tetracyclines and macrolides emphasizing the necessity of periodic testing of antibiotic susceptibility within a geographic region. In accordance with previously published *in vivo* experiments high effectiveness of pleuromutilins was observed in the *in vitro* examinations, suggesting the potential of this antibiotic group in the therapy of *M. bovis* infections.

Traditionally, antibiotic susceptibility examination of mycoplasmas is technically demanding, time-consuming and rarely performed in diagnostic laboratories, thereby, empirical treatment is often introduced which might lead to therapeutic failure and development of resistance to critically important antimicrobials. Information about the genetic background of antibiotic resistance is essential for the development of genetic-based diagnostic assays and also for the prevention of the spread of resistance to antibiotics. Whole genome sequencing and

sequence comparison of 35 *M. bovis* field isolates and 36 laboratory-derived antibiotic resistant mutants revealed molecular markers responsible for the high MICs to certain antibiotics. Mutations associated with high MICs to fluoroquinolones were found at the quinolone resistance determining regions of *gyrA* and *parC* genes, while in the case of tetracyclines alterations were described on genes encoding the 16S rRNA, which form the primary tetracycline binding site. A single transversion in the *rrs1* gene resulted in ≥256 µg/ml MIC to spectinomycin, while several mutations were identified in genes encoding the 23S rRNA, which were responsible for high MICs to the 50S inhibitors: macrolides, lincomycin, florfenicol and pleuromutilins. Some of these mutations led to cross-resistance to other classes of antibiotics with a similar mechanism of action.

For the simultaneous detection of multiple mutations responsible for high MICs to fluoroquinolones, tetracyclines, spectinomycin, macrolides, lincomycin, florfenicol and pleuromutilins in *M. bovis*, 9 mismatch amplification mutation assays and 7 high resolution melt tests were designed and evaluated. The reported methods proved to be highly reliable tested on the DNA of *M. bovis* pure cultures or on clinical samples containing high copy numbers of *M. bovis*, assuming the usefulness of the assays during the therapy of clinical *M. bovis* infections. For the most reliable antibiotic resistance determination, especially in the case of clinical samples, the regions examined by the MAMA and HRM tests are overlapping. The presented method is rapid, highly cost-effective and can provide the antibiogram of *M. bovis* to 12 antibiotics of 7 antimicrobial groups, thus it may represent a suitable alternative for the conventional antibiotic susceptibility tests and it can promote the effectiveness of the therapy.

Összefoglalás

A *Mycoplasma bovis* világszerte elterjedt kórokozó, szarvasmarhákban tüdő-, tőgy- és ízületi gyulladást idézhet elő. Európa és Észak-Amerika szarvasmarha-állományaiban az általa okozott gazdasági károk igen jelentősek. A század elejére a hazai szarvasmarha állományok csaknem kétharmada, 2008-ra pedig az állományok 100%-a szeropozitívnak bizonyult.

Egy hatékony genotipizáló rendszer elengedhetetlen a fertőzés nyomon követésére és járványtani vizsgálataihoz. Mind a háztartási gének vizsgálatán alapuló MLST (multi-locus sequence typing), mind pedig a variábilis számú tandem ismétlődések elemzésén alapuló MLVA (multi-locus variable-number tandem-repeat analysis) módszerekkel a hazai *M. bovis* populáció nagy genetikai változékonyságát tártuk fel. Mindkét módszerrel két fő kládot különböztettünk meg, számos elágazással és alcsoporttal, azonban a két tipizáló módszer eredményei között csekély átfedést találtunk. Ebből kifolyólag a *M. bovis* törzsek jellemzésére a két rendszer együttes használatát javasoljuk: az MLST közepes felbontású, egymástól viszonylag távoli izolátumok tipizálására, míg az MLVA nagyobb felbontású, egymással közel rokon izolátumok rokonsági viszonyainak feltárására alkalmas módszer.

A *M. bovis* okozta fertőzések megelőzésére jelenleg nem áll rendelkezésre hatékony vakcina, ezért a megfelelő tartási körülmények és az antibiotikum terápia a védekezés legfőbb eszközei. A rutin diagnosztikában az antibiotikumok *in vivo* hatékonysága *in vitro* antibiotikum érzékenységi vizsgálatokkal, a minimális gátló koncentráció értékek (MIC; minimum inhibitory concentration) meghatározásával becsülhető. Hazai *M. bovis* törzsek antibiotikum érzékenységének megállapítására eddig nem készült átfogó vizsgálat. Nyolc antibiotikum család 15 antibiotikumával szemben végzett *in vitro* vizsgálataink alapján a fluorokinolonok bizonyultak a legígéretesebb antimikrobiális szereknek a *M. bovis* okozta hazai fertőzések kezelésére. A mycoplasma fertőzések gyógykezelése során gyakran használt antibiotikumokkal (elsősorban a tetraciklinekkel és makrolidokkal) szemben növekvő rezisztenciát mutattunk ki. Eredményeink kiemelik a rendszeres antibiotikum érzékenységi vizsgálatok szükségességét. Korábbi *in vivo* vizsgálatokkal összhangban a pleuromutilinek hatékonyan gátolták a baktérium növekedését *in vitro* vizsgálataink során, így ez az antibiotikumcsoport a későbbiekben alkalmas lehet terápiás célokra.

A mycoplasma törzsek antibiotikum érzékenységének meghatározása a klasszikus mikrohígításos módszerrel pénz- és időigényes feladat, ezért a diagnosztikai laboratóriumokban ritkán alkalmazzák. A gyakorlatban a tapasztalati alapon kiválasztott, gyakran nem megfelelő terápiás szer a kezelés hatástalanságához vezethet, illetve kedvez az antibiotikum rezisztencia kialakulásának is. Az antibiotikum rezisztencia genetikai hátterének ismerete elengedhetetlen a gyors molekuláris tesztek fejlesztéséhez, illetve az

antibiotikum rezisztencia terjedésének megelőzéséhez. Vizsgálataink során 35 hazai *M. bovis* izolátum és 36 *in vitro* szelektált antibiotikum rezisztens törzs teljes genom szekvenálását végeztük el. Magas MIC értékekért felelős pontmutációkat a fluorokinolonok esetén a DNS-giráz, illetve a topoizomeráz IV A alegységein (GyrA és ParC) találtunk. Emelkedett tetraciklin MIC értékeket okozó mutációkat azonosítottunk a 16S rRNS-t kódoló géneken, továbbá a spektinomicinre ≥256 µg/ml MIC értékkel rendelkező valamennyi törzs hordozta az *rrs1* gén egy pontmutációját. A bakteriális riboszóma 50S alegységét gátló antibiotikumok (makrolidok, lincomycin, pleuromutilinek, florfenikol) esetében a 23S rRNS-t kódoló géneken találtunk magas MIC értékekkel összefüggő mutációkat, melyek közül néhány keresztrezisztenciát okozott a hasonló hatásmechanizmusú szerekkel szemben.

A fluorokinolonokkal, tetraciklinekkel, spektinomycinnel, makrolidokkal, linkomycinnel, florfenikollal és pleuromutilinekkel szemben magas MIC értékekért felelős mutációk egyidejű kimutatására 9 nagy felbontású olvadáspont analízist (HRM, high resolution melt) és 7 pontmutációk kimutatására alkalmas MAMA (mismatch amplification mutation assay) rendszert fejlesztettünk ki. A tesztek megbízhatóan működtek tiszta *M. bovis* tenyészeteken és nagy mennyiségű *M. bovis* DNS-t tartalmazó klinikai minták esetén is, így a rendszerek hasznosak lehetnek a klinikai tüneteket előidéző *M. bovis* fertőzések kezelésekor. A MAMA és HRM tesztek által vizsgált régiók átfednek, így növelve a módszerek megbízhatóságát, különösen klinikai mintákon alkalmazva. Az általunk fejlesztett rendszerek lehetővé teszik a különböző antibiotikum-érzékenységgel rendelkező *M. bovis* törzsek gyors és költséghatékony elkülönítését 7 antibiotikum család 12 antibiotikumával szemben, így alkalmasak lehetnek a klasszikus antibiotikum érzékenységi vizsgálatok helyettesítésére, növelve a gyógykezelés hatékonyságát.

2. Introduction

2.1. History and taxonomy

Mycoplasma bovis belongs to the class *Mollicutes* (named after the cell wall-less characteristic; from the Latin mollis, soft; cutis, skin), to the order *Mycoplasmatales*, to the family *Myoplasmataceae* and the genus *Mycoplasma* (Maniloff 2002). The term *Mycoplasma* (from the Greek mykes, fungus; plasma, something formed) was first used by Frank in 1889 describing structures within the root nodules of legumes because of their morphological similarities to fungi. The name was later adopted by Nowak to describe the causative agent of contagious bovine pleuropneumonia. In 1898 *Mycoplasma mycoides* subsp. *mycoides* was reported as the first successfully cultivated mycoplasma by Nocard and Roux. In 1956 the taxonomic name *Mycoplasma* was recommended by Edward and Freundt because it drew attention to the one generally accepted property of the organism, namely its plasticity (Krass and Gardner 1973).

Up to now, over one hundred recognised Mycoplasma taxa have been described including 25 species infecting cattle (Nicholas et al. 2016, Maniloff 2002). *M. bovis*, one of the most significant pathogens in cattle, was first isolated in 1961 in the USA from a case of severe mastitis (Hale et al. 1962). It was formerly named *M. agalactiae* subsp. *bovis* because of the similarities in many aspects with the small ruminant pathogen, *M. agalactiae*. Based on double immunodiffusion and growth inhibition tests Jain et al. (1967) suggested the term *M. bovimastitidis* since no close relationship to *M. agalactiae* had been demonstrated. However, these authors did not present sufficient data to categorize this pathogen as a distinct species. In 1976, the agent was described as the cause of pneumonia and arthritis in gnotobiotic calves (Gourlay et al. 1976) and in the same year it was classified as a separate species with the name used in still based on DNA-DNA hybridization experiments (Askaa and Erno 1976).

2.2. Properties of the agent

M. bovis as all members of the class *Mollicutes* is a prokaryote of Gram-positive lineage that evolved from Clostridium-like bacteria by gene deletion. The lack of cell wall and the tri-layered cell membrane results in pleomorphic shapes and interferes with Gram staining (Caswell and Archambault 2008).

The first published genome sequence of *M. bovis* for the type strain PG45 (NCTC 10131) is 1,003,404 base pairs (bp) in length and has a 29.3% G+C content containing 826 open

reading frames (Wise et al. 2011). The small genome size and the limited biosynthetic capacity of mycoplasmas may explain their dependency on the host, the complex media containing essential nutrients required for cultivation and their limited survival in the environment (Caswell and Archambault 2008).

The cell size of mycoplasmas is about 0.2-0.3 μm in diameter and most of them are facultative anaerobes and require sterols for growth in media (Caswell and Archambault 2008). Biochemical properties of *M. bovis* is similar to *M. agalactiae* as it does not ferment glucose or hydrolyse arginine but use organic acids (lactate and pyruvate) for energy sources, which acidifies the media and gives an orange-yellow colour to the broth (when containing phenol red pH indicator) without turbidity during cultivation (Miles et al. 1988, Nicholas and Ayling 2003). Colonies of *M. bovis* on solid media often have a typical fried-egg appearance but it can produce films and spots as a result of lipolytic activity (Nicholas and Ayling 2003).

Despite the cell-wall less characteristic, in the environment *M. bovis* can survive for long periods if protected from sunlight and desiccation. As several other *Mycoplasma* species, *M. bovis* produces biofilms, which increase the bacterial resistance to heat and desiccation (McAuliffe et al. 2006). It is able to survive at 4°C for nearly 2 months in sponges and milk, over 2 weeks on wood and in water and it can remain infectious for years in frozen sperm (Nicholas and Ayling 2003); although at higher environmental temperatures survival time drops considerably (Pfutzner and Sachse 1996).

2.3. Geographic distribution

M. bovis have spread via animal movements (global transportation of animals and sperm) to numerous countries of Europe (including Spain [1967], France [1974], Great Britain [1974], Czechoslovakia [1975], Germany [1977], Denmark [1981], Switzerland [1983], Northern Ireland [1993] and the Republic of Ireland [1994]) and even of the world (Israel [1964], Australia [1970], Morocco [1988], South Korea [1989], Brazil [1989], Chile [2000], South Africa [2005], and China [2008]) (Nicholas 2011), with the exception of Finland (Härtel et al. 2004, Autio et al. 2007), Norway (Gulliksen et al. 2009), and New Zeeland (Taylor et al. 2009), which seem to be *M. bovis* free. Estimations of the prevalence of infection vary widely between reports; however the prevalences reported in former studies (in the 1980s and ‘90s) are generally lower compared to the prevalences observed in more recent studies (Caswell and Archambault 2008).

In Hungary *M. bovis* infection was first reported in 1975 (Romváry et al. 1975). By the beginning of this century, the average seropositivity rate of individual animals was found to be 11.3% and the overall rate of seropositive herds was 64.7% tested by enzyme-linked immunosorbent assay (ELISA) (Tenk et al. 2004), which values increased to 82.9% in individual level and 100% on farm level by 2008 (Fodor et al. 2017).

2.4. Epidemiology

2.4.1. Shedding, infection routes and transmission of the disease

M. bovis is highly adapted to cattle but occasionally it has been isolated from deer, goats, pigs and even from human (Ojo and Ikede 1976, Madoff et al. 1979, Dyer et al. 2004, Spergser et al. 2013). Feedlot bison is also considered to be susceptible to *M. bovis* infections, as the pathogen is capable to cause primary disease and outbreaks in this species (Dyer et al. 2008).

The main source of *M. bovis* infection is usually the introduction of clinically or subclinically affected cattle into naive herds. Infected cattle shed the bacteria via the respiratory tract from months to years, acting as reservoirs of the infection within a herd (Nicholas and Ayling 2003, Pfutzner 1990). Increased excretion of *M. bovis* was observed in cattle with clinical disease (Bennett and Jasper 1977). Stressful events such as transportation or cold stress are also associated with increased rates of nasal shedding (Boothby et al. 1983).

The major routes of transmission of the pathogen are the udder-to-udder spread, and by aerosols, by nose-to-nose contact, or indirectly via feed, water, housing, or other fomites (Jasper et al. 1973, Gonzalez et al. 1992, Maunsell et al. 2011). In the epidemiology of the agent ingestion of infected milk is also important for young calves (Bennett and Jasper 1977), while congenital infections seem to occur infrequently (Pfutzner and Sachse 1996). Artificial insemination with infected semen can be another common route of infection (Pfutzner 1990).

M. bovis colonizes the mucosal surfaces of the respiratory tract, teat canal or the genital tract of contact animals where it can persist without causing symptoms (Nicholas and Ayling 2003). The development of the clinical signs depends on other pathogens and environmental factors (i.e. herd size) as well.

Coinfection of *M. bovis*-associated pneumonia is common mainly with *Mannheimia haemolytica*, *Pasteurella multocida, Arcanobacterium pyogenes or M. arginini*. Less frequently, coinfection occurs with other pathogens including *Histophilus somni, Bovine herpesvirus*-1*, Bovine respiratory syncytial virus, Bovine parainfluenza*-3 *virus* or *Bovine viral diarrhea virus* (Stipkovits et al. 2000b, Gagea et al. 2006, Fulton et al. 2009, Caswell et al.

2010). In these cases *M. bovis* is thought to be the predisposing factor in the infections, possibly by compromising host defences, hence enabling the invasion by other bacterial pathogens (Nicholas and Ayling 2003).

2.4.2. Molecular epidemiology

Molecular typing methods are expected to reveal new insights into the epidemiology of *M. bovis* by the identification of infection sources and routes and by the exploration of genetic relatedness both within herds and across larger populations. The implementation of new control strategies also could be enabled by the molecular typing methods (Nicholas et al. 2016).

Initially, several DNA fingerprinting techniques including random amplified polymorphic DNA (RAPD) (McAuliffe et al. 2004), amplified fragment length polymorphism (AFLP) (Kusiluka et al. 2000, McAuliffe et al. 2004), pulsed-field gel electrophoresis (PFGE) (McAuliffe et al. 2004, Punyapornwithaya et al. 2010), insertion sequence (IS) typing (Miles et al. 2005, Thomas et al. 2005) or arbitrary primed polymerase chain reaction (AP-PCR) (Butler et al. 2001) were used to compare *M. bovis* isolates. However, these techniques have several disadvantages, such as the poor reproducibility of the results with the RAPD method or the need for special equipment and the time consuming nature of PFGE analysis (McAuliffe et al. 2004, Caswell and Archambault 2008).

Recently, several multi-locus sequence typing (MLST) systems (Manso-Silván et al. 2012, Becker et al. 2015, Register et al. 2015, Rosales et al. 2015) and a multi-locus variable-number tandem repeat (VNTR) analysis (MLVA) (Pinho et al. 2012) have been developed for the genetic characterization of *M. bovis* isolates. MLST schemes were originally designed to capture the intermediate-level evolutionary relationships between bacterial isolates (e.g. on a country or continent level) because it focuses on sequence analysis of housekeeping genes known to be poorly variable. MLVA, based on the inherent size variability of repeat regions, was proposed to detect the quick mutation events (e.g. during epidemics) within bacteria species providing a higher degree of reproducibility than PFGE or AFLP (Keim et al. 2007, Margos et al. 2011, Pinho et al. 2012, Becker et al. 2015).

Attempts to develop protein based typing methods of *M. bovis* have been also described. Expression profiles of the variable surface protein (Vsp) were not related to any of the typing patterns of AFLP, RAPD or PFGE because of recombination events in the Vsp locus. This incongruence implies that its use for subtyping strains with an epidemiological concordance is limited (McAuliffe et al. 2004, Caswell and Archambault 2008). By contrast, the pattern of total protein obtained by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass

Spectrometry (MALDI-TOF MS) was proved to be promising for subtyping *M. bovis* strains (Pereyre et al. 2013, Becker et al. 2015).

The considerable genomic heterogeneity reported among *M. bovis* field isolates is often hard to interpret, as apart from the ability of mycoplasmas to create genetically diverse populations, there is an extensive trade of cattle between different regions, which can also contribute to this high variablilty (Maunsell and Donovan 2009, Nicholas et al. 2016).

Nevertheless, MLST examination of *M. bovis* isolates revealed that the strains originating from bisons are genetically distinct from strains infecting cattle, and that these host-specific genotypes cause outbreaks in the North American bison population (Register et al. 2015).

Identical genotypes were frequently identified by PFGE in *M. bovis* strains originating from different organs and the milk of the same host, suggesting the internal transmission of the agent, but the presence of multiple genotypes within one animal was also described (Biddle et al. 2005). Generally, closely related strains or identical genotypes are isolated from the different pathological sites of an infected animal or from multiple animals within a herd, but endemically infected open herds can harbour numerous genetically diverse strains (Kusiluka et al. 2000, Butler et al. 2001, Maunsell and Donovan 2009).

Correlations between epidemiological data and the genotypes of the strains have been described in several cases. Typing study based on AFLP and RAPD from the United Kingdom showed two distinct clusters of pneumonic *M. bovis* strains. The isolates which have been introduced from the continental countries of Europe after the removement of the restrictions on animal transfer due to the creation of the European Union market formed one group. The other group of isolates complied the genotypes which were present previously in the country (McAuliffe et al. 2004, Nicholas et al. 2016).

The presence of two main clusters was also observed in the French, Swiss and Austrian *M. bovis* populations (Becker et al. 2015, Bürki et al. 2016). Genotypes of the French strains mainly isolated from respiratory cases showed correlation with the year of isolation determined by MALDI-TOF, MLST and MLVA methods. Evenmore, the group of the recent French isolates consisted of more homogenous genotypes and the strains were characterised by multidrug resistant phenotypes suggesting the spread of a multiresistant clone (Gautier-Bouchardon et al. 2014, Becker et al. 2015). MLST typing of Swiss and Austrian *M. bovis* isolates revealed accordance also in the clinical signs beside the temporal connection within the cluster of the genotypes (Bürki et al. 2016). A unique genotype was identified by using MLVA and RAPD causing outbreaks of the Austrian Alpine dairy herds in 2007 and 2009, which crossed the species barrier by infecting pigs and became widespread in that region (Spergser et al. 2013).

Global MLST analysis of *M. bovis* isolates revealed two main clonal complexes, one including most of the British and German isolates and the other containing more heterogeneous isolates from Europe, Asia and Australia (Rosales et al. 2015).

2.5. Pathogenesis

M. bovis has virulence factors that enable the colonization and the persistence on mucosal surfaces, the invasion of host cells, persistence and dissemination of the agent at the predilection sites of the disease and the evasion of the aggressive immune response. Despite the clear evidence of the pathogenic potential of *M. bovis,* there is poor understanding of the molecular mechanism of its pathogenesis. Molecules involved in adherence, antigenic variation, immunomodulation, production of secondary metabolites and biofilm formation are likely to be important in the pathogenesis (Maunsell et al. 2011, Bürki et al. 2015).

One of the first steps during *M. bovis* infection is the adherence to bovine tracheobronchial epithelial cells. This adhesion presumably facilitates colonization of the lung, and it is mediated by membrane proteins including Vsps - a family of immunodominant lipoproteins on bacterial surface - and unrelated proteins, such as P26 and pMB67 (Sachse et al. 1996, Behrens et al. 1996, Thomas et al. 2003b, Thomas et al. 2003c). Cytoadherence is differing among the strains and correlating with pathogenicity and the number of *in vitro* passages (Thomas et al. 2003b, Thomas et al. 2003c).

After the colonization of the respiratory tract, *M. bovis* is able to invade to immune cells, which contribute to the dissemination of the bacteria to different infection sites of the host (Van Der Merwe et al. 2010).

As *M. bovis* develops chronic infection in the lung and disseminates to other organs it should be able to evade the host's immune defences. Antigenic variation is thought to be one way of the continuous immune evasion that *M. bovis* applies (Caswell et al. 2010, Bürki et al. 2015). Vsps are responsible for the highly variable antigenic profiles of *M. bovis.* These are unrelated to the geographical origin, site of isolation or type of clinical disease and are variable among different subclones within the same strain (Rosengarten et al. 1994, Bürki et al. 2015). Extensive variation in the sequence, size, number and expression of Vsps was described. *M. bovis* field isolates have variation in *vsp* reiterated coding sequences as well as in strain specific repetitive sequences (Nussbaum et al. 2002). Genes of Vsps contain repetitive and specific *vsp* inversion sequences favouring chromosomal recombination events, which could lead to duplications, inversions or deletions and result in gain or loss of surface antigens (Behrens et al. 1994, Lysnyansky et al. 1996). The genome of the *M. bovis*

type strain (PG45) comprises 13, while the Chinese strain HB0801 contain 6 different *vsp* genes (Behrens et al. 1994, Lysnyansky et al. 1996, Lysnyansky et al. 1999, Qi et al. 2012). The co-expression of several Vsps leads to surface mosaic with different structural and antigenic features (Lysnyansky et al. 1999). Vsp variants can be selected by exposure to antibodies (Le Grand et al. 1996).

Besides alteration of the surface antigens, *M. bovis* has other mechanisms to evade the immune responses, such as inducing the apoptosis of lymphocytes (Vanden Bush and Rosenbusch 2002), down-regulation of lymphocyte proliferation (Van Der Merwe et al. 2010), inhibiting the oxidative burst of neutrophils by binding these cells (Thomas et al. 1991) or expressing anti-inflammatory cytokines, which result in poor opsonisation (Mulongo et al. 2014, Bürki et al. 2015).

M. bovis generates secondary metabolites, such as hydrogen peroxide to damage the host cell. The amount of peroxide produced varies among different isolates and *in vitro* passages of strains led to a reduction of hydrogen peroxide levels (Khan et al. 2005). Biofilm formation of *M. bovis* may contribute to the persistence of this pathogen besides increasing its resistance to desiccation and heat stress (McAuliffe et al. 2006).

2.6. Clinical signs and pathology

Pneumonia, arthritis, mastitis, otitis media or any combination of these are the typical clinical signs of *M. bovis* infections. Additionally, less common clinical manifestations may occur affecting various organs in the animals (Maunsell and Donovan 2009).

In naturally infected animals which contacted with calves showing clinical disease the incubation period for the development of respiratory signs was 2 weeks (Adegboye et al. 1996). Under experimental conditions, respiratory disease and arthritis were recorded 8-10 days after *M. bovis* infection (Stipkovits et al. 2000a, Caswell and Archambault 2008).

2.6.1. Pneumonia

The clinical symptoms of *M. bovis*-associated pneumonia are usually indistinguishable from pneumonia caused by other pathogens, and consist of fever, loss of appetite, nasal discharge, coughing, hyperpnoea, dyspnoea and depression (Stipkovits et al. 2000b, Maunsell et al. 2011). Features, such as chronicity, poor response to antibiotic treatment or accompanying arthritis or otitis media can suggest *M. bovis* infection (Caswell and Archambault 2008).

Up to date, four forms of pneumonia have been associated with *M. bovis* infection: the pathogen alone can develop caseonecrotic pneumonia and suppurative pneumonia without

necrosis, while *M. bovis* co-infection with other bacteria (eg. *A. pyogenes*, *H. somni*, *M. haemolytica*, *P. multocida*) can result in bronchopneumonia with foci of coagulation necrosis and chronic bronchopneumonia with abscessation (Caswell and Archambault 2008). The most characteristic lesion of *M. bovis* induced pneumonia in calf is bilateral, multifocal caseonecrotic bronchopneumonia (Figure 1). The diameters of foci vary between 1 mm and several centimetres, and they involve 20-90% of the total lung tissue (Shahriar et al. 2002, Khodakaram-Tafti and López 2004, Gagea et al. 2006). Histologically, these foci have a central eosinophilic necrotic material, and are surrounded by necrotic cells, neutrophils, lymphocytes and macrophages. *M. bovis* antigen is visible mostly at the margin of the caseonecrotic area, extracellularly and within phagocytes and in the oedematous alveoli (Shahriar et al. 2002, Khodakaram-Tafti and López 2004, Gagea et al. 2006, Caswell et al. 2010).

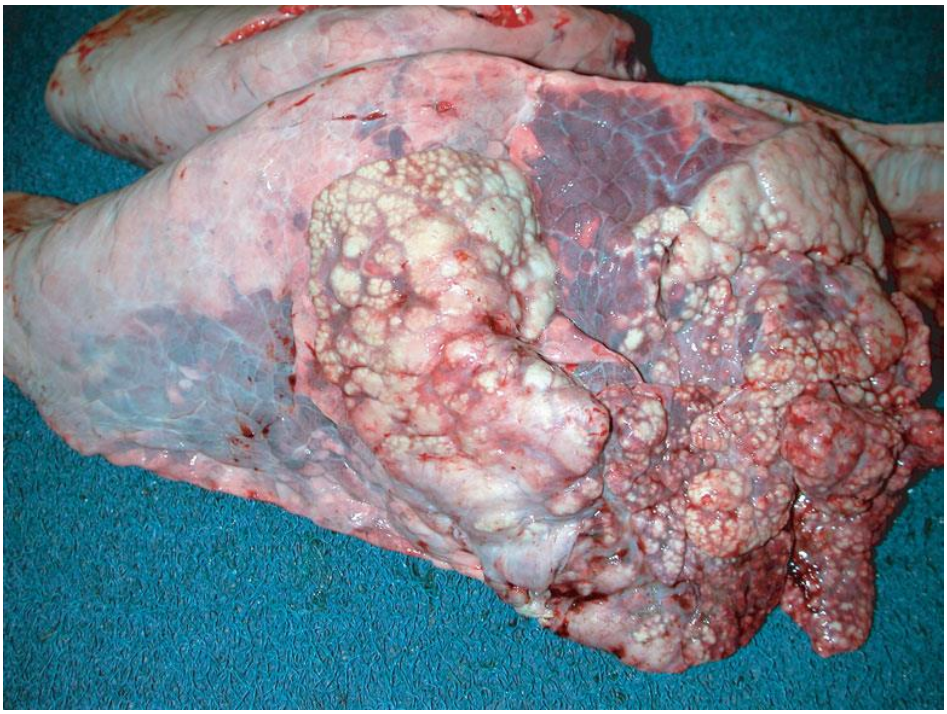

Figure 1. *M. bovis* pneumonia with multifocal greyish-white nodules in the cranioventral areas of the lung
(Gagea et al. 2006, Caswell and Archambault 2008)

2.6.2. Arthritis

M. bovis-induced arthritis can occur at any age of the cattle, but it is typical in preweaned calves and it is accompanied by respiratory disease (Stipkovits et al. 1993, Gagea et al. 2006, Maunsell and Donovan 2009, Maunsell et al. 2011). Clinically fever, lameness, joint swelling and pain can be observed in the acute phase. Large rotator joints (hip, stifle, hock, shoulder, elbow and carpal) are commonly affected. Poor response to antibiotic therapy is

also a common feature of the disease. Lesions in the joints are characterized by necrotizing fibrinosuppurative arthritis and tenosynovitis. In chronic cases the affected joints contain yellowish-white fibrinous or caseous material in thickened joint capsule. Involvement of the adjacent ligaments and tendons is common (Gagea et al. 2006, Maunsell and Donovan 2009, Maunsell et al. 2011).

Histologically, several erosions on the articular cartilage are observed with hyperplasia, mixed inflammatory cell infiltration and necrosis of the synovial membran. *M. bovis* antigen can be detected in the area of inflammatory infiltration, intraarticular inflammatory exudates and at the foci of caseous necrosis (Gagea et al. 2006, Maunsell and Donovan 2009).

2.6.3. Mastitis

Presentation of mycoplasma mastitis can vary from endemic subclinical diseases to severe clinical outbreaks (González and Wilson 2003, Maunsell et al. 2011). *M. bovis*-associated mastitis usually includes the inflammation of more than one quarter of the udder, a drastic decrease in milk production, high contagiousness, no response to antibiotic treatment and increase in purulent, abnormal secretion (Nicholas et al. 2016).

Returning to milk production of the infected cows is possible but it is a slow process, and it is accompanied by the shedding of *M. bovis* (González and Wilson 2003). Concurrent arthritis or respiratory disease or both are frequently diagnosed (Wilson et al. 2007, Maunsell et al. 2011).

2.6.4. Otitis media

Otitis media associated with *M. bovis* infection can occur sporadically, as enzootic disease or as outbreaks. Clinical signs consist of ear pain, evidenced by head shaking and scratching, ear drop and other neurological signs including cranial nerve VIII deficits, twitching, and/or incoordination. Purulent ear discharge, epiphora, anorexia and listlessness were also observed (Lamm et al. 2004, Francoz et al. 2004, Maunsell et al. 2011).

Affected ears are often filled with caseous or suppurative exudate which can accompained by osteolysis of surrounding bones (Lamm et al. 2004, Maunsell and Donovan 2009). Prolonged antimicrobial therapy is usually successful in the elimination of infection (Francoz et al. 2004).

2.6.5. Other diseases

M. bovis can be isolated from the conjunctiva of infected cattle, although its association with infectious keratoconjunctivitis is uncommon (Alberti et al. 2006). Meningitis can develop as a consequence of otitis media (Stipkovits et al. 1993, Maeda et al. 2003, Maunsell et al. 2011).

In an unusual presentation of *M. bovis* infection, subcutaneous decubital abscesses over the brisket and joints were reported (Kinde et al. 1993). *M. bovis* was also isolated from myocarditis (Haines et al. 2004). In these unusual cases *M. bovis* is mainly considered as a predisposing or coinfecting agent.
In experimental cases *M. bovis* has been associated with abortion in cows and seminal vesiculitis in bulls (LaFaunce and McEntee 1982, Bocklisch et al. 1986, Pfutzner and Sachse 1996).

2.7. Diagnosis

As the clinical and pathological signs are not phatogonomic for *M. bovis* infections, laboratory diagnosis is based on the isolation of the agent and/or by demonstration of its presence by molecular (polymerase chain reaction, PCR) and/or by immunological methods (ELISA; immunohistochemistry) (Nicholas and Ayling 2003, Maunsell and Donovan 2009).

2.7.1. Sampling

Sample handling and transport are particularly important to ensure the survival of *M. bovis*, therefore samples should be transported and processed as soon as possible and should be kept chilled (Maunsell and Donovan 2009). In pneumonic cattle, bronchoalveolar lavage fluid or the affected lung tissue are more suitable samples for the diagnosis of *M. bovis* infections compared to nasal swabs (Thomas et al. 2002). Nasal cultures did not correlate with the presence of *M. bovis* in the lower respiratory tract or with the manifestation of the clinical disease (Allen et al. 1991, Thomas et al. 2002). An adequate sample for the detection of this agent is milk, synovia, eye swab or semen, washings of the prepuce and genital discharge in the case of mastitis, arthritis, keratoconjunctivitis or infertility, respectively (Nicholas and Ayling 2003). If swabs are used, wooden-shaft cotton swabs should be avoided during the sampling, because they are potentially inhibitory to mycoplasmal growth. Instead, the use of Dacron, calcium alginate or polyester swabs are preferred, which are transported in the adequate liquid medium (Murray and Baron 2003). As mycoplasmas are intracellular bacteria, vigorous swabbing is recommended to harvest as many cells as possible (Caswell and Archambault 2008).

2.7.2. Cultivation

Although *M. bovis* is less fastidious to culture than some other pathogenic mycoplasmas, the isolation procedures require complex media, specialized equipment and technical skills (Caswell et al. 2010). *M. bovis* can grow in various media but all of them should contain

yeast extract, tryptone (amino acid sources), serum (sterol source), glucose and/or pyruvate (energy source), penicillin or other β-lactam antibiotics (selective agent) and phenol red or other pH indicator (for the detection of the growth) (Hannan 2000, Caswell and Archambault 2008). Broth cultures are incubated at 37°C under atmospheric conditions and growth of the bacteria is often apparent after 48 hours, but incubation up to 10 days is recommended before the sample is considered negative. Agar plates are incubated at 37°C under 5% to 10% CO_2 atmosphere until visible colonies (2-4 days) appear. Colonies are examined by stereomicroscope and show 0.1 to 0.5 mm diameter with typical fried-egg appearance (Murray and Baron 2003, Maunsell et al. 2011) (Figure 2). To obtain pure *M. bovis* culture of a field isolate, the filtration-cloning method is recommended. In such cases broth cultures are filtered through a 220-450 nm pore-size membrane to remove microcolonies and plated on agar until visible colonies appear. Then, a single colony is inoculated into the broth media. Conventionally, isolates should be cloned three times by filtration to obtain pure cultures (Tully 1983), but it is not always suggested because of the high risk of mutations related to adaptation to *in vitro* growth (Caswell and Archambault 2008).

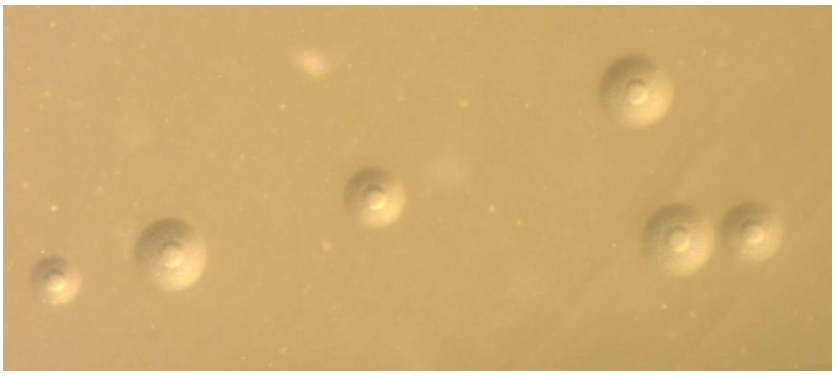

Figure 2. Colonies of *M. bovis* on solid media with typical fried-egg morphology (x30 magnification)

Culture positive isolates can then be identified by serological or molecular methods. Alternatively, a selective diagnostic medium is available for the detection of *M. bovis*, with red colonies appearing in several days (Nicholas et al. 2016).

The traditional mycoplasma isolation method has the advantage of high sensitivity coupled with the possibility of isolation of multiple mycoplasma species from the same specimen. However, cultivation of mycoplasmas is a slow, labour intensive process, which requires specialized reagents and considerable technical expertise. Numerous factors can influence the isolation method, like the antibiotic treatment of the herd, the presence of faster growing opportunistic mycoplasmas (ie. *M. bovirhinis*), the overgrowth by secondary pathogens, the presence of antibodies or preservatives or the autolyzation of the samples; all leading to obtain false negative results (Caswell et al. 2010, Nicholas 2011).

2.7.3. Molecular methods

Molecular techniques are particularly appropriate in situations which interfere with mycoplasma isolation (listed above). Despite the increasing number of novel molecular techniques for the identification of *M. bovis*, e.g. loop-mediated isothermal amplification (LAMP) (Higa et al. 2016), currently PCR remains the most useful, rapid and simple method of detecting specific mycoplasmas (Nicholas et al. 2016). Various PCR systems have been described for the detection of *M. bovis* DNA and the most challenging objective of these assays was the accurate discrimination of *M. bovis* from *M. agalactiae* (Bashiruddin et al. 2005). Only 8 nucleotides differ between the sequences of the 16S rRNA of these two species (Mattsson et al. 1994). Hence PCR assays targeting the 16S rRNA gene do not reliably distinguish *M. bovis* and *M. agalactiae* unless additional methods are used, such as melting temperature analysis of the PCR products or denaturing gradient gel electrophoresis of the amplicons (Cai et al. 2005, McAuliffe et al. 2005). Both conventional and real-time PCR systems have been developed to target *uvrC* (Subramaniam et al. 1998; Rossetti et al. 2010), *oppD/F* (Hotzel et al. 1996, Sachse et al. 2010), *polC* (Marenda et al. 2005), *vsp* (Ghadersohi et al. 1997, Hayman and Hirst 2003, Tenk et al. 2006) and *fusA* (Boonyayatra et al. 2012) genes to provide the direct identification of *M. bovis.* The listed assays are specific to the species level and are applicable on mycoplasma strains isolated by routine culture method as well as on clinical samples collected for the direct detection.

For the enhancement of *M. bovis* detection in milk samples, antigen capture by monoclonal antibodies (Hotzel et al. 1999), DNA-binding filter membranes (Hotzel et al. 1996) or nested PCR system (Pinnow et al. 2001) have been used.

The principle advantages of these molecular techniques are their high specificity, rapid turnaround time, cost efficiency and the compatibility with testing other pathogens (Caswell et al. 2010).

2.7.4. Immunological methods

Serology is a useful tool for the detection of *M. bovis* exposure in herd level. *M. bovis* has both lipid and protein antigens which can elicit antibody responses, and antibody levels remain high in the hosts for several months (Nicholas and Ayling 2003). It should be noted that individual animal titers poorly correlate with the severness of the disease (Le Grand et al. 2002) and maternal antibodies result in high titers in calves (Virtala et al 2000). The animals in which *M. bovis* is found only in the nasal cavity seroconversion rarely occurs, therefore the specific antibodies are undetectable. Similarly to the molecular methods, immunological tests are also capable to detect the infection even when the *in vitro* cultivation failed (Nicholas and Ayling 2003).

The principal method used for serodiagnosis of *M. bovis* infection is an indirect ELISA. Kits are commercially available and with the identification and characterization of novel antigens increasing number of tests have been described by local laboratories (Dénes et al. 2003; Sun et al. 2014; Wawegama et al. 2016; Khan et al. 2016).

Indirect hemagglutination test is also applicable to demonstrate the presence of specific antibodies to *M. bovis* in the serum or in other body fluids (Maunsell and Donovan 2009).

Immunohistochemical demonstration of *M. bovis* antigen has the advantage of revealing the location of the bacteria within a lesion and is applicable on fixed tissue, allowing retrospective examinations (Maunsell and Donovan 2009).

2.8. Control of the disease

2.8.1. Vaccination

M. bovis has the ability to evade the immune responses of the host by altering its surface proteins and by inducing immunomodulatory effects, which may partially explain why vaccination does not give full protection in calves against *M. bovis*-associated diseases (Maunsell and Donovan 2009). In certain instances the effect of *M. bovis* vaccines have appeared promising in experimental studies (Nicholas et al. 2002, Zhang et al. 2014, Dudek et al. 2016) but they proved to be ineffective or even exacerbated the disease under field conditions (Nicholas et al. 2006, Maunsell et al. 2009, Soehnlen et al. 2011).

Despite of the limited efficacy evidenced under field conditions, some bacterin-based vaccines are licensed for marketing in the USA and UK, mainly for the pneumonic form of the *M. bovis* infections (Maunsell and Donovan 2009, Nicholas et al. 2016).

2.8.2. Management

Since no effective vaccine is available against *M. bovis*, adequate management practices are promoted in the control of the disease caused by this pathogen (Caswell and Archambault 2008).

The most effective control strategy to prevent *M. bovis* infection is the maintenance of closed herd by testing each cattle before entry and keeping them separated. In feedlot cattle, where this type of biosecurity is not practical the prevention strategy focuses on maximizing the health of the anilmals by strengthening the immune and respiratory systems by proper ventilation, good nutrition and controlling other respiratory pathogens by vaccination. Also other sources of stress, like overcrowding or cold stress should be minimized (Maunsell and Donovan 2009).

In *M. bovis* infected herds the potential sources of exposure should be controlled by pasteurizing milk, avoiding colostrum pooling and by sanitizing the equipment. Segregation of calves with clinical signs from healthy animals and the “all-in, all-out” practice are also recommended. The promptly treatment with effective antimicrobial drugs of animals with clinical disease is also a suggested control strategy, but metaphylactic use of antibiotics is generally undesirable. Culling of animals is recommended when the animal welfare is compromised (Maunsell and Donovan 2009).

2.8.3. Treatment

Beside management practices, efforts to control *M. bovis* infections often rely on antimicrobial treatments in the early stages of the disease. Mycoplasmas are intrinsically resistant to β-lactam antimicrobials and sulphonamides, because they do not possess a cell wall and do not synthesize folic acid. In addition, mycoplasmas are also naturally resistant to polymyxins, trimethoprim, nalidixic acid and rifampin limiting the number of applicable antimicrobial agents during the therapy (Taylor-Robinson and Bébéar 1997, Lysnyansky and Ayling 2016). Mainly the protein or nucleic acid synthesis inhibitor antimicrobial classes are active against *M. bovis*, but against the few antimicrobials licensed for treatment, an increasing resistance is detected (Gautier-Bouchardon et al. 2014, Ayling et al. 2014, Lysnyansky and Ayling 2016).

Antibiotic therapy of mastitis has often failed, but antimicrobial treatment of pneumonia was successful in several cases and it may help reducing the economic losses (Nicholas and Ayling 2003, Nicholas et al. 2016). In routine practice, the potential effectiveness of the antimicrobials *in vivo* can be assessed by *in vitro* susceptibility testing and the determination of the minimum inhibitory concentration (MIC) values. However, interpretation of the results of the *in vitro* examinations should be handled with caution as *in vivo* factors, like the concentration of the applied antibiotics in the target organs and cells or biofilm formation of the bacteria can also influence the efficiency of the treatment (Reeve-Johnson 1999, McAuliffe et al. 2006). Guidelines of *in vitro* testing of animal pathogen mycoplasma species have been published (Hannan 2000), but the lack of standard quality control strains and breakpoints makes the interpretation of antibiotic susceptibility difficult. Another disadvantage is that the determination of the antibiotic susceptibility of mycoplasmas can take several weeks because of the time-consuming isolation methods prior to the *in vitro* susceptibility tests. Thereby, empirical treatment is often introduced, which can lead to therapeutic failure and the development of resistance to critically important antimicrobials (Lysnyansky and Ayling 2016).

Control of *M. bovis* infection requires early diagnosis and treatment with antimicrobials such as fluoroquinolones, tetracyclines, macrolides or florfenicol (Apley and Coetzee 2013, Lysnyansky and Ayling 2016).

Fluoroquinolones have mycoplasmacidal effect by acting on topoisomerases which inhibit the DNA synthesis of the bacteria (Piddock 1999). Although fluoroquinolones are considered to be effective agents in the therapy of bovine mycoplasmosis, strains with high MICs (≥8 μg/ml) have been reported from several countries (Thomas et al. 2003a, Rosenbusch et al. 2005, Gerchman et al. 2009, Uemura et al. 2010, Gautier-Bouchardon et al. 2014, Ayling et al. 2014). The *in vivo* use of fluoroquinolones is controversial. In experimental trials the oral use of enrofloxacin against *M. bovis*-associated pneumonia mitigated the clinical signs in calves and marbofloxacin was proved to be effective in the treatment of bovine respiratory disease (Thomas et al. 2001, Stipkovits et al. 2005). On the contrary, the monthly fluoroquinolone treatment repeated over three months did not prevent the development of respiratory disease caused by *M. bovis* in an other report (Nicholas and Ayling 2003). In mycoplasmas development of resistance to the fluoroquinolones is usually due to target mutations in quinolone resistance-determining regions (QRDRs) of the genes encoding topoisomerases, but enhanced efflux of the drug by the overexpression of efflux pumps has been also described (Raherison et al. 2002, Lysnyansky and Ayling 2016).

Tetracyclines and spectinomycin inhibit protein synthesis by primarily binding to the 30S subunit of the ribosome (Noah et al. 1999). Increasing resistance to them has been reported from several countries (Ayling et al. 2014, Gautier-Bouchardon et al. 2014, Heuvelink et al. 2016). In clinical use, oxytetracycline treatment resulted in improvement in calves with pneumonia partially associated with *M. bovis* (Musser et al. 1996) and it is also a recommended antimicrobial agent in the therapy of otitis media and interna (Bertone et al. 2015). Treatment with spectinomycin did not alter the clinical course of the disease in calves with *M. bovis* and *P. multocida* pneumonia, although the numbers of both bacteria in the lung were reduced in the treated calves (Poumarat et al. 2001). In pathogenic mycoplasmas, tetracycline and spectinomycin resistance are mainly achieved by point mutations in the 16S rRNA gene, altering the tetracycline or spectinomycin binding sites (Dégrange et al. 2008b, Schnee et al. 2014, Amram et al. 2015), but tetracycline resistance of *M. hominis* and Ureaplasmas are mainly associated with ribosomal protection by *tet*(M) determinants (Roberts et al. 1985, Dégrange et al. 2008a, Mardassi et al. 2012).

Macrolides and lincosamides act on the 50S subunit of the ribosome, inhibiting the protein synthesis. Macrolides have a preferential distribution in lungs and can penetrate phagocytic cells therefore allowing treatment of respiratory and intracellular infections (Reeve-Johnson 1999). Nowadays these antibiotics are losing their efficiency against *M. bovis* in many countries of the world (Ayling et al. 2014, Gautier-Bouchardon et al. 2014, Heuvelink et al.

2016, Kong et al. 2016) by acquiring one or more point mutations at the macrolide binding site in domains II and/or domains V on the 23S rRNA (Lerner et al. 2014, Kong et al. 2016). Mutations in L4 and L22 ribosomal proteins involved in forming the macrolide-binding site were shown to be responsible for macrolide and lincosamide resistance of *in vitro* selected *M. pneumoniae* strains but the significance of these mutations in *M. bovis* has not been clarified (Pereyre et al. 2004). Clinical efficacy of tilmicosin against respiratory diseases was demonstrated in early studies, while later it was demonstrated ineffective in the treatment of *M. bovis*-associated diseases suggesting the spread of acquired resistance to tilmicosin due to the overuse of the antibiotic (Gourlay et al. 1989, Picavet et al. 1991, Musser et al. 1996, Haines et al. 2001, Hendrick et al. 2013). Tulathromycin, a relatively new semi-synthetic macrolide was highly effective in the treatment and prevention of *M. bovis*-associated respiratory diseases in calves. Efficacy of the agent was regardless of the MIC of the strains (1 or >64 µg/ml) (Godinho et al. 2005a) which can be explained by the antibiotic's slow elimination and high distribution in the lung (Benchaoui et al. 2004).

Pleuromutilins and florfenicol, the fluoro-derivative of chloramphenicol, are mycoplasmastatic antibiotics commonly used in the veterinary field. They bind the 50S ribosomal subunit preventing protein synthesis (Schwarz et al. 2004, Van Duijkeren et al. 2014). Up to now, low MICs to pleuromutilins have been reported, while MIC values of florfenicol show slight increase (Thomas et al. 2003a, Gautier-Bouchardon et al. 2014, Ayling et al. 2014, Lysnyansky and Ayling 2016). In clinical use, florfenicol was an effective in the treatment of respiratory diseases partially associated with mycoplasmas (Godinho et al. 2005b). The efficacy of valnemulin against *M. bovis* infection was demonstrated *in vivo* under field and experimental conditions, but pleuromutilins are not registered for cattle (Stipkovits et al. 2001, Stipkovits et al. 2005). In mycoplasmas point mutations of the 23S rRNA genes taking part in the formation of the active pocket of pleuromutilin derivatives were associated with elevated MICs (Li et al. 2010, Van Duijkeren et al. 2014).

2.9. Economic impact

It is difficult to measure the economic impact of *M. bovis* infections, as the clinical and pathological signs are unspecific and the diseases attributed to this agent commonly involve interactions of more than one pathogen (Caswell and Archambault 2008). Costs of mycoplasma infections include the reduced production, treatment and labour expenses, increased mortality and premature culling, implementation of diagnostic, control and preventive measures (Maunsell et al. 2011). Within these limitations, the respiratory diseases due to this pathogen are estimated to be responsible for up to one third of the economic losses in the cattle industry in Europe (approximately €144-192 million per year). In the United States the estimated costs are $32 million per year as a result of reduced weight gain, and $108 million per year in the case of calves of milking cattle with *M. bovis*-associated mastitis (Nicholas and Ayling 2003). In addition, the prolonged treatment of chronic *M. bovis*-associated diseases with a variety of antibiotics contributes to the development and spread of antimicrobial resistance, which are notable non-economic costs (Caswell and Archambault 2008).

3. Aims of the study

The aims of the study were:

Ad 1. to genetically characterize the Hungarian *M. bovis* population with the MLST and MLVA methods in order to evaluate and compare these two typing systems and to better understand the epidemiology of *M. bovis* in Hungary.

Ad 2. to determine the *in vitro* susceptibility profile of the Hungarian *M. bovis* isolates to fifteen antibiotics of eight antimicrobial groups that could potentially be used in the therapy.

Ad. 3. to investigate the molecular mechanisms involved in the resistance of *M. bovis* and to identify mutations responsible for the high MICs to seven different antimicrobial families (fluoroquinolones, tetracyclines, aminocyclitol, macrolides, lincosamide, phenicol and pleuromutilins) by using whole-genome sequencing of field isolates and laboratory-derived mutants.

Ad. 4. to develop and to characterize rapid and cost-effective real-time PCR based assays for the simultaneous determination of antibiotic susceptibility profile of *M. bovis* isolates in the case of seven antimicrobial groups.

4. Materials and methods

4.1. *Mycoplasma bovis* isolates

4.1.1. Nomenclature of *Mycoplasma bovis* isolates in the present study

Following the literature, the current study refers to the *M. bovis* type strain (NCTC 10131) as PG45. Isolates collected between 2010 and summer, 2013 have "MYC" identifier (n=35; Table 1, Figure 3). The examinations of this study were performed in chronological order thus the number of the examined isolates may vary between different studies (eg. in Chapter 4.2. n=31).

Clinical samples (lung and nasal swab) collected from autumn, 2013 to 2016 were denominated as "BM" (n=30). *M. bovis* was isolated (n=20) from clinical samples for further analysis (Chapter 4.5. and Table 2).

Laboratory-derived antibiotic resistant mutants (n=36) are named after the parent *M. bovis* isolate (PG45 / MYC52 / MYC53) and the drug used for the selection (eg. tylosin resistant MYC53).

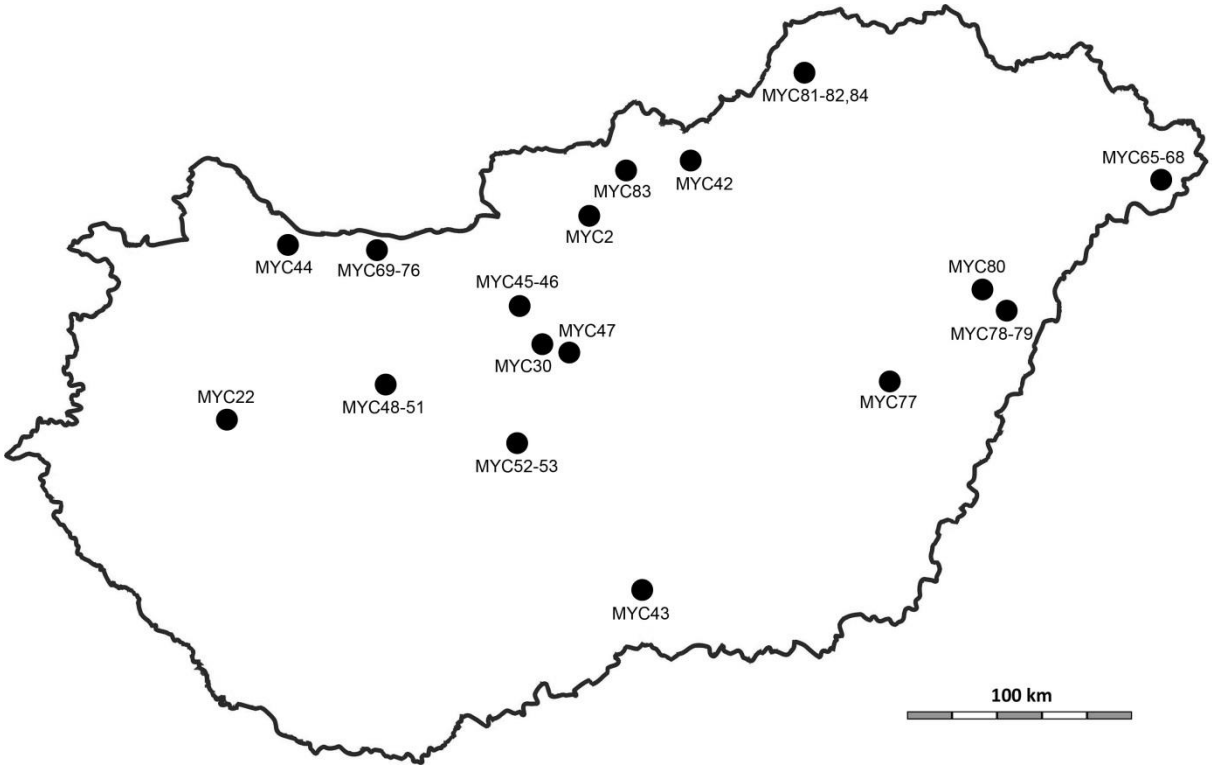

Figure 3. Map of Hungary showing the geographical origins of the 35 *M. bovis* isolates from 2010-2013.

Table 1. Background data of *M. bovis* isolates and the examinations performed on them

No.	Sample ID	Origin of herd	Date	Host	Sample source	MLST	MLVA	AB	WGS
1	MYC2	Püspökhatvan	2011	cattle	lung	X	X	X	X
2	MYC22	Sümeg	2012	cattle	lung	X	X	X	X
3	MYC30	Bugyi	2012	cattle	lung	X	X	X	X
4	MYC42	Nemti	2012	cattle	lung	X	X	X	X
5	MYC43	Zsana	2012	cattle	lung	X	X	X	X
6	MYC44	Győrszentiván	2012	cattle	lung	X	X	X	X
7	MYC45	Budapest	2012	cattle	lung	X	X	X	X
8	MYC46	Budapest	2012	cattle	lung	X	X	X	X
9	MYC47	Dabas	2012	cattle	lung	X	X	X	X
10	MYC48	Ősi	2012	cattle	nasal swab	X	X	X	X
11	MYC49	Ősi	2012	cattle	nasal swab	X	X	X	X
12	MYC50	Ősi	2012	cattle	lung	X	X	X	X
13	MYC51	Ősi	2012	cattle	nasal swab	X	X	X	X
14	MYC52	Solt	2012	cattle	lung	X	X	X	X
15	MYC53	Solt	2012	cattle	lung	X	X	X	X
16	MYC65	Csengersima	2012	cattle	nasal swab	X	X	X	X
17	MYC66	Csengersima	2012	cattle	nasal swab	X	X	X	X
18	MYC67	Csengersima	2012	cattle	lung	X	X	X	X
19	MYC68	Csengersima	2012	cattle	lung	X	X	X	X
20	MYC69	Komárom	2013	cattle	nasal swab	X	X	X	X
21	MYC70	Komárom	2013	cattle	nasal swab	X	X	X	X
22	MYC71	Komárom	2013	cattle	nasal swab	X	X	X	X
23	MYC72	Komárom	2013	cattle	nasal swab	X	X	X	X
24	MYC73	Komárom	2013	cattle	nasal swab	X	X	X	X
25	MYC74	Komárom	2013	cattle	nasal swab	X	X	X	X
26	MYC75	Komárom	2013	cattle	nasal swab	X	X	X	X
27	MYC76	Komárom	2013	cattle	nasal swab	X	X	X	X
28	MYC77	Kertészsziget	2010	cattle	lung	X	X	X	X
29	MYC78	Hosszúpályi	2011	cattle	lung	X	X	X	X
30	MYC79	Hosszúpályi	2011	cattle	lung	X	X	X	X
31	MYC80	Ebes	2011	cattle	lymph node	X	X	X	X
32	MYC81	Felsőnyárád	2013	cattle	lung			X	X
33	MYC82	Felsőnyárád	2013	cattle	nasal swab			X	X
34	MYC83	Alsótold	2013	cattle	lung			X	X
35	MYC84	Felsőnyárád	2013	cattle	nasal swab			X	X
36	PG45	Connecticut	1961	cattle	mastitis/milk	X	X	X	X
37	02035[a]	Saudi Arabia	<2002	cattle	not known	X			
38	95035[a]	Cameroon	1995	cattle	nasal swab	X			
39	88091[a]	Turkey	1988	goat	lung	X			
40	8790[a]	Ethiopia	1987	goat	lung	X			

[a] Manso-Silván et al. 2012. Abbreviations: No: number; MLST: multi-locus sequence typing; MLVA: multi-locus variable number of tandem repeats analysis; AB: antibiotic susceptibility; WGS: whole genome sequencing

Table 2. Background information of the 30 clinical samples included in the current study.

No.	Sample ID	Origin	Date	Sample type	DNA of clinical samples		*M. bovis* isolation
					M. bovis specific PCR[a]	Universal Mycoplasma PCR[b]	
1	BM121	Tápiószecső	2013	lung	+++	+	+
2	BM122	Nagyecsed	2013	lung	+++	mixed	+
3	BM124	Kapuvár	2013	lung	+++	mixed	+
4	BM218	Vány	2015	lung	+++	mixed	+
5	BM239	Abony	2015	lung	+++	mixed	+
6	BM274	Kapuvár	2015	lung	+++	mixed	+
7	BM338	Hács	2015	lung	+++	+	+
8	BM343	Hács	2015	lung	+++	mixed	+
9	BM367	Bugyi	2016	lung	+++	mixed	+
10	BM375	Takácsi	2016	lung	+++	mixed	+
11	BM381	Somlószőlős	2016	lung	+++	+	+
12	BM385	Hács	2016	lung	+++	mixed	+
13	BM388	Somogytúr	2016	lung	+++	mixed	+
14	BM396	Malomsok	2016	lung	+++	+	+
15	BM237	Zalaszentmihály	2015	lung	++	mixed	+
16	BM272	Berettyóújfalu	2015	lung	++	+	-
17	BM394	Bugyi	2016	lung	++	mixed	+
18	BM270	Somogytúr	2015	lung	+	mixed	+
19	BM354	Kocsér	2016	lung	+	mixed	+
20	BM356	Kocsér	2016	lung	+	mixed	+
21	BM140	Csengersima	2014	lung	-	mixed	-
22	BM302	Nagykanizsa	2015	lung	-	-	-
23	BM323	Felsőrajk	2016	lung	-	-	-
24	BM346	Alsóörs	2016	lung	-	-	-
25	BM348	Zalalövő	2016	lung	-	mixed	-
26	BM357	Kocsér	2016	nasal swab	-	-	+
27	BM368	Kisszentmárton	2016	lung	-	mixed	-
28	BM369	Bögöte	2016	lung	-	mixed	-
29	BM373	Kaposvár	2016	lung	-	mixed	-
30	BM416	Dunaegyháza	2016	lung	-	-	-

[a] According to Subramaniam et al. (1998); Weak positive „+" results were distinguished from medium „++" and strong positive „+++" samples. [b] According to Lauerman et al. (1995); "Mixed" indicates the presence of more than one *Mycoplasma* sp. and was distinguished from simple positive "+" results.

4.1.2. Isolation method of *Mycoplasma bovis*

Nasal swabs, lung samples and a single lymph node were collected from live and dead animals from different parts of Hungary between 2010 and 2013 (Table 1, Figure 3). Ethical approval was not required as all samples were collected during routine diagnostic examinations or necropsies. The samples were inoculated into 2 ml of Mycoplasma broth medium (pH 7.8) (Thermo Fisher Scientific Inc. /Oxoid Inc./, Waltham, MA) supplemented with 0.5% (w/v) sodium pyruvate, 0.5% (w/v) glucose and 0.005% (w/v) phenol red and cultured at 37°C in a 5% CO_2 atmosphere. Following colour change (red to yellow shift) the

cultures were inoculated onto solid Mycoplasma media (Thermo Fisher Scientific Inc. /Oxoid Inc./) and were incubated at 37°C and 5% CO_2 for 3 days, until visible colonies appeared. Mixed cultures were filter cloned only once to exclude contaminant *Mycoplasma* species and to minimize *in vitro* mutations of the isolates. Mixed primary cultures which failed to be purified by a single filter cloning were excluded (data not shown).

4.1.3. Molecular identification of *Mycoplasma bovis*

DNA extraction was performed on 200 µl of Mycoplasma broth culture using the QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions for Gram-negative bacteria.

All isolates were identified by PCR amplifying a 1626 bp long part of the *uvrC* gene encoding the subunit C of excinuclease ABC of *M. bovis*. The system utilised the following primer pair: MBOUVRC2-L: 5′-TTA CGC AAG AGA ATG CTT CA-3′; MBOUVRC2-R: 5′-TAG GAA AGC ACC CTA TTG AT-3′ (Subramaniam et al. 1998). PCR was performed in 25 µl total volume, containing 1 µl target DNA, 5 µl of 5X Green GoTaq Flexi Buffer (Promega, Inc., Madison, WI), 2.5 µl of $MgCl_2$ (25 mM; Promega, Inc.), 0.75 µl of dNTP (10 mM; Qiagen Inc.), 2 µl of each primer (10 pmol/µl), and 0.25 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega, Inc.). The PCR consisted of initial denaturation for 5 min at 95°C followed by 35 amplification cycles of denaturation for 15 sec at 95°C, primer annealing at 48°C for 60 sec and extension at 72°C for 90 sec. The final extension step was performed for 5 min at 72°C.

The purity of the cultures (i.e. to exclude *M. arginini* or other *Mycoplasma* spp. contamination) was confirmed by an universal mycoplasma PCR system targeting the 16S/23S rRNA intergenic spacer region in Mollicutes (Lauerman et al. 1995). PCR was performed in 25 µl total volume, containing 1 µl target DNA, 5 µl of 5X Green GoTaq Flexi Buffer (Promega, Inc.), 2.5 µl $MgCl_2$ (25 mM; Promega, Inc.), 0.5 µl dNTP (10 mM; Fermentas), 1 µl of each primer (10 pmol/µl), and 0.25 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega, Inc.). The PCR consisted of initial denaturation for 5 min at 95°C followed by 35 amplification cycles of denaturation for 30 sec at 95°C, primer annealing at 54°C for 30 sec, and extension at 72°C for 60 sec. The final extension step was performed for 5 min at 72°C.

All conventional PCRs were performed in a Biometra–T Personal thermal cycler (Biometra, Analytik Jena AG, Germany). The DNA of the *M. bovis* type strain (PG45, NCTC 10131) served as a positive control, while nuclease free water was used as negative control. After amplification, 5 µl of each sample, control and 100-bp DNA ladder (GeneRuler 100 bp Plus, Thermo Fisher Scientific Inc.) were loaded in 1% agarose gel (SeaKem LE Agarose, Lonza Inc., USA) containing GR Safe Nucleic Acid stain (Lab Supply Mall, InnoVita Inc.,

Gaithersburg, MD) for electrophoresis. The products visualized in ultra violet light and photographically documented (Kodak Inc., USA).

Amplicons of universal mycoplasma PCR were sequenced on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). The reading errors of the chromatograms were corrected with programs of the Lasergene package (DNASTAR Inc., Madison, WI) and nucleic acid database was searched using the BLASTN program in GenBank.

4.2. Genotyping of the Hungarian *Mycoplasma bovis* isolates

4.2.1. Multi-locus sequence typing

The typing of 31 *M. bovis* isolates from Hungary was performed by using a previously published MLST assay (Manso-Silván et al. 2012) (Table 1). In addition, four *M. bovis* strains (strain IDs: 02035, 95035, 88091 and 8790; GenBank accession numbers: KF926436-KF926475 and KJ438174-KJ438257), for which MLST sequences were available in GenBank, were also included in the study for comparison (Table 1). The *M. agalactiae* type strain PG2, isolated from a sheep in 1931, Spain was included as an outgroup. The MLST analysis was based on four housekeeping genes (*fusA*, *gyrB*, *lepA*, *rpoB*) and was performed by using the amplification primers and PCR conditions described by Manso-Silván et al. (2012) (Table 3). The reaction mixture was prepared in 25 µl total volume containing 10 to 100 ng of target DNA diluted in nuclease-free water, 5 µl of 5X Green GoTaq Flexi Buffer (Promega, Inc., Madison, WI), 2.5 µl of $MgCl_2$ (25 mM; Promega), 0.75 µl of dNTP (10 mM; Qiagen Inc.), 1 µl of each primer (10 pmol/µl), and 0.25 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega, Inc.). The PCR consisted of initial denaturation for 2 min at 94°C followed by 35 amplification cycles of denaturation for 15 sec at 94°C, primer annealing according to Table 3 for 30 sec, and extension at 72°C for 90 sec. The final extension step was performed for 5 min at 72°C.

PCR products were isolated from agarose gel (QIAquick gel extraction kit; Qiagen Inc.), and direct cycle sequencing was performed with the primers used for amplification on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems). The reading errors of the chromatograms were corrected with the SeqMan program (Lasergene package, DNASTAR Inc., Madison, WI). Sequences were trimmed, concatenated and aligned with all published sequences using the BioEdit 7.2.2 software (Hall 1999).

Table 3. Primers and loci used for MLST of *M. bovis* (Manso-Silván et al. 2012)

Locus	Primer name	Primer sequence (5'→3')	Annealing temperature (°C)	Amplicon length (bp)	Analysed sequence length (bp)
MLST-*fusA*	FusA-F	TATGATGAAAGTTCTAGATGGTG	47	808	660
	FusA-R	TTTACCTTCAACATCAGCAC			
MLST-*gyrB*	GyrB-F	GCTGGCGGTAAATTTGATTC	52	725	630
	GyrB-R	TCAAAAATCGGATCWGGGTG			
MLST-*lepA*	LepA-F	TTTTTAGACCAAATGGATTTAG	52	813	660
	LepA-R	TAAGCTCTTTTTAAGTTCGG			
MLST-*rpoB*	RpoB-F	ATGGTTTTTTACAAACTCCA	52	806	645
	RpoB-R	AGATACGTTTGGAATGTCTC			

Phylogenetic analysis of the concatenated sequences (2595 bp length) containing the four housekeeping genes was conducted with the neighbour-joining method using pairwise distances and 1000 bootstraps in the MEGA 5.05 software (Tamura et al. 2011). The average evolutionary divergence of the concatenated sequences was also estimated with MEGA 5.05 both within and between *M. bovis* clades. Analyses were conducted using the Maximum Composite Likelihood model with standard error estimated through 1000 bootstrap replicates. The rate variation among sites was modelled using gamma distribution (shape parameter = 1) including all codon positions. A recombination analysis was performed on the concatenated sequence alignment using the RDP 4 software (Martin et al. 2010). The default selection of detection methods (RDP, GeneConv, and MaxChi) and general settings were used to perform the analyses.

4.2.2. Multi-locus variable number tandem repeat analysis

Thirty-one Hungarian isolates were screened with an MLVA system based on nine TRs described by Pinho et al. (2012) (Table 4). This was done in order to generate an overview of the genetic relatedness of the Hungarian isolates which is comparable with the MLST results, and to determine the level of genetic diversity within each subclade identified by MLST.

All PCRs were performed in 25 µl total volume as described above in MLST (chapter 4.2.1.). Reaction mixes were first heated to 95°C for 5 min to denature DNA and activate the polymerase, then cycled at 95°C for 60 sec, 54-60°C according to Table 4 for 60 sec, and 72°C for 60 sec for 30 cycles, and finally, incubated at 72°C for 10 min for a final polymerase extension step.

Table 4. Primers and tandem repeat loci used for MLVA of *M. bovis* (Pinho et al. 2012)

Tandem repeat	Primer name	Primer sequence (5'→3')	Annealing temperature (°C)	Amplicon size of PG45 (bp)	Period size (bp)
TR14	14-F 14-R	AACATTGTTGACACAATGCCATC CGCTGCTTTTTCGTCCTCC	56	555	84
TR29	29-F 29-R	ATCTAATCAGTAATTGCATGCTTAG TCCTAATAAGTAGACAACGAACAG	54	857	116
TR30	30-F 30-R	TGTTTCAGCAGGTATAGAGTCAG GATAGCTGCTTCATGTGATGG	55	217	51
TR31	31-F 31-R	ACTTAAATTGTGAGTAATAGTTCCACTC GCAATATTTTTTTGATCATATCTCGCATC	56	987	102
TR35	35-F 35-R	GTTGTATCATTTTGAATGGGGTCTATC TCGCTAGCTATGCCACTAGTA	56	242	45
TR40-41	40-41-F 40-41-R	TCCAAGAATTTATAAAGGAGTACTTGG CTGATGCTTCTTTTCCGTATTCC	56	661	39
TR49-51	49-51-F 49-51-R	GAAAGCATCCTTTTGTTCTTCTATGAT TAGTAAATCCAAACGAAACAGATGATG	56	209	45
TR52	52-F 52-R	GCATCATGTCAAAACAAGAATCGAACAGA CTTATGACCAAGGCGACCACGA	60	667	102
TR59	59-F 59-R	TGAATCTTGATCTTTTTGGTCTGAGT CTAGTTGCATCGCCGAAGC	57	428	138

After amplification, 2-5 µl of each reaction mixture was subjected to electrophoresis (8 V/cm) in 2% standard agarose gel (SeaKem LE Agarose, Lonza Inc.), and the amplified DNA products were visualized with GR Safe nucleic acid gel stain (Lab Supply Mall, InnoVita Inc.). Depending on the length of the tandem repeat units a 100-bp or a 20-bp DNA ladder (GeneRuler 100 bp Plus or O'RangeRuler 20 bp, Thermo Fisher Scientific Inc.) was used as molecular weight marker. Electrophoresis was performed until the yellow dye had run for at least 20 cm. Stained gels were visualized by UV light, photographically documented (Kodak Inc.) and band sizes were estimated with the help of the Kodak MI SE software package (Kodak Inc.).

The band size estimates were converted to numbers of repeat units (Pinho et al. 2012). The clustering analysis was performed with a neighbour-joining method based on pairwise distances in the MEGA5.05 software (Tamura et al. 2011).

4.2.3. Discriminatory power of the typing methods

The discriminatory power of the different typing schemes was calculated using Simpson's index of diversity with 95% confidence intervals (Hunter and Gaston 1988). The quantitative level of congruence and respective confidence intervals between the two typing methods was calculated based on the data of the thirty-one isolates and PG45 analysed with both methods using the adjusted Rand and Wallace coefficients (Carriço et al. 2006). An online tool was used to perform these calculations (Comparing Partitions Website).

4.3. Antibiotic susceptibility testing

4.3.1. Antimicrobial agents and *Mycoplasma bovis* isolates

The susceptibility of 36 *M. bovis* isolates (35 isolates from Hungary and the PG45, Table 1) was determined by microbroth dilution method to 15 antimicrobial agents listed in Table 5.

The antimicrobial agents were originated from VETRANAL, Sigma-Aldrich, Germany, except tulathromycin (Pfizer Inc., NY, USA). They were diluted and stored according to the recommendations of Hannan (2000). Stock solutions of 1 mg/ml enrofloxacin, danofloxacin and marbofloxacin were prepared in 0.1 M NaOH; stock solutions of 1 mg/ml florfenicol, gamithromycin and tulathromycin were prepared in 96% ethanol and in sterile distilled water; and the rest of the stock solutions of 1 mg/ml were prepared in sterile distilled water. All aliquots were stored at -70°C until needed and twofold dilutions were freshly prepared for each microtest according to Table 5.

Table 5. Antibiotics and concentration ranges used in susceptibility examinations of *M. bovis* isolates

Antibiotic group	Antibiotic	Concentration range tested (µg/ml)
Fluoroquinolones	Danofloxacin	0.039-10
	Enrofloxacin	0.039-10
	Marbofloxacin	0.039-10
Tetracyclines	Oxytetracycline	0.25-64
	Tetracycline	0.25-64
Aminoglycoside	Gentamicin	0.25-64
Aminocyclitol	Spectinomycin	1-256
Macrolides	Tylosin	0.5-128
	Tilmicosin	0.5-128
	Gamithromycin	0.5-128
	Tulathromycin	0.5-128
Lincosamide	Lincomycin	0.25-64
Phenicol	Florfenicol	0.125-32
Pleuromutilins	Tiamulin	0.039-10
	Valnemulin	0.039-10

The number of colour changing units (CCU) was calculated by microplate dilution method, from the lowest dilution showing colour change after one week of incubation (Ter Laak et al. 1993, Hannan 2000). Aliquots of purified cultures were stored frozen at -70°C until required.

4.3.2. Microbroth dilution method

The microbroth dilution test was performed as recommended by (Hannan 2000) using 10^4-10^5 CCU/ml of each isolate. The 96-wells microtiter plates contained nine vertical columns of 100 µl of twofold dilutions of the antibiotic and 100 µl of mycoplasma inoculum. The plates were designed to contain pH control (Column 10; broth medium adjusted to pH 6.8), sterility control (Column 11; broth medium without antibiotic and Mycoplasma inoculum) and growth control (Column 12; broth medium without antibiotic) as well.

Mycoplasma broth medium (pH 7.8) (Thermo Fisher Scientific Inc. /Oxoid Inc./) supplemented with 0.5% (w/v) sodium pyruvate, 0.5% (w/v) glucose and 0.005% (w/v) phenol red was used as culture medium. The duplicates of three clinical isolates and the duplicate of the type strain (*M. bovis* PG45, NCTC 10131) were tested on each 96-well microtiter plates. The CCU of the inoculum for each tested isolate was confirmed at the time of antibiotic testing by a separate microplate titration.

The final MIC value was defined as the lowest concentration of the antibiotic that completely inhibits the growth in the broth (no pH and colour change) after one week incubation period at 37°C (Ter Laak et al. 1993, Hannan 2000). MIC_{50} and MIC_{90} values were defined as the lowest concentrations that inhibit 50% and 90% of the bacteria isolates. The type strain (*M. bovis* PG45, NCTC 10131) was used the quality control of the MIC determination.

4.4. Identification of mutations responsible for the high MICs to seven antimicrobial families

4.4.1. Selection of antibiotic resistant mutants of *Mycoplasma bovis*

The molecular mechanisms of resistance to 12 antibiotics of 7 antimicrobial families (danofloxacin, enrofloxacin, marbofloxacin, tetracycline, oxytetracycline, spectinomycin, tilmicosin, tylosin, lincomycin, florfenicol, tiamulin and valnemulin) were investigated. Low variability of gentamycin MIC values of the Hungarian *M. bovis* isolates was detected. In addition, information about the genetic background of gentamycin resistance is limited. Examinations with tulathromycin and gamithromycin were included later in the study than with the rest of the antibiotics, therefore investigation of *in vitro* development and genetic background of gentamycin, tulathromycin and gamithromycin resistance were not performed.

The selection of antibiotic-resistant mutants was performed, including the *M. bovis* reference strain (PG45, NCTC 10131) and two field isolates (MYC52 and MYC53) with low MIC values to most of the tested antibiotics (Table S1). The selection was carried out by serial passages of the isolates in Mycoplasma broth medium (Thermo Fisher Scientific Inc. /Oxoid Inc./) containing subinhibitory concentrations (increasing in twofold dilutions) of each of the examined antibiotics (Pereyre et al. 2004, Gruson et al. 2005). The culture containing the highest antibiotic concentration with detectable growth (red to yellow shift) was used to inoculate another antibiotic dilution panel for the following passage series. Passages were performed until MIC values reached ≥10 µg/ml for fluoroquinolones and pleuromutilins, ≥64 µg/ml for tetracyclines and lincomycin, ≥128 µg/ml for florfenicol and ≥256 µg/ml for spectinomycin.

Resistant mutants were subcultivated in antibiotic-free medium at least five times and MICs of all drugs were determined again by the end of the passages to check whether the phenotype was stable without selection pressure. Cross-resistance was also examined with microbroth dilution tests among the 12 examined drugs.

4.4.2. Next generation sequencing

M. bovis genomic DNAs of the 35 Hungarian isolates, the reference strain (PG45, NCTC 10131) and the 36 *in vitro* selected mutants were extracted from 200 µl of logarithmic-phase broth cultures using QIAamp DNA Mini Kit (Qiagen Inc.) according to the manufacturer's instructions.

Two PCR systems were used for the specific amplification of the 23S rRNA genes *rrl1* and *rrl2*, and the 16S rRNA genes *rrs1* and *rrs2* (Table 6). PCR amplification of *rrl1* and *rrs1* genes was performed with MB-282-F and MB-rrl-3R primers according to Lerner et al.

(2014). For the amplification of *rrl2* and *rrs2* genes, a PCR system of Lerner et al. (2014) was modified in this study. Reaction was carried out in 50 µl final volumes containing 0.4 µl TaKaRa Taq polymerase (5U/ µl; Takara Bio Inc., Otsu, Japan), 5µl 10x PCR Buffer containing 15 mM $MgCl_2$ (Takara Bio Inc.), 4 µl dNTP Mixture (2.5 mM; Takara Bio Inc.), 2.5 µl from each primer (10 pM/µl) and 1 µl of a DNA template.

PCR reactions were carried out in T100 series thermocycler (Bio-Rad, Hercules, CA, USA) according to the following parameters: 95°C for 5 min, 30 cycles of 95°C for 15 sec, 56°C for 30 sec, 72°C for 4 min followed by 72°C for 10 min. Products for sequencing were purified by QIAquick Gel Extraction kit (Qiagen Inc.) according to the manufacturer's instructions.

Table 6. Primers used for the specific amplification and separation of the 23S rRNA genes of *M. bovis* (Lerner et al. 2014)

Amplification target	Primer name	Primer sequence (5'→3')	Annealing temperature (°C)	Amplicon length (bp)
rrl1 and *rrs1*	MB-282-F MB-rrl-3R	GGATATCTAACGCCGTGTCT GTACTGGTCAGCTCAACAC	56	5041
rrl2 and *rrs2*	MB-rrs4-F2* MB-287-R	GCATGTCGAGCGATGATAGC CTAATTCCAAGTGCCACTAGCG	56	5247

* New forward primer was designed in the present study

Next-generation sequencing of the 142 PCR products (containing separated *rrl* and *rrs* genes) and 71 whole genomes (35 field and 36 mutant isolates) were performed on Ion Torrent platform as previously described (Rónai et al. 2015).

One hundred ng of DNA was subjected to enzymatic fragmentation using the reagents supplied in the NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent™ kit (New England Biolabs, Hitchin, United Kingdom) according to the manufacturer's instructions. The adaptor ligation was performed using reagents from the same kit, whereas barcoded adaptors were retrieved from the Ion Xpress™ Barcode Adapters (Life Technologies Inc., Waltham, MA, USA).

The barcoded library DNA samples were column purified using the Gel/PCR DNA Fragments Extraction kit (Geneaid Biotech Ltd., Taipei, Taiwan). Then the eluted library DNA was run on 2% precast gel (Thermo Fisher Scientific). Products between 300 and 350 bp were directly used without further purification in the PCR mixture of the NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent™ kit (New England Biolabs).

Library amplification was performed in 12 cycles and the products were purified by the Gel/PCR DNA Fragments Extraction kit (Geneaid). The library DNA was eluted in 50 µl nuclease free water and quantified fluorometrically on Qubit® 2.0 equipment using the

Qubit® dsDNA BR Assay kit (Invitrogen). Subsequently, the library DNA was diluted to 10-14 pM and then clonally amplified by emulsion PCR. This step was carried out according to the manufacturer's protocol using the Ion PGM Template kit on OneTouch v2 instrument. Enrichment of the templated beads (on Ion One Touch ES machine) and further steps of pre-sequencing set-up were performed according to the 200 bp protocol of the manufacturer. The sequencing protocol recommended for Ion PGM™ Sequencing Kit on a 316 chip was strictly followed.

4.4.3. Sequence analysis

Sequence analysis and alignment were performed using DNASTAR software, version 12.1.0.145 (Lasergene Inc., Madison, Wisconsin, USA). Sequence data were mapped to *M. bovis* PG45 complete genome sequences (Genbank accession No.: CP002188, NC_014760) obtained from GenBank. Putative single nucleotide polymorphisms (SNPs) between the reference genome and the genomes of 35 field isolates and the 36 antibiotic resistant mutants selected *in vitro* were identified under the following settings: mer size: 19 nt, minimum match percentage: 93, minimum alignment length: 25, maximum gap size: 30, match score:10, mismatch penalty: 20, gap penalty: 50, gap extension penalty: 10, alignment cutoff: 200, SNP calculation method: haploid bayesian, P not reference percentage: 75%, Phred score (Q call): 30, SNP percentage: 75–100%, coverage depth minimum: 5. An average number of reads and read lengths were 184 450 and 177 bp for whole genomes and 4 985 and 169 bp for PCR products. The overall coverage and the depth of coverage were 31.9 and 30.3 for whole genome per isolate and 174.7 and 113.6 for the amplicons per isolate. The validity of SNPs was confirmed by manual examination of the assembled sequences.

The numbering of nucleotide and amino acid positions is based on genes and proteins of *Escherichia coli* strain K12 substrain MG1655 (GenBank accession number CP014225) unless indicated otherwise. Nomenclature for *rrn* genes was used throughout the present study as suggested by (Amram et al. 2016): *rrs1*/*rrs2* instead of *rrs3*/*rrs4* and *rrl1*/*rrl2* instead of *rrl3*/*rrl4* genes.

4.5. Development of molecular assays for the rapid detection of antibiotic susceptibility of *Mycoplasma bovis*

4.5.1. MAMA and HRM design

Mismatch amplification mutation assay (MAMA) is a PCR-based molecular biological technique used for SNP discrimination in many bacteria (Birdsell et al. 2012). In brief, MAMAs are based on allele-specific primers that are SNP specific at the 3' end. A single destabilizing mismatch at the 3' end of each allele-specific primer enhances the discrimination capacity of the assay. One of the allele-specific primers is marked with an additional 15-20 bp long GC-clamp that increases the melting temperature and the size of the amplicon as well. The temperature shift can be easily detected in the presence of intercalating fluorescent dye on a real-time PCR platform (Melt-MAMA) and the difference in the sizes of the amplicons can be observed in 3% agarose gel electrophoresis (Agarose-MAMA). In the present study, MAMAs were designed and tested for the detection of SNPs related to antibiotic resistance (Table 7). Besides SNPs, regions with several mutations were identified in resistant isolates. These "hot-spot" regions of genes related to antibiotic resistance were targeted by high resolution melt (HRM) assays (Table 8). HRM is based on thermodynamic differences between small amplicons, therefore it is a suitable technique for the analysis of polymorphic SNPs (Palais et al. 2005).

Melt-MAMA and HRM assays were optimized for Applied Biosystems Step-One Plus real-time PCR system with StepOne Software version 2.3 (Thermo Fisher Scientific). All MAMA and HRM primers were designed and tested on the *M. bovis* reference strain (PG45, NCTC 10131), on the 35 field isolates (Table 1) and on the 36 antibiotic resistant mutants selected *in vitro* (Table S1). Primer melting temperature (T_m) and general suitability were calculated using the NetPrimer software (Premier Biosoft International, Palo Alto, CA).

The primer sequences and thermocycler parameters for the assays can be found in Tables 6 and 7. PCR mixture of Melt-MAMAs and HRMs were identical and composed of 2 µl 5X Color-less GoTaq Flexi Buffer (Promega Inc.), 1 µl $MgCl_2$ (25mM), 0.3 µl dNTP (10 mM, Qiagen Inc.), 0.5 µl EvaGreen (20X, Biotium Inc., Hayward, CA), primers (10 pmol/µl, Table 7 or 7), 0.08 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; Promega Inc.) and 1 µl DNA template with a final volume of 10 µl. Thermocycling parameters were 95°C for 10 min, followed by 34 cycles of 95°C for 15 sec and 60°C for 1 min. Endpoint PCR products were subjected to melt analysis using the following dissociation protocol: 95°C for 15 sec, followed by incremental temperature ramping (0.3°C for Melt-MAMA, 0.1°C for HRM) from 60°C to 95°C. EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature.

Table 7. Primers and parameters of MAMAs designed in this study.

Gene	SNP[a]	Antibiotic	Primer name	Primer sequence (5'→3')	Primer[b]	T_{ann} (°C)
gyrA	C248T	FQs	gyrA-248-res	ggggcggggcggggCACCATTGCTTCATAAACCGCAA	0.15	60
			gyrA-248-sen	CACCATTGCTTCATAAACCGTAG	0.15	
			gyrA-248-con	GTGCAAGAATTGTTGGTGATGTTT	0.15	
parC	G239T	FQs	parC-239-res	ggggcggggcggggCTAACCATTGCRTYATAAATAGCAA	0.15	60
			parC-239-sen	CTAACCATTGCRTYATAAATAGTAC	0.15	
			parC-239-con	GAAATCAGCTCGTGTTGTCGG	0.15	
parC	G250A	FQs	parC-250A-res	CATGGYGATAGTTCTATTTGTA	0.15	60
			parC-250-sen	ggggcggggcggggcCATGGYGATAGTTCTATTTCTG	0.15	
			parC-250-con	TTTCACTCTTGCCCCATC	0.15	
rrs1/rrs2	A965T	TETs	rrs-965-res	ggggcggggcggggcTGGAGCATGTGGTTTAATTTAAT	0.15	60
			rrs-965-sen	TGGAGCATGTGGTTTAATTTCAA	0.6	
			rrs-965-con	AGCTTTGCAGAAGATGTCAAGAGT	0.15	
rrs1/rrs2	A967T	TETs	rrs-967-res	ggggcggggcggggcAGTGGGTAAGGTTCTACGCGCAA	0.6	60
			rrs-967-sen	AGTGGGTAAGGTTCTACGCGAAT	0.15	
			rrs-967-con	ACTTAAAGGAATTGACGGGGATC	0.15	
rrs1/rrs2	G1058C	TETs	rrs-1058-res	ggggcggggcggggACAGAATGACAGATGGTGCAAGC	0.15	60
			rrs-1058-sen	ACAGAATGACAGATGGTGCAGGG	0.6	
			rrs-1058-con	TCGTTGCAGGACTTAACCGAAC	0.15	
rrs1/rrs2	C1192A	Sc	rrs-1192-res	ggggcggggcggggGTAAGAGGCATGATGRTTTGGCT	0.15	60
			rrs-1192-sen	GTAAGAGGCATGATGRTTTGTCG	0.3	
			rrs-1192-con	CTCTAAGGAGACTGCCCGAGTAATC	0.15	
rrl1/rrl2	G748A	MACs	rrl-748-res	ggggcggggcggggcGAGGGTCGAACCGTAGTACATTA	0.6	60
			rrl-748-sen	GAGGGTCGAACCGTAGTACAGTG	0.15	
			rrl-748-con	TGGAATTTCTCCGCTATTCACAA	0.15	
rrl1/rrl2	A2059G	MACs/L	rrl-2059-res	ggggcggggcggggcTATAGTAAAGCTCCATGGGGTCATC	0.15	60
			rrl-2059-sen	TATAGTAAAGCTCCATGGGGTCGTT	0.15	
			rrl-2059-con	ATGGTCCCAGTGAAAACGCTG	0.15	

Resistant or sensitive phenotypes are labelled with "res" or "sen" abbreviation, while "con" means consensus in the primer name. [a] according to *E. coli* numbering; [b] Primer (10 pmol/µl) volume in 10 µl reaction mixture (µl).
T_{ann}= annealing temperature; FQs=fluoroquinolones; TETs=tetracyclines; Sc=spectinomycin; MACs=macrolides; L=lincomycin.

Table 8. Primers and parameters of HRMs designed in this study.

Assay name	Antibiotic	Amplicon[a]	Primer name	Primer sequence (5'→3')	T_{ann} (°C)
gyrA	FQs	213-286	HRM-GYRA-F	TGATGTTTTAGGTAAGTATCACCCTCA	60
			HRM-GYRA-R	AATCTTGAGCCATACGCACCA	
rrs1/2-A	TETs	993-1005	HRM-RRSA-F	GCACAAGCGGTGGAGCAT	60
			HRM-RRSA-R	TTGCAGAAGATGTCAAGAGTGGG	
rrs1/2-B	TETs, Sc	1139-1251	HRM-RRSB-F	GTTGAGCACTCTAAGGAGACTGCC	60
			HRM-RRSB-R	TTGTACCGTCCATTGTAGCACG	
rrl1/2-A	MACs	672-778	HRM-RRLA-F	CCGAAACCAGGTGACCTATTCA	60
			HRM-RRLA-R	CCGCTATTCACAAGTCATCCG	
rrl1/2-B	50S inhibitors	2005-2101	HRM-RRLB-F	GACTCGGTGAAATTATGGTCCC	60
			HRM-RRLB-R	CAAATTCCAATACGAAGTTATAGTAAAGC	
rrl1/2-C	50S inhibitors	2473-2524	HRM-RRLC-F	GATCACATCGACGGCAAGGT	60
			HRM-RRLC-R	CTCCAGGATGCGATGAGCC	
rrl1/2-D	50S inhibitors	2557-2636	HRM-RRLD-F	CGCCCATTAAAGCGGTACG	60
			HRM-RRLD-R	TATTCCAACGCCCACATCAGA	

[a] according to *E. coli* nucleotide numbering; T_{ann}= annealing temperature; FQs=fluoroquinolones; TETs=tetracyclines; Sc=spectinomycin; MACs=macrolides.

Agarose-MAMAs were performed in C1000™ Touch Thermal Cycler (Bio-Rad Laboratories, Inc.) in 25 µl total volume containing 1 µl target DNA diluted in 5 µl 5X Green GoTaq Flexi Buffer (Promega Inc.), 2.5 µl $MgCl_2$ (25mM), 0.5 µl dNTP (10 mM, Qiagen Inc.), primers (10 pmol/µl) according to Table 7 and 0.25 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; Promega Inc.) under the following PCR conditions: 94°C for 5 min followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The final elongation step was performed at 72°C for 5 min. Electrophoresis was carried out in 3% agarose gel (MetaPhor Agarose, Lonza Group Ltd., Basel, Switzerland) and a 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific Inc.) was used as molecular weight marker.

Products of the PCR systems (Table 6) diluted in nuclease-free water (1:100) were applied as target DNA in the case of MAMA and HRM tests for the detection of mutations found on gene duplicates (*rrl1*/*rrl2* and *rrs1*/*rrs2*).

All specimens were tested in duplicate. HRM profiles were analysed using High Resolution Melt software, version 3.0.1 (Thermo Fisher Scientific Inc.). Fluorescent values were normalized according to user-adjustable pre-melting and post-melting temperature intervals.

4.5.2. MAMA and HRM validation

Initially, evaluation of the MAMA and HRM assays was performed on DNAs extracted from purified culture of *M. bovis* field isolates (n=35) and laboratory derived mutants (n=9 to fluoroquinolones; n=9 to 30S inhibitors; n=18 to 50S inhibitors) with known sequence and MIC values (Tables 11-13 and Table S3).

In order to test the sensitivity of the assays, tenfold dilutions of each genotype were used in the range of 10^6-10^0 copy number/µl. Template copy number was calculated with the help of an online tool (Staroscik 2004) by measuring the concentration of DNA of pure *M. bovis* cultures by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.). The lowest DNA concentrations (template copy number) yielding T_m specific to the genotype were considered the detection limit of the assays.

The specificity of the assays was tested by including the following bovine *Mycoplasma* species in the analysis: *M. alkalescens*, *M. alvi*, *M. arginini*, *M. bovigenitalium*, *M. bovirhinis*, *M. californicum*, *M. canadense*, *M. dispar* and *M. verecundum*. The genetically closely related species, *M. agalactiae* was also included.

In order to test the intra-run repeatability at least four replicates of each genotype were examined within the same run. For inter-run reproducibility test, the duplicate of at least one sample from each genotype was examined in separate tests.

For further evaluation of the assays, HRM and melt-MAMA tests were challanged with the DNA of clinical samples (n=30; Table 2). Genotype calls were checked with microbroth

dilution method and genotype assignment of the *M. bovis* isolates originating from the same clinical samples as the examined DNA (n=20). The DNA of a nasal swab and 29 lung samples (Table 2) was extracted with Qiamp DNA Mini kit (Qiagen Inc.) according to the manufacturer's instructions. Before performing all the melt-MAMA and HRM assays on the 30 clinical samples the presence of DNA of *M. bovis* or other *Mycoplasma* spp. was checked with the two PCR systems mentioned in chapter 4.1.2. (Lauerman et al. 1995, Subramaniam et al. 1998).

Rate of the false results gained in the assays was calculated with the following formula:

$$\text{false result rate} = \frac{100 \times \text{number of misclassified samples}}{\text{total number of samples}}$$

The *M. bovis* pure isolates were considered misclassified if their genotype calls were not identical with their sequence and MIC data, while the clinical samples were considered misclassified when their genotype calls differed from the genotype calls and MICs of the isolates purified from the clinical samples.

5. Results

5.1. Genetic diversity of the Hungarian *Mycoplasma bovis* isolates

Hungarian *M. bovis* isolates were clustered into two clades (A and B) by MLST (Figure 4). Two isolates (MYC52-53) had the same sequence type (ST) as the reference strain PG45 (NCTC 10131) and were closely related to a strain from Saudi Arabia, while the other Hungarian isolates formed a novel clade with five subclades. Isolates originating from the same herds had the same STs and were assigned to the same subclades by MLST except for MYC65 isolate.

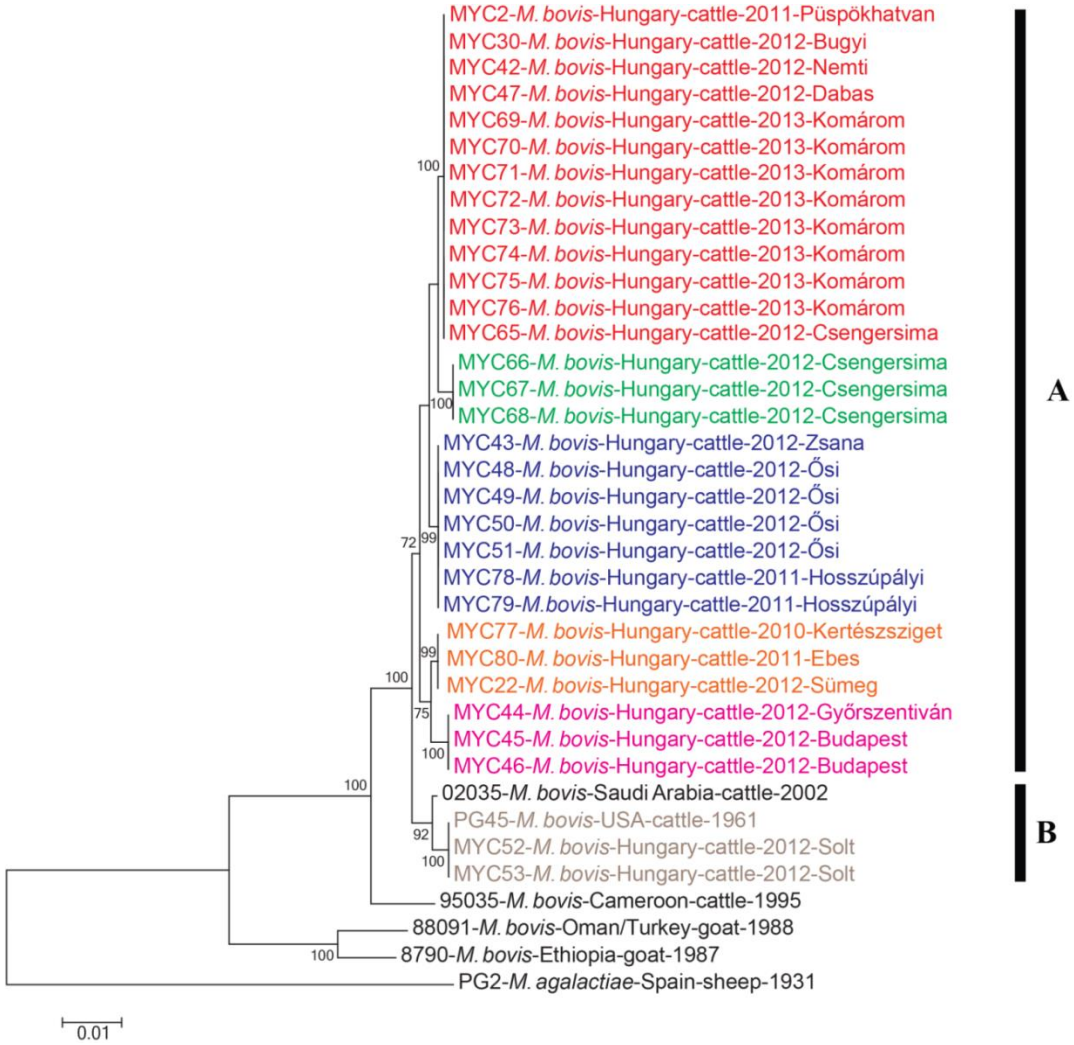

Figure 4. Genetic relationships between *M. bovis* isolates based on MLST.
Neighbour-joining phylogenetic tree showing relationships between the concatenated partial sequences of four housekeeping genes obtained from *M. bovis* isolates. Letters "A" and "B" indicate the two main clades. Bootstrap values of neighbour-joining (1000 replicates) of >70 are shown. The scale bar represents the average number of substitutions per site. Colours indicate the subclades formed by Hungarian isolates.

The within-group means of genetic distances between the concatenated sequences were 0.001 (±0.001 standard error, SE) in the Hungarian clade (Clade A) and 0.001 (± 0.000 SE) in the PG45 clade (Clade B). The mean values of between-clade genetic distances for the concatenated sequences ranged from 0.003 to 0.012 (Table 9). The recombination analyses on the alignment of the concatenated MLST loci did not reveal any recombination events.
MLST sequences of the Hungarian *M. bovis* isolates were deposited to GenBank with the following accession numbers: KF926436-KF926475 and KJ438174-KJ438257.

Table 9. Estimates of average evolutionary divergence of concatenated sequence pairs between and within *M. bovis* clades.

	Evolutionary divergence					
	Clade A	Clade B	M.b.-Cameroon	M.b.-Oman/Turkey	M.b.-Ethiopia	Distance (SE)
Clade A						0.001 (0.001)
Clade B	0.003					0.001 (0.000)
M.b.-Cameroon	0.006	0.005				n/c
M.b.-Oman/Turkey	0.012	0.012	0.010			n/c
M.b.-Ethiopia	0.012	0.012	0.010	0.003		n/c
M. agalactiae	0.030	0.030	0.029	0.029	0.027	n/c

The numbers of base substitutions per site are shown as calculated from the average substitutions of all sequence pairs between (matrix) and within (column) groups. Standard error estimates are shown in parentheses. n/c: not calculated.

The Hungarian *M. bovis* isolates and the reference strain PG45 formed two major clades with several subclades and branches by MLVA (Figure 5). One main group comprised three isolates (MYC52-53 and 80) and the reference strain PG45 while the other main branch contained the rest of the isolates. Isolates originating from the same herd were generally clustered together or close to each other (MYC45-46, MYC52-53, MYC65-68, MYC69-76, MYC 78-79), but within herd divergence was also detected (MYC48-51). MLVA profiles of the isolates are presented in Table S2.

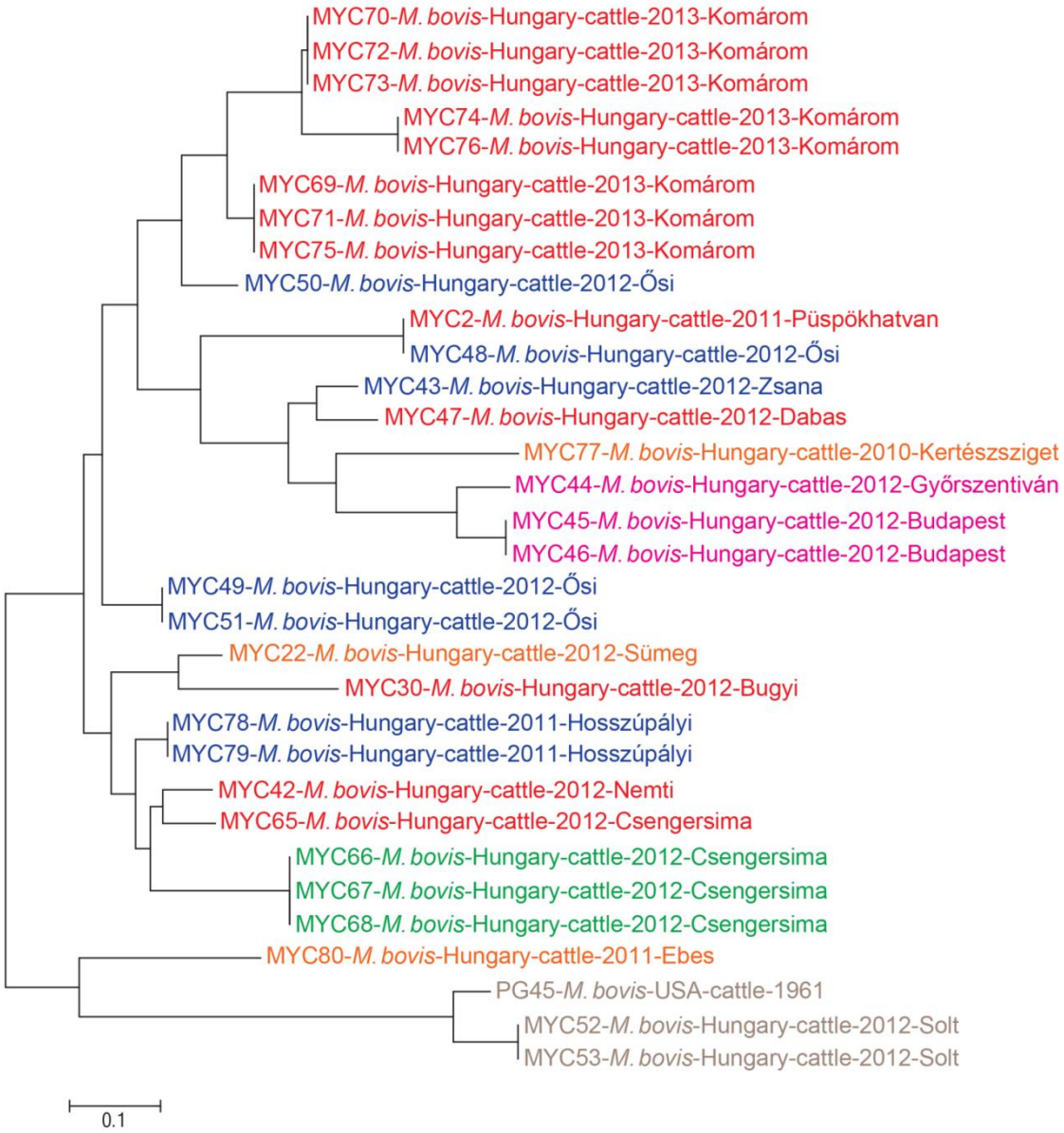

Figure 5. Genetic relationships between 31 Hungarian isolates and the reference strain PG45 based on MLVA.

Dendrogram was constructed with the neighbour-joining method based on pairwise distances. The scale bar represents the average number of substitutions per site. Colours indicate the subclades determined by MLST.

Six STs were discriminated by MLST and twenty different MLVA profiles were identified among the analysed isolates and PG45. The Simpson's index of diversity was 0.776 (CI: 0.678-0.874) in MLST and 0.970 (CI: 0.952-0.987) in MLVA. The value of the Adjusted Rand coefficient was 0.178, the Adjusted Wallance coefficient MLVA → MLST was 0.099 (CI: 0.004-0.194) and the Adjusted Wallance coefficient MLST → MLVA was 0.914 (CI: 0.828-1.000). These values indicate poor concordance between the results of the two typing systems. Correlation was not found between the genotype and source (lung, nasal swab or lymph node) of the *M. bovis* isolates. MLST alignment, MLVA data and phylogenetic trees were deposited in Dryad Digital Repository [doi: 10.5061/dryad.f4ks8].

5.2. Antibiotic susceptibility of the Hungarian *Myoplasma bovis* isolates

The MIC values of *M. bovis* type strain PG45 determined in the present study were identical with values previously obtained for danofloxacin, enrofloxacin, marbofloxacin, spectinomycin, tilmicosin and tylosin using the microbroth dilution method (Table S2) (Ter Laak et al. 1993, Gerchman et al. 2009). The MIC value of PG45 (2 µg/ml) for oxytetracycline was within the range of previously published studies applying microbroth dilution test (0.1 / 0.125 / 0.16 / 4 µg/ml) (Ter Laak et al. 1993, Hannan 2000, Rosenbusch et al. 2005; Gerchman et al. 2009). The MIC values of PG45 for lincomycin and tiamulin (1 µg/ml and 0.078 µg/ml, respectively) were slightly higher than in previous studies (0.25 µg/ml for lincomycin; ≤0.015 µg/ml and 0.05 µg/ml for tiamulin) (Ter Laak et al. 1993, Hannan 2000, Gerchman et al. 2009). For gentamicin, gamithromycin, tulathromycin, tetracycline, florfenicol and valnemulin data of PG45 determined by microbroth dilution test were not available. The results for the *M. bovis* type strain PG45 were consistent throughout the present study.

The MIC values of the fifteen antimicrobial agents obtained from the examinations of the Hungarian *M. bovis* isolates are shown on Figure 6, Figure 7, Table 10 and Table S3. Isolates with elevated MIC values were found in the case of all applied antibiotics with the exception of pleuromutilins. Fluoroquinolones and pleuromutilins were found to be the most active compounds *in vitro*.

Table 10. Summary of range, mode, MIC_{50} and MIC_{90} values of the 35 *M. bovis* isolates obtained from cattle in Hungary. All values are expressed as µg/ml.

		Range	Mode	MIC_{50}	MIC_{90}
Fluoroquinolones	Danofloxacin	0.078 - ≥10	0.156	0.156	0.312
	Enrofloxacin	0.078 - ≥10	0.156	0.156	0.312
	Marbofloxacin	0.312 - ≥10	0.625	0.625	0.625
Aminoglycosid	Gentamicin	2 - 16	4	4	8
Aminocyclitol	Spectinomycin	2 - ≥256	≥256	≥256	≥256
Tetracyclines	Oxytetracycline	2 - ≥64	≥64	≥64	≥64
	Tetracycline	≥0.25 - 16	8	8	16
Macrolides	Gamithromycin	8 - ≥128	16	16	≥128
	Tilmicosin	≥0.5 - ≥128	≥128	≥128	≥128
	Tylosin	≥0.5 - ≥128	≥128	≥128	≥128
	Tulathromycin	2 - ≥128	16	16	≥128
Phenicol	Florfenicol	4 - 8	4	4	8
Lincosamide	Lincomycin	0.5 - ≥64	≥64	≥64	≥64
Pleuromutilin	Tiamulin	0.039 – 0.625	0.156	0.156	0.312
	Valnemulin	≤0.039 - ≤0.039	≤0.039	≤0.039	≤0.039

The antibiotic susceptibility profiles of the Hungarian isolates were consistent within the tested group of fluoroquinolones. Three isolates (MYC44, MYC45 and MYC46) had high MIC values (≥10 µg/ml) to danofloxacin, enrofloxacin and marbofloxacin, while the rest of the isolates were inhibited by these antimicrobial agents with MICs ≤0.312 or 0.625 µg/ml (Figure 6 and 7A-C).

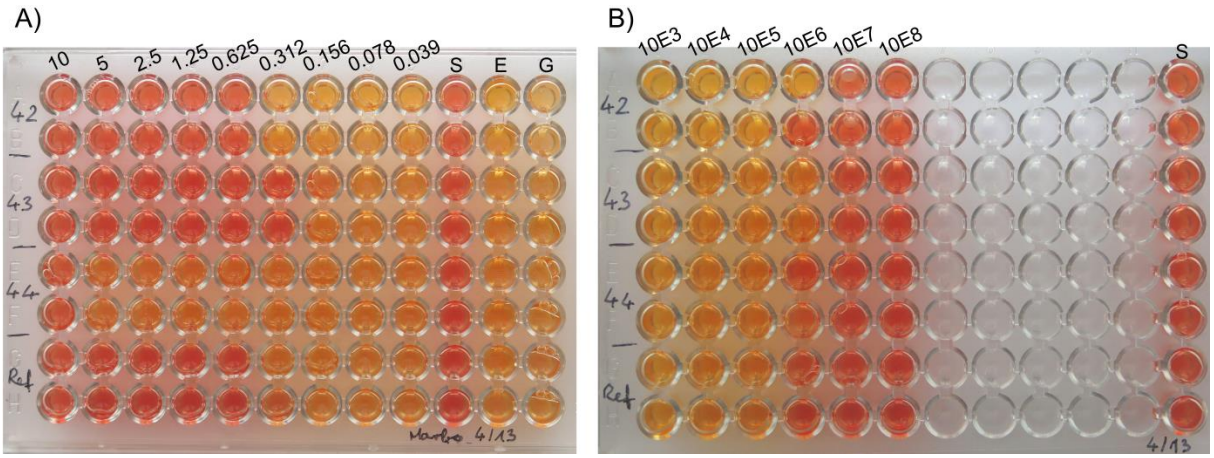

Figure 6. MIC determination of *M. bovis* isolates by microbroth dilution method (A) and simultaneously performed viable counting method (B).
MIC values (expressed as µg/ml) to marbofloxacin were 0.625 µg/ml, 0.312 µg/ml, 10 µg/ml and 0.625 µg/ml for MYC42, MYC43, MYC44 and *M. bovis* reference strain (PG45, NCTC 10131), respectively. Abbreviations: S= sterility control, E= end point control, G= growth control

The MICs for gentamicin clustered steadily around the MIC_{50} value (4 µg/ml) (Figure 7D). MIC values of spectinomycin divided the isolates into two distinct populations, with 48% of isolates yielding MICs of ≤4 µg/ml and the rest clustering with MICs ≥256 µg/ml (Figure 7E).
Two *M. bovis* isolates (MYC52 and MYC53) originating from the same herd were inhibited by both tetracyclines and macrolides with low MIC values (Table S3). MICs of the remaining isolates were high to the tested tetracyclines with MIC_{90} values ≥64 µg/ml to oxytetracycline and 16 µg/ml to tetracycline (Figure 7F-G).
Among the macrolides, the MICs of tilmicosin showed a bimodal distribution, as two isolates yielded MICs ≤0.5 µg/ml, while the rest yielded MICs ≥128 µg/ml, and gradual distribution of MICs of tylosin were observed. The MICs of the examined new generation macrolides (gamithromycin and tulathromycin) showed a comparable distribution with peaks at MIC_{50} (16 µg/ml) and MIC_{90} (≥128 µg/ml) (Figure 7H-K).
MICs for lincomycin also clustered the isolates into two groups, one with MICs ≤2 µg/ml and another with MICs ≥64 µg/ml (Figure 7L). The narrow range of MIC values (4-8 µg/ml) of florfenicol is demonstrated on Figure 7M. MICs of pleuromutilins to all of the examined Hungarian isolates were low with MIC_{90} values of 0.312 µg/ml for tiamulin and ≤0.039 µg/ml for valnemulin (Figure 7N-O).
Isolates originating from the same herd showed similar antibiotic susceptibility profiles (Table S3).

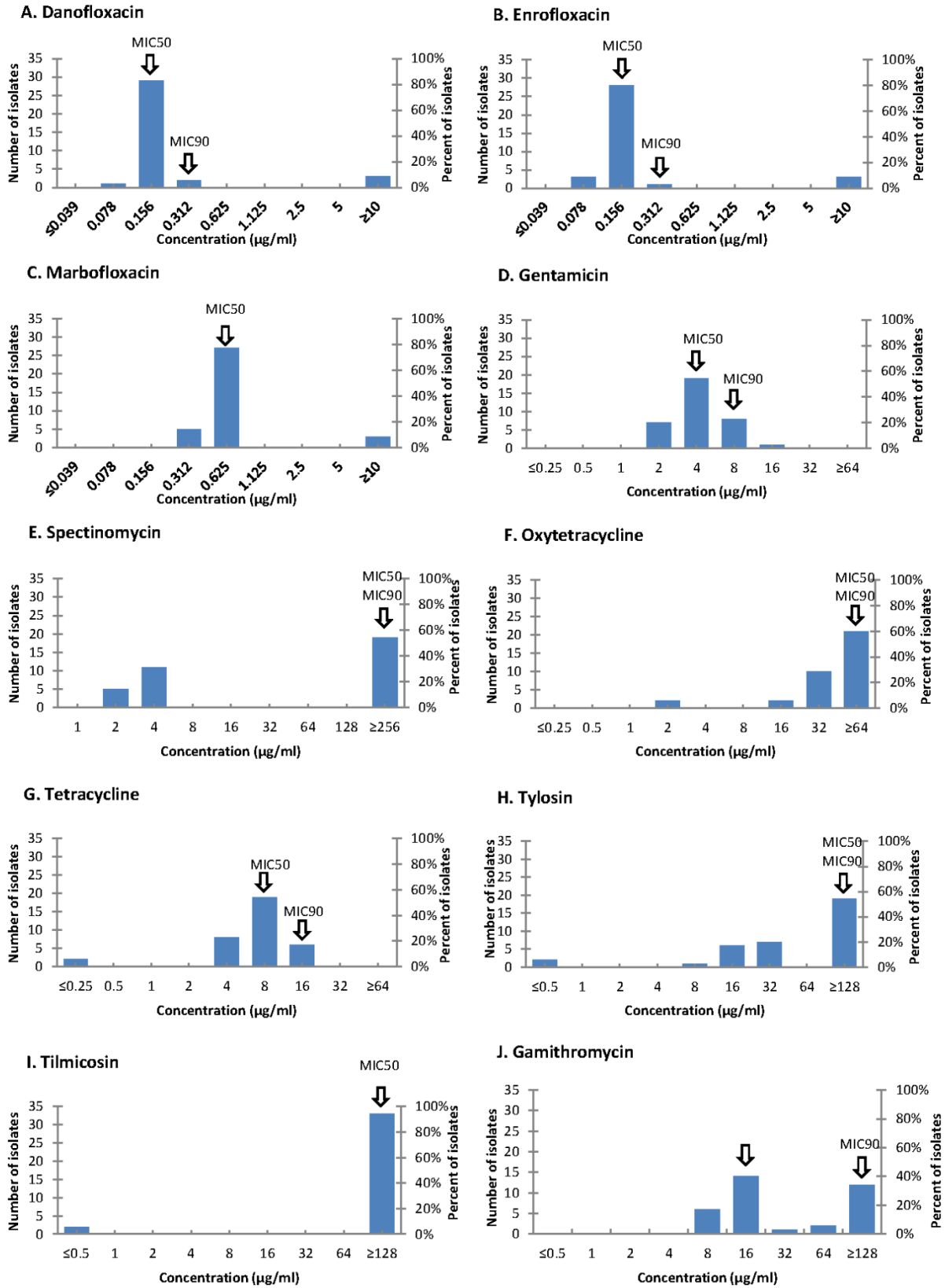

Figure 7. MIC distribution of the 35 Hungarian isolates for each antibiotic tested in the study. Arrows indicate the MIC_{50} and MIC_{90} values.

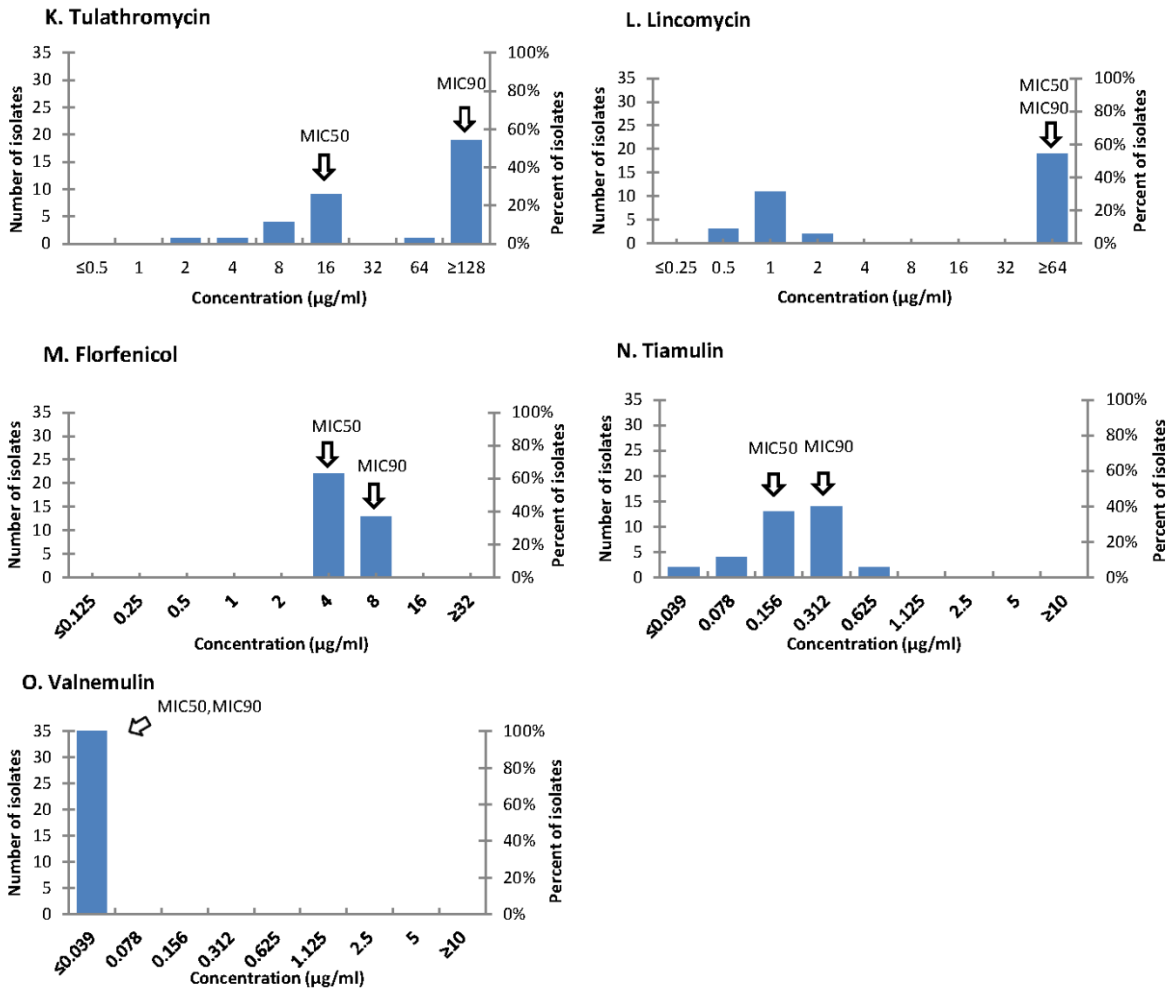

Figure 7 (continued). MIC distribution of the 35 Hungarian isolates for each antibiotic tested in the study.

Arrows indicate the MIC_{50} and MIC_{90} values.

5.3. Mutations responsible for the high MICs to seven antimicrobial groups

5.3.1. Fluoroquinolones

Mutants with high MICs to fluoroquinolones (≥10 µg/ml) were selected *in vitro* for danofloxacin, enrofloxacin and marbofloxacin by 7-10, 4-8 and 5-7 passages of the parental strains PG45, MYC52 and MYC53, respectively (Figure 8, Table S1). Mutants remained highly resistant to the selector antibiotic after serial passages in antibiotic-free medium. Moreover, in each case cross-resistance (≥10 µg/ml) developed in the selected mutants to the other fluoroquinolones examined as well.

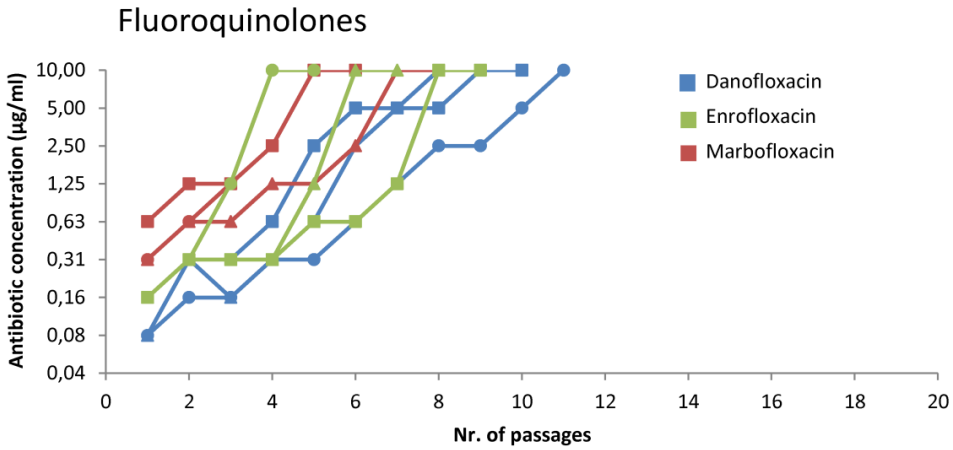

Figure 8. Development of resistance to fluoroquinolones in *in vitro* selected mutant strains. Fluoroquinolone concentrations used during the *in vitro* selection of resistant *M. bovis* isolates plotted versus the number of passages. Symbols mark the parental isolates: triangle: *M. bovis* reference strain (PG45, NCTC 10131), circle: MYC52, square: MYC53.

Comparing whole genome sequences of isolates with extremely high MICs (≥10 µg/ml) and low MIC values (≤0.625 µg/ml) to fluoroquinolones revealed mutations in the QRDRs of the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes (Table 11). All *M. bovis* field isolates (MYC44-MYC46) (Table S3) and laboratory-derived mutants with MIC ≥10 µg/ml (PG45, MYC52-53) harboured mutations in *gyrA*, *gyrB* and *parC* genes. No fluoroquinolone resistance related mutations were detected in *parE*.

Table 11. Mutations detected in the *gyrA*, *gyrB* and *parC*-QRDRs of *M. bovis* field isolates and *in vitro* selected mutants with high MICs to fluoroquinolones.

	Mutations associated with high MICs in the current and/or previous studies								Mutations with unknown effect				
	gene	*gyrA*				*parC*			*gyrA*		*gyrB*		
Strain	NT[a]	244	248	259	260	232	239	250	1860	2038	109	968	1278
	AA[b]	82	83	87	87	78	80	84	620	680	37	320	423
PG45		G (Asp)	C (Ser)[c]	G (Glu)[c]	A (Glu)[c]	G (Gly)[c]	G (Ser)[c]	G (Asp)[c]	T (Ile)	G (Asp)	C (His)	T (Ala)	T (Asn)
MYC44-46			T (Phe)				T (Ile)		C (Ile)			C (Val)	
$MYC52_{danofloxacin}$		A (Asn)						A (Asn)					A (Lys)
$MYC53_{danofloxacin}$		A (Asn)		A (Lys)				A (Asn)					
$PG45_{danofloxacin}$			T (Phe)			T (Cys)							
$MYC52_{marbofloxacin}$			A (Tyr)					T (Tyr)			T (Asn)		
$MYC53_{marbofloxacin}$				A (Lys)				C (His)					
$PG45_{marbofloxacin}$				A (Lys)			T (Ile)						
$MYC52_{enrofloxacin}$				A (Lys)			T (Ile)						
$MYC53_{enrofloxacin}$					G (Gly)			A (Asn)		C (His)			
$PG45_{enrofloxacin}$				A (Lys)			T (Ile)	A (Asn)					

[a] *E. coli* nucleotide numbering; [b] *E. coli* amino acid numbering; [c] Altered positions related to high MICs to fluoroquinolones already described in *M. bovis* isolates (Lysnyansky et al. 2009, Sato et al. 2013, Mustafa et al. 2013, Khalil et al. 2015).
PG45: *M. bovis* reference strain NCTC 10131 (MIC 0.156 µg/ml to danofloxacin and enrofloxacin, 0.625 µg/ml to marbofloxacin);
MYC44-46: Hungarian field isolates with MICs of ≥10 µg/ml to all tested fluoroquinolones;
Abbreviation of antibiotic resistant mutants selected *in vitro*: $Strain_{drug\ used\ for\ selection}$

In *gyrA* gene of the three field isolaets (MYC44-46) with elevated MICs to fluoroquinolones a silent mutation (T1860C) and a non-synonymous mutation (C248T) resulting in Ser83Phe amino acid change were observed. In the *in vitro* selected mutants, the 248 position of *gyrA* gene was surrounded with additional SNPs (G244A, C248T, C248A, G259A and A260G) and caused amino acid mutations of Asp82Asn, Ser83Tyr, Ser83Phe, Glu87Lys and Glu87Gly, respectively. In the case of enrofloxacin resistant MYC53 mutant a single substitution G2038C, causing amino acid alteration Asp680His, was also observed.

In *gyrB*, a substitution at *E. coli* nucleotide position 968 was present. This nucleotide was found in *M. bovis* isolates MYC44-46, but not in the selected fluoroquinolone resistant mutants results in Val320Ala alteration. In addition, marbofloxacin and danofloxacin resistant MYC52 mutants harboured point mutations at C109A and T1278A, causing His37Asn and Ile423Asn amino acid alterations.

The SNP G239T of *parC* gene of field isolates MYC44-46 caused Ser80Ile alteration. Resistant mutant strains showed the same (G239T) and the following additional mutations of this region of *parC*: G232T, G250A, G250C and G250T resulting in Gly78Cys, Asp84Asn, Asp84His and Asp84Tyr changes (Table 11).

Sequences related to high MICs to fluoroquinolones were deposited to GenBank with the following accession numbers: KR493099-KR493133 and KX462228-KX462236 for *gyrA*, KR493134-KR493141 and KT218638-KT218664 for *gyrB*, KR493142-KR493176 and KX462237-KX462245 for *parC* genes.

5.3.2. 30S inhibitors: tetracyclines and an aminocyclitol

Mutants with notably increased MICs (≥64 µg/ml for tetracyclines and ≥256 µg/ml for spectinomycin) were obtained with all the selector 30S inhibitory antibiotics (Figure 9, Table S1). Tetracycline, oxytetracycline and spectinomycin resistant strains were selected after 13-18, 9-15 and 2-3 passages, respectively. Resistance to spectinomycin evolved more rapidly in the isolates than to tetracyclines. All mutants kept their high-level resistance after serial passages in antibiotic-free medium to the applied antibiotic with the exception of tetracycline resistant PG45 mutant (MIC to tetracycline decreased to 8 µg/ml). Cross-resistance between the examined tetracyclines was observed in the mutants. A fourfold increase in MICs to spectinomycin was noticed in tetracycline selected mutants in comparison to the parental isolates (16 µg/ml vs 4 µg/ml). The same results were obtained with spectinomycin selected mutants, which had a fourfold increase in their MICs to tetracycline (from 0.25 µg/ml to 1 µg/ml) (Table S2).

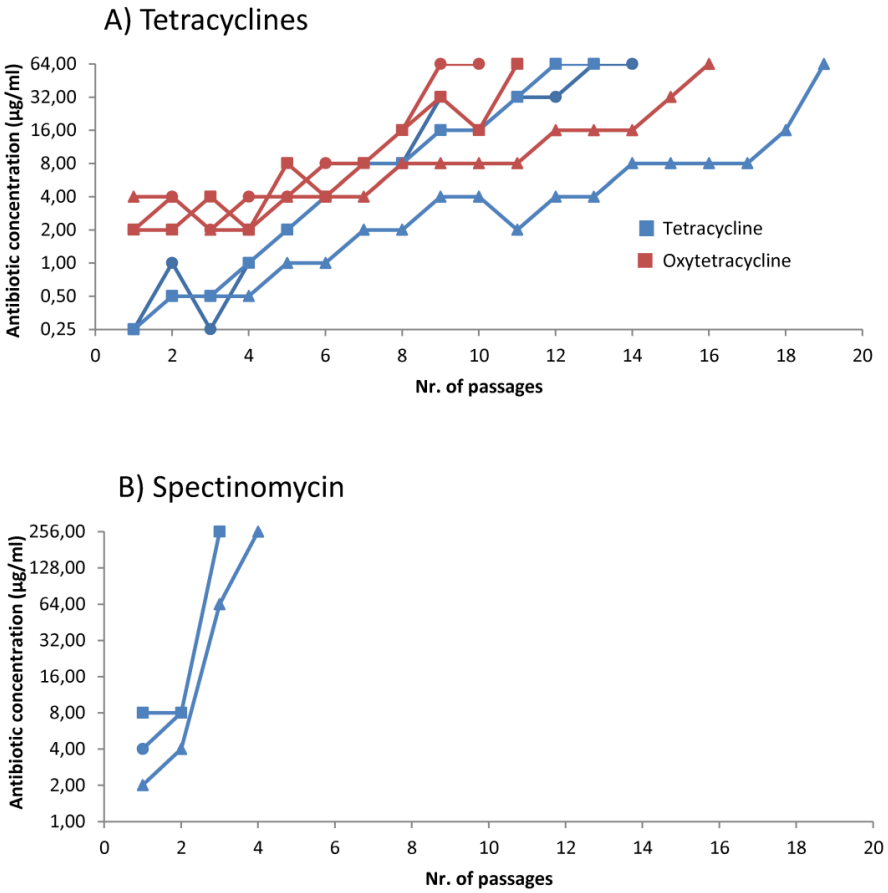

Figure 9. Development of resistance to tetracyclines and spectinomycin in *in vitro* selected mutant strains.

Antibiotic concentrations used during the *in vitro* selection of resistant *M. bovis* isolates plotted versus the number of passages. Symbols mark the parental isolates: triangle: *M. bovis* reference strain (PG45, NCTC 10131), circle: MYC52, square: MYC53.

Mutations related to increased MICs to tetracyclines and spectinomycin were discovered in *rrs1* and *rrs2* genes encoding the 16S rRNA (Table 12).

Mutations A965T and A967T in both *rrs1* and *rrs2* genes were identified in all field isolates with MICs of ≥16 µg/ml and ≥4 µg/ml (33/35 isolates) to oxytetracycline and tetracycline, respectively. In tetracycline resistant mutant strains (with the exception of PG45) mutations were observed at positions 962-967, 1058, 1195, 1196 of the Tet-1 tetracycline binding site located in the 30S ribosomal subunit.

The following SNPs located in the 16S rRNA region not closely associated with Tet-1 or other tetracycline binding sites were also found in all field isolates and *in vitro* selected mutants with elevated MICs to tetracyclines: G1012A, A1153G and A1268G in *rrs2* allele. The same substitutions were identified in the *rrs1* allele of tylosin resistant MYC52 and PG45 strains as well. No *tet*(M) determinants and derivatives have been identified in the genome of the examined isolates.

Table 12. Mutations detected in *rrs1* and *rrs2* genes of *M. bovis* field isolates and *in vitro* selected mutants with high MICs to tetracyclines or spectinomycin.

Strain	Mutations associated with high MICs in the current and/or previous studies									Mutations with unknown effect				
	gene[a]	*rrs1-rrs2*								*rrs1-rrs2*				
	NT[b]	962-964	965	966	967	1058	1192	1195	1196	1199	335	859	1012	1153	1268
PG45		TGA-TGA	A-A[c]	G-G[c]	A-A[c]	G-G[c]	C-C[d]	C-C	A-A	T-T	C-C	C-C	A-G	G-A	G-A
MYC52-53															
MYC2,42,65-76			T-T		T-T						T-C		A-A	G-G	G-G
MYC80			T-T		T-T		A-A						A-A	G-G	G-G
MYC30,44-47,77,82-83			T-T		T-T		A-A			C-T			A-A	G-G	G-G
MYC22,43,48-51,78-79,81,84			T-T		T-T		A-A			C-C			A-A	G-G	G-G
MYC82												C-A	A-A	G-G	G-G
$MYC52_{tetracycline}$			T-T		T-T	C-C									
$MYC53_{tetracycline}$			T-T					CC-CC	T-T						
$PG45_{tetracycline}$															
$MYC52_{oxytetracycline}$		del-del													
$MYC53_{oxytetracycline}$			T-T	T-T				T-T							
$PG45_{oxytetracycline}$				T-T				T-T							
$MYC52_{spectinomycin}$							T-C								
$MYC53_{spectinomycin}$							T-C								
$PG45_{spectinomycin}$							T-C								

[a] both SNPs of *rrs*1 and *rrs*2 genes are given at the certain nucleotide positions, respectively; [b] *E. coli* nucleotide numbering; [c] Altered positions related to high MICs to tetracyclines already described in *M. bovis* isolates (Amram et al. 2015) or in other *Mycoplasma* species (Dégrange et al. 2008b); [d] Altered positions related to high MICs to spectinomycin (Schnee et al. 2014) already described in *M. bovis.*

PG45: *M. bovis* reference strain NCTC 10131 (MIC 0.25 µg/ml to tetracycline, 2 µg/ml to oxytetracycline; 4 µg/ml to spectinomycin);

MYC52-53: MIC 0.25 µg/ml to tetracycline, 2 µg/ml to oxytetracycline; 4 µg/ml to spectinomycin;

MYC2,42,65-76: MIC ≥4 µg/ml to tetracycline, MIC ≥16 µg/ml to oxytetracycline, MIC≤ 4 µg/ml to spectinomycin;

MYC22,30,43-51,77-84: MIC ≥4 µg/ml to tetracycline, MIC ≥16 µg/ml to oxytetracycline and MIC >256 µg/ml to spectinomycin;

Abbreviation of antibiotic resistant mutants selected *in vitro*: $Strain_{drug\ used\ for\ selection}$

All field isolates with high MICs to spectinomycin (≥256 µg/ml; 19/35 isolates) as well as *in vitro* selected mutants harboured a single mutation at position 1192 of *rrs1* gene: C to A in field isolates and C to T in mutant strains, respectively. It should be noted that the substitution C335T was also identified within the *rrs1* and *rrs2* alleles of field isolates with MIC ≤4 µg/ml to spectinomycin but not in the reference strain PG45 (Table 12).
Genbank accession numbers of sequences related to 30S inhibitor resistance are presented here: KX462352-KX462395 for *rrs1* and KX462396-KX462439 for *rrs2* genes.

5.3.3. 50S inhibitors: macrolides, lincosamides, phenicols and pleuromutilins

Resistant mutants to tylosin and tilmicosin were obtained after 3-6 and 2-5 passages, respectively and remained stable after passages without the selector antibiotics (Figure 10A, Table S1). Cross-resistance between tylosin and tilmicosin was observed in all macrolide resistant mutants. At least eightfold increase to florfenicol (from 4 µg/ml to >32 µg/ml) were detected in two cases (tylosin resistant mutants MYC53 and PG45) and highly elevated MICs to lincomycin (from 0.5-1 µg/ml to >64 µg/ml) and tiamulin (from 0.078-0.156 µg/ml to 0.625-1.25 µg/ml, respectively) were observed in the cases of the tilmicosin resistant MYC52 and MYC53 mutants.
Mutations of *rrl1* and *rrl2* genes detected in *M. bovis* isolates with elevated MICs to 50S inhibitors are listed in Table 13. In field isolates (14/35 isolates) with MICs ≥128 µg/ml to tilmicosin and 8-32 µg/ml to tylosin substitutions A534T, T1248C, C1371T in *rrl2* and substitution G748A in both *rrl1* and *rrl2* were detected. In the Hungarian isolates with high MICs to tylosin (≥128 µg/ml; 33/35 isolates) the additional substitutions G954A in *rrl1* and A2059G in both genes were observed. In macrolide resistant mutants (MICs ≥128 µg/ml to tylosin and tilmicosin) the following mutations were found: A534T, G748A, G748C, C1371T, A2059G, A2059T, A2063T, C2067T and G2823A as well as insertion GTG after nucleotide 753 (Table 13).
No mutations associated with high MICs to macrolides were found in field isolates in genes encoding ribosomal proteins L4 and L22. Only tylosin resistant MYC52 harboured a substitution C191T (according to *M. bovis* PG45 numbering) causing alteration Ala64Val of L4 protein (according to PG45 numbering).

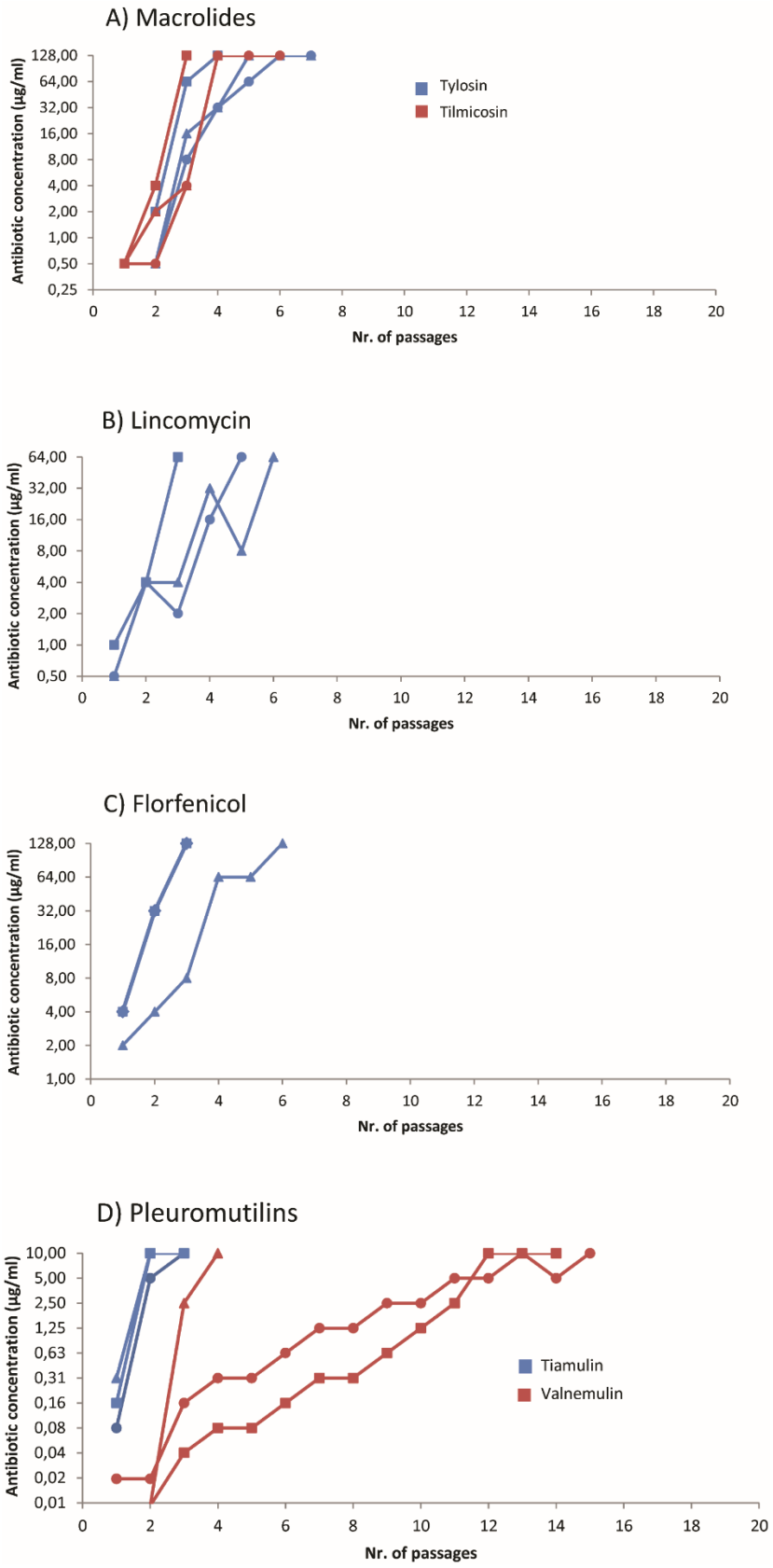

Figure 10. Development of resistance to 50S inhibitors in *in vitro* selected mutant strains. Antibiotic concentrations used during the *in vitro* selection of resistant *M. bovis* isolates are plotted versus the number of passages. Symbols mark the parental isolates: triangle: *M. bovis* reference strain (PG45, NCTC 10131), circle: MYC52, square: MYC53.

Lincomycin resistant *M. bovis* mutant strains were obtained after 2-5 passages (Figure 10B, Table S1). Only lincomycin resistant PG45 mutant decreased its level of resistance from MIC >64 µg/ml to 16 µg/ml after serial passages in antibiotic-free medium. Lincomycin resistant mutant strains (with the exception of the lincomycin resistant PG45 strain) showed highly increased MICs to tylosin (from 0.5 µg/ml to >128 µg/ml), tilmicosin (from 0.5 µg/ml to >128 µg/ml) and tiamulin (from 0.078-0.0156 µg/ml to 1.25-5 µg/ml). In one case (MYC53) slightly elevated MIC of valnemulin (from <0.039 to 0.039 µg/ml) was also observed.
All field isolates with MICs ≥64 µg/ml to lincomycin harboured mutations A2059G in both *rrl1* and *rrl2* alleles and only MYC53 mutant harboured this alteration in one *rrl1* gene (Table 13). Both lincomycin resistant MYC52 and PG45 mutants possessed the substitution A2060G in *rrl1* gene and an additional SNP (C2612T) in MYC52 was also found. Mutants, which showed high level cross-resistance to lincomycin (≥64 µg/ml) also had a substitution at position either 2059 or 2060 of *rrl1* confirming the role of these nucleotides in lincomycin resistance: A2059G in tilmicosin resistant MYC52, A2059T in tilmicosin resistant MYC53 and A2060G in valnemulin resistant MYC53 (Table 13).

Stable, florfenicol resistant mutant strains (>32 µg/ml) were selected after 2-5 passages (Figure 10C, Table S1). In florfenicol resistant PG45 notable changes in the MICs of tylosin (from ≤0.5 to ≥128 µg/ml) and tilmicosin (from ≤0.5 to 16 µg/ml) and twofold change in the MIC of lincomycin (from 1 to 2 µg/ml) were found. In contrast, in florfenicol resistant MYC52 and MYC53 strains only a slight increase in the MICs of tylosin (from ≤0.5 to 2 and 4 µg/ml) and tilmicosin (from ≤0.5 to 8 µg/ml) was observed, but significant increase of MICs of lincomycin (from ≤0.5 and 1 to 8 µg/ml), tiamulin (from 0.078 and 0.156 to 0.625 and 10 µg/ml) and valnemulin (from ≤0.039 to 0.039 and 10 µg/ml) was identified.
Eleven laboratory selected mutants were described with high MICs to florfenicol (≥16 µg/ml), among them, seven isolates harboured mutation G2062T or A2063T in at least one allele of the 23S rRNA gene (Table 13, Table S1). In addition, the florfenicol resistant MYC52 mutant in which the substitution G2506A was described showed a cross-resistance to tiamulin.

Table 13. Mutations detected in *rrl1* and *rrl2* genes of *M. bovis* field isolates and *in vitro* selected mutants with high MICs to macrolides, lincomycin, florfenicol or pleuromutilins.

Strain	Mutations associated with high MICs in the current and/or previous studies													Mutations with unknown effect							
	gene[a]	*rrl1-rrl2*													*rrl1-rrl2*							
	NT[b]	748	752	2035	2059	2060	2062	2063	2067	2448	2500	2506	2611	2612	171	316	534	538	954	1248	1371	2823
PG45		G-G[c,d]	- -	C-C[e]	A-A[c,d]	A-A	G-G[d]	A-A[d]	C-C	G-G[e]	C-C[e]	G-G[e]	C-C[d]	C-C	C-C	C-C	A-A	A-A	G-G	T-T	T-C	G-G
MYC52-53																	T-A					
MYC2,42,66-76		A-A															T-T			T-C	T-T	
MYC65		A-A														T-C	T-T			T-C	T-T	
MYC22,30,43-51,77,79,81-84		A-A			G-G												T-T		A-G	T-C	T-T	
MYC80		A-A			G-G												T-T	G-A	A-G	T-C	T-T	
$MYC52_{tylosin}$		A-A	GTG-GTG														T-T				T-T	
$MYC53_{tylosin}$								T-A									T-A					
$PG45_{tylosin}$								T-T	T-T								T-T				T-T	
$MYC52_{tilmicosin}$		C-G			G-A												T-A					
$MYC53_{tilmicosin}$					T-A												T-A					
$PG45_{tilmicosin}$		A-G															T-A					A-G
$MYC52_{lincomycin}$						G-A								T-C			T-A					
$MYC53_{lincomycin}$					T-A												T-A					
$PG45_{lincomycin}$						G-A											T-A					
$MYC52_{florfenicol}$							T-G										T-A					
$MYC53_{florfenicol}$												A-G					T-A					
$PG45_{florfenicol}$								T-A									T-A					
$MYC52_{tiamulin}$							T-G										T-A					
$MYC53_{tiamulin}$										A-G							T-A					
$PG45_{tiamulin}$							T-G										T-A					
$MYC52_{valnemulin}$																	T-T					
$MYC53_{valnemulin}$				A-C		G-A					A-C		T-C			C-T	T-A					
$PG45_{valnemulin}$							T-G								C-T		T-A					

[a] both SNPs of *rrl*1 and *rrl*2 genes are given at the certain nucleotide positions, respectively; [b] *E. coli* nucleotide numbering; [c] Altered positions related to high MICs to macrolides in *M. bovis* (Lerner et al. 2014); [d] positions related to high MICs to macrolides and lincomycin in other *Mycoplasma* species (Matsuoka et al. 2004, Pereyre et al. 2004, Kobayashi et al. 2005, Hong et al. 2013, Lysnyansky et al. 2015). [e] Neighbouring mutated positions already described in *M. gallisepticum* (Li et al. 2010) and in other bacteria species related to phenicol and pleuromutilin resistance (Long et al. 2009, Hidalgo et al. 2011);

PG45: *M. bovis* reference strain NCTC 10131 (MIC 0.5 µg/ml to tylosin and tilmicosin, and 1 µg/ml to lincomycin);

MYC52-53: MIC 0.5 µg/ml to tylosin and tilmicosin, ≤1 µg/ml to lincomycin;

MYC2,42,65-76: MIC ≥128 µg/ml to tilmicosin, ≤32 µg/ml to tylosin and ≤2 µg/ml to lincomycin;

MYC22,30,43-51,77-84: MIC ≥128 µg/ml to tylosin and tilmicosin and ≥64 µg/ml to lincomycin;

Abbreviation of antibiotic resistant mutants selected *in vitro*: $Strain_{drug\ used\ for\ selection}$

Tiamulin and valnemulin resistant mutant strains were successfully obtained after 2-3 and 3-14 passages (Figure 10D, Table S1). All tiamulin resistant mutant strains showed cross-resistance to florfenicol (>32 µg/ml) and elevated MICs to lincomycin (4-16 µg/ml). Tiamulin resistant PG45 became resistant to all tested 50S inhibitors with the exception of tylosin. Evolvement of valnemulin resistance strongly differed among the isolates: PG45 became resistant after 3 passages, while MYC52 and MYC53 needed 14 and 10 passages, respectively. After five passages on antibiotic-free medium, the MIC value (0.078 µg/ml) of valnemulin of the mutant MYC52 decreased. Highly elevated MICs to all tested 50S inhibitors developed in valnemulin resistant MYC53 mutant.

In tiamulin and valnemulin resistant mutant strains the substitutions C2035A, A2060G, G2062T and C2500A were found, which positions are closely associated with the pleuromutilin binding sites of the 23S rRNA genes. Other mutations were observed in a region not related to the antibiotic binding pocket (C171T and C316A). No chromosomal mutations of *rrl* genes were detected in tiamulin resistant MYC53 and valnemulin resistant MYC52 isolates.

The C/T mutation was observed in the neighbouring positions 2611 and 2612 in valnemulin resistant MYC53 and in lincomycin resistant MYC52 mutant strains, respectively (Table 13).

Sequences related to 50S inhibitor resistance were deposited to GenBank with the following accession numbers: KX462246-KX462298 for *rrl1*, KX462299-KX462351 for *rrl2.*

5.4. Rapid detection of antibiotic susceptibility of *Mycoplasma bovis*

5.4.1. Validation

Genotype assignment by HRM (with restrictions listed in chapters 5.4.2-5.4.4.) was concordant with results of MAMAs and IonTorrent sequencing for all DNA isolated from the purified cultures of *M. bovis* field isolates (n=35) and laboratory derived mutants (n=36) and the data were in accordance with the MIC values determined earlier by conventional broth microdilution method (Tables 11-13, Tables S3-S4). Melting temperatures and sizes of amplicons are listed in Table 14 for MAMA and Table 15 for HRM analysis. All HRM assays, melt- and agarose-MAMAs clearly differentiated the sensitive and resistant genotypes (Figures 11-15). Cross-reactions tested with other bovine mycoplasmas are listed in Tables 14-15.

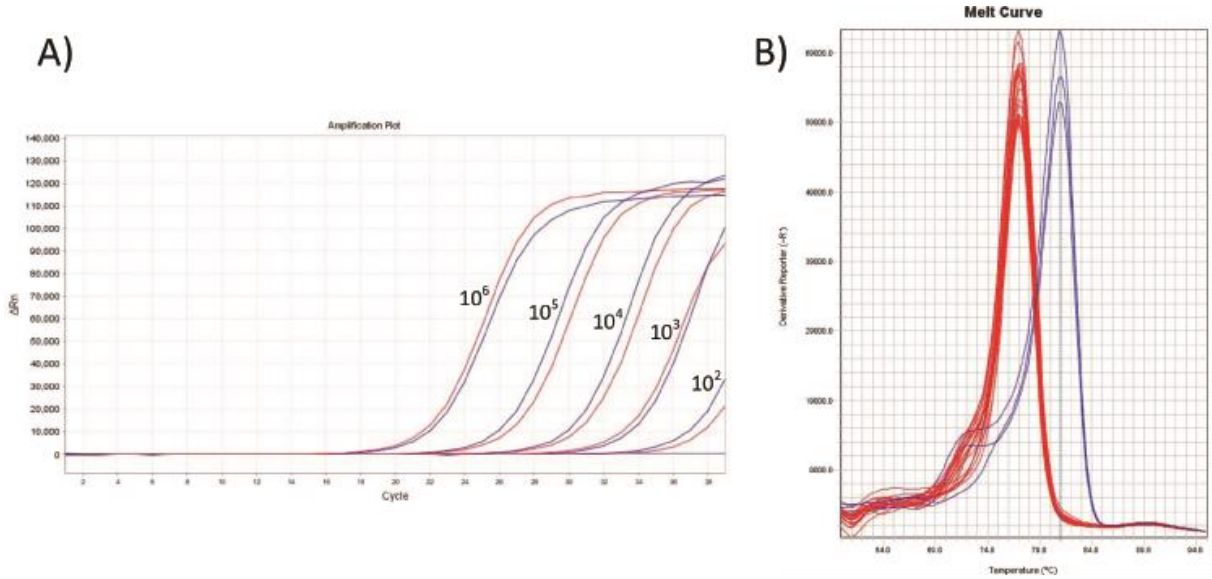

Figure 11. Amplification plot and melting curves of melt-MAMA (*parC*-G239T substitution).
A) Amplification plot of dilution series of *M. bovis* reference strain (PG45, NCTC10131) (red line) and a fluoroquinolone resistant field isolate MYC44 (blue line) showing the sensitivity of the assay.
B) Melting curves show melting temperatures of fluoroquinolone sensitive isolates (T_m=77.0°C) and resistant isolates (T_m=80.7°C).

For further evaluation of the assays, genotype assessments of clinical samples (n=30) were compared with genotype call and MIC values of the *M. bovis* isolates (n=20) originated from *M. bovis* positive samples (Table 2 and Table S4-S6). In the case of clinical sample BM272 the species specific PCR (Subramaniam et al. 1998) detected *M. bovis* DNA; however, the isolation failed due to the autolysed condition of the specimen. The clinical sample BM357 was the only nasal swab included in the present study. Although the DNA of *M. bovis* was

not detected with conventional PCR method (Subramaniam et al. 1998), the bacterium was isolated from the sample, and the repeated DNA detection from the clinical sample with the presented real-time PCR assays of higher sensitivity (eg. MAMA rrl1/2-G748A) was successful (Table S3-S6).

The reproducibility of genotype calls of all molecular assays designed in the present study was 100.0% tested in both intra- and inter-run settings. In the independent runs slight shifts in the melting temperatures were detected during the melt curve analysis of PCR amplicons; however, the melt curve shapes and temperature differences remained unchanged.

All samples reached a plateau before the end of the real-time PCR amplification stage. Overshot amplification curves were excluded from further analysis and the run was repeated with the diluted DNA. When the shape of the melt curve of the clinical isolates was bimodal the result was interpreted to be ambiguous (Figure S1), as bimodal melt curve suggests the presence of more than one amplicon.

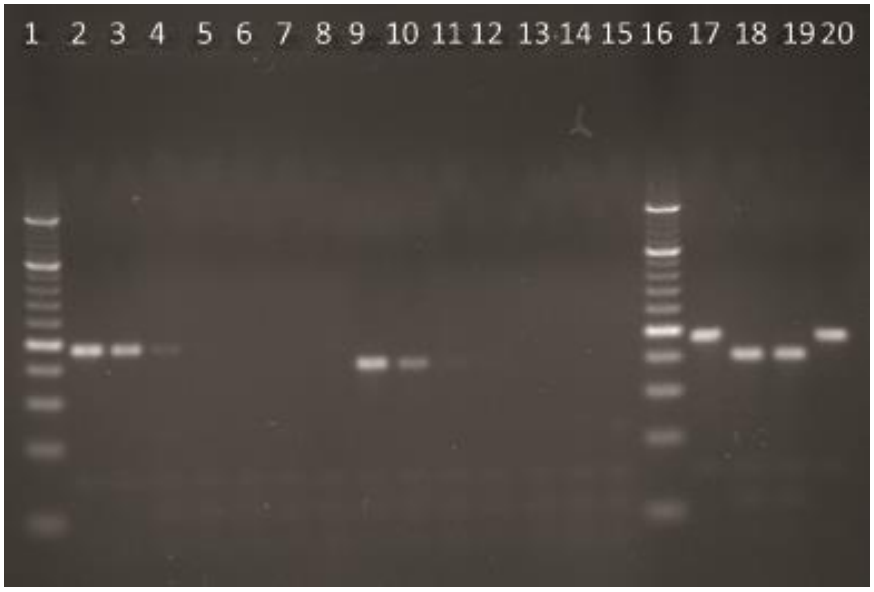

Figure 12. PCR product sizes of agarose MAMA for the detection of G239T of *parC* gene.
Electrophoresis was performed in 3% agarose gel (MetaPhor Agarose, Lonza Group Ltd.). Lanes 1 and 16: 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific Inc.); Lanes 2-8: serial dilution (10^6-10^0 copy number/µl) of fluororoquinolone resistant MYC44; Lanes 9-15 serial dilution (10^6-10^0 copy number/µl) of fluoroquinolone sensitive *M. bovis* reference strain (PG45, NCTC10131); Lane 17: resistant isolate MYC44 (95 bp); Lane 18: sensitive strain PG45 (81 bp); Lane 19: sensitive isolate MYC80 (81 bp); Lane 20: resistant isolate MYC46 (95 bp).

Table 14. Results of MAMAs designed in this study.

Gene	SNP	Antibiotic	Genotype[a]	T_m (°C)	Amplicon length (bp)	NTC	Sensitivity (copy number/reaction)[b]	Cross-reaction[b]	False result rate[b]	
									isolates	clinical samples
*gyr*A	C248T	FQs	R S	81.4 76.8	88 74	-	10^3 10^3	-	0% (0/64)	10.0% (3/30)
*par*C	G239T	FQs	R S	80.7 77.0	95 81	-	10^2 10^2	-	0% (0/64)	13.3% (4/30)
*par*C	G250A	FQs	R S	74.4 80.7	53 68	Ct 36 T_m 71.1°C	10^2 10^4	-	0% (0/64)	6.7% (2/30)
rrs1/*rrs*2	A965T	TETs	R S	81.0 77.6	82 68	Ct 33 T_m 74.1°C	10^3 10^2	*M. agalactiae, M. alvi, M. bovigenitalum, M. bovirhinis, M. californium, M. dispar, M. verecundum*	0% (0/64)	23.3% (7/30)
*rrs*1/*rrs*2	A967T	TETs	R S	85.0 80.4	96 81	Ct 34 T_m 82.6°C	10^2 10^3	*M. agalactiae, M. alkalescens, M. alvi, M. arginini, M. bovirhinis, M. canadense, M. verecundum*	0% (0/64)	6.7% (2/30)
*rrs*1/*rrs*2	G1058C	TETs	R S	84.2 81.7	85 70	Ct 35 T_m 76.8°C	10^3 10^2	*M. agalactiae, M. alvi, M. arginini, M. bovigenitalum, M. bovirhinis, M. californium, M. canadense, M. dispar, M. verecundum*	0% (0/64)	20.0% (6/30)
*rrs*1/*rrs*2	C1192A	Sc	R S	83.5 79.3	85 70	Ct 29 T_m 75.5°C	10^5 10^5	*M. agalactiae, M. alvi, M. bovirhinis, M. californium*	0% (0/64)	46.7% (14/30)
*rrl*1/*rrl*2	G748A	MACs	R S	83.3 78.9	78 63	-	10^2 10^2	*M. agalactiae, M. verecundum*	0% (0/73)	0% (0/30)
*rrl*1/*rrl*2	A2059G	MACs/L	R S	84.0 81.1	80 65	Ct 32 T_m 82.0°C	10^4 10^3	*M. agalaciaet, M. alvi, M. bovirhinis, M. californium, M. dispar, M. verecundum*	0% (0/73)	40.0% (12/30)

[a] Genotypes "R" (resistant) and "S" (sensitive) are named after the primers. [b] Values refer to Melt-MAMAs. Abbreviations: T_m= melting temperature; SNP= single nucleotide polymorphism; NTC=negative control; FQs=fluoroquinolones; TETs=tetracyclines; Sc=spectinomycin; MACs=macrolides; L=lincomycin; Ct=cyclic threshold.

Table 15. Results of HRM assays designed in this study.

Assay name	Antibiotic	Genotypes obtained by HRM (mutations and T_m [°C])					Cross-reaction	Sensitivity (copy number/reaction)[a]	False result rate	
		S_{wt}	R_F	R_{M1}	R_{M2}	R_{M3}			isolates	clinical samples
gyrA	FQs	- 77.26 CI95:77.23-77.29	C248T 76.93 CI95:76.89-76.97	G244A + G259A 75.90 CI95:75.89-75.90	G244A / C248A / G259A 76.59 CI95:76.54-76.64	A260G 77.70 CI95:77.69-77.70	*M. bovirhinis, M. bovigenitalium*	10^3	0% (0/64)	16.7% (5/30)
rrs1/2-A	TETs	- 79.08 CI95:79.05-79.11	A965T + A967T 79.24 CI95:79.21-79.26	A965T + G966T / G966T 78.6 CI95:78.54-78.61	TGA deletion 78.80 CI95:78.79-78.01	-	*M. agalactiae, M. bovigenitalium, M. californicum*	10^2	0% (0/64)	20.0% (6/30)
rrs1/2-B	TETs, Sc	- 83.80 CI95:83.78-83.82	C1192A / C1192T / C1195T / C1195CC + A1196T 83.43 CI95:83.40-83.46	-	-	-	*M. agalactiae, M. alkalescens, M. alvi, M. arginini, M. bovirhinis, M. californium, M. canadense, M. dispar, M. verecundum*	10^2	0% (0/64)	23.3% (7/30)
rrl1/2-A	MACs	- 82.14 CI95:82.11-82.17	G748A 81.75 CI95:81.73-81.77	GTG insertion 82.0 CI95:81.99-82.00	-	-	*M. agalactiae* *M. alvi*	10^2	0% (0/73)	3.3% (1/30)
rrl1/2-B	50S inhibitors	- 80.85 CI95:80.80-80.90	A2059G / A2060G 81.19 CI95:81.14-81.24	A2063T + C2067T / G2062T 80.4 CI95:80.34-80.45		-	*M. agalactiae, M. alvi, M. bovirhinis, M. californium, M. dispar, M. verecundum*	10^2	0% (0/73)	20.0% (6/30)
rrl1/2-C	50S inhibitors	- 81.79 CI95: 81.76-81.82	-	C2500A / G2506A 81.23 CI95:81.18-81.30	-	-	*M. alvi, M. bovirhinis, M. californium, M. canadense, M. verecundum*	10^3	0% (0/73)	6.7% (2/30)
rrl1/2-D	50S inhibitors	- 82.38 CI95:82.35-82.41	-	C2611T / C2612T 82.02 CI95:81.95-82.10	-	-	*M. agalactiae, M. arginini, M. bovigenitalium, M. canadense, M. dispar*	10^2	0% (0/73)	13.3% (4/30)

[a] Sensitivity of each genotype was identical within an assay. Abbreviations: FQs=fluoroquinolones; TETs=tetracyclines; Sc=spectinomycin; MACs=macrolides; T_m= melting temperature; S_{wt}: antibiotic sensitive wild type; R_F: genotypes of antibiotic resistant field isolates; R_{M1}-R_{M3}: genotypes of antibiotic resistant mutants selected *in vitro*.

5.4.2. Detection of fluoroquinolone resistance

For the detection of fluoroquinolone resistance three MAMAs (gyrA-C248T; parC-G239T; parC-G250A) and one HRM system (gyrA-213-286) were designed (Tables 14-15). Due to silent mutations (T234C and C252T) the region 232-250 of *parC* was inadequate for designing HRM assays. The presented MAMA and HRM assays are able to identify 8 out of the 10 tested key substitutions (5/5 in *gyrA* and 3/5 in *parC* gene) associated with high MICs to fluoroquinolones (Table 11). All four SNPs found in *M. bovis* field strains with increased MICs to fluoroquinolones are distinguishable with the described assays, and the assays MAMA-gyrA-C248T and MAMA-parC-G250A are able to detect the substitutions C248A of *gyrA* and G250T of *parC* (mutations found in the mutant strains only) as well (Table S4).

The HRM assay of the nucleotide region 213-286 of *gyrA* gene discriminated the following genotypes harbouring the listed mutations: S_{wt} (sensitive) – wild type; R_F (antibiotic resistant genotype developed under field conditions) – C248T; R_{M1} (mutant) – G244A and G259A; R_{M2} – G244A or C248A or G259A; and R_{M3} – A260G (Table 15 and Figure 13). Normalization interval of 71.6-72.0 and 80.6-81.0°C was used in the analysis of the amplicons.

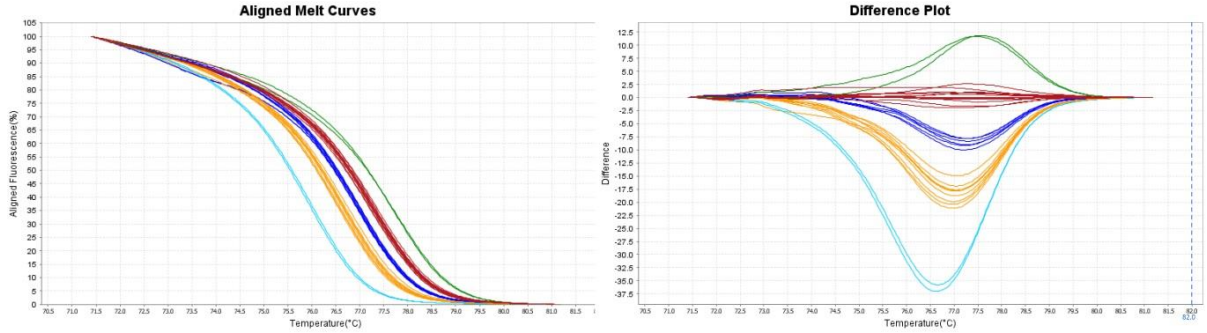

Figure 13. HRM analyses for the detection of fluoroquinolone resistance. Aligned melt curves and difference plots of the assay gyrA (nucleotide region 213-286 of *gyrA*).

Red: S_{wt} T_m=77.26°C; Dark blue: R_F T_m=76.93°C, C248T; Light blue: R_{M1} T_m=75.90°C, G244A + G259A; Orange: R_{M2} T_m=76.59°C, G244A / C248A / G259A; Green: R_{M3} T_m=77.70°C, A260G.

Abbreviations: S_{wt}: antibiotic sensitive genotypes, wild type; R_F: genotype of antibiotic resistant field isolates; R_{M1-M3}: genotypes of antibiotic resistant mutants selected *in vitro*.

The sensitivity of real-time PCR based methods (melt-MAMA and HRM) was 10^2-10^4 copy numbers/reaction depending on the assay (Tables 14-15). The sensitivity of agarose-MAMAs showed a sensitivity of 10^3-10^5 copies/reaction.

Out of the 20 *M. bovis* isolates, three isolates (BM239, BM385 and BM388) achieved high MICs (≥5 µg/ml) to the tested fluoroquinolones and harboured mutations in both *gyrA* and *parC* genes, while two isolates (BM124 and BM274) with slightly elevated MICs (1.25 µg/ml to marbofloxacin and 0.625 µg/ml to danofloxacin and enrofloxacin) harboured mutations in

only *gyrA* (Table S4). False result rate of the assays which were performed on clinical samples varied between 6.7% and 16.7% (Tables 14-15).

5.4.3. Detection of resistance to 30S inhibitors

Three SNPs (A965T, A967T, G1058C) responsible for elevated MICs to tetracyclines and one (C1192A) for spectinomycin were targeted with MAMA assays. In addition, two regions (872-1000 for tetracycline and 1139-1251 for tetracycline and spectinomycin) of *rrs1*/*rrs2* genes were selected to design HRM assays for the detection of resistance to 30S inhibitors (Tables 14-15). All 4 substitutions of *M. bovis* field strains with high MICs to 30S inhibitors and 6 out of 7 mutations present in *in vitro* selected mutant strains (Table 12) can be indicated by the above listed MAMA and HRM assays (Table S5).

Genotypes differentiated by the rrs1/2-A HRM assay (nucleotide region 993-1005 of *rrs1*/*rrs2*) are as follows: S_{wt} – wild type; R_F – A965T and A967T; R_{M1} – G966T or A965T and G966T; and R_{M2} – with TGA deletion before nucleotide 965 (Figure 14A).

The HRM assay rrs1/2-B (nucleotide region 1139-1251 of *rrs1*/*rrs2*) designed to detect mutations associated with high MICs to spectinomycin (C1192A) and tetracycline (T1199C) discriminated two genotypes (S_{wt} and R_F). R_F genotype was detected in isolates with elevated MICs to spectinomycin or tetracyclines harbouring one of the following mutations: C1192A or C1192T or C1195T or C1195CC + A1196T (Figure 14B). However, the isolates with low MICs to spectinomycin developed under field conditions and also the isolates with MICs ≥256 µg/ml to spectinomycin which harbour both substitutions C1192A and T1199C were all designated genotype S_{wt}. Because of the thermodynamic differences the substitution C1192A and T1199C can only be detected by HRM assay when they occur independently. Therefore, the combined use of the MAMA assay identifying the substitution C1192A of the *rrs1*/*2* genes (rrs1/2-C1192A) and of the HRM assay specific for the nucleotide region 1139-1251 of the *rrs1*/*2* genes is needed to detect mutations associated with elevated MICs to spectinomycin or tetracyclines. Normalization intervals were adjusted to 73.4-73.8 and 82.4-82.6°C; 79.6-80.0 and 86.3-86.7°C for the analysis of rrs1/2-A and rrs1/2-B assays.

The sensitivity of melt-MAMA and HRM assays was 10^2-10^3 copy numbers/reaction, while agarose-MAMAs showed 10^3-10^5 copies/reaction sensitivity. MAMA rrs1/2-C1192A was the only exception, as the sensitivity of the assay was 10^5 copies/reaction either performed on real-time PCR platform or by agarose gel electrophoresis. False result rate of the assays using the DNA of clinical samples varied between 6.7% and 23.3% for tetracyclines and it was 53.3% for spectinomycin (MAMA rrs1/2-C1192A) (Tables 14-15).

All 20 *M. bovis* isolates originating from the 30 clinical samples had high MIC values to tetracyclines (≥4 µg/ml to tetracycline and 16 µg/ml to oxytetracycline) which were in

harmony with the results of the molecular methods (Table S5). All 20 isolates harboured the mutations A965T and A967T, 10 isolates harboured T1199C on at least one of the *rrs* genes and none of them harboured the substitution G1058C. Fifteen isolates showed high MICs to spectinomycin (≥256 µg/ml) determined by microbroth dilution test and all of them were identified as genotype R by the MAMA test rrs1/2-C1192A (Table S5).

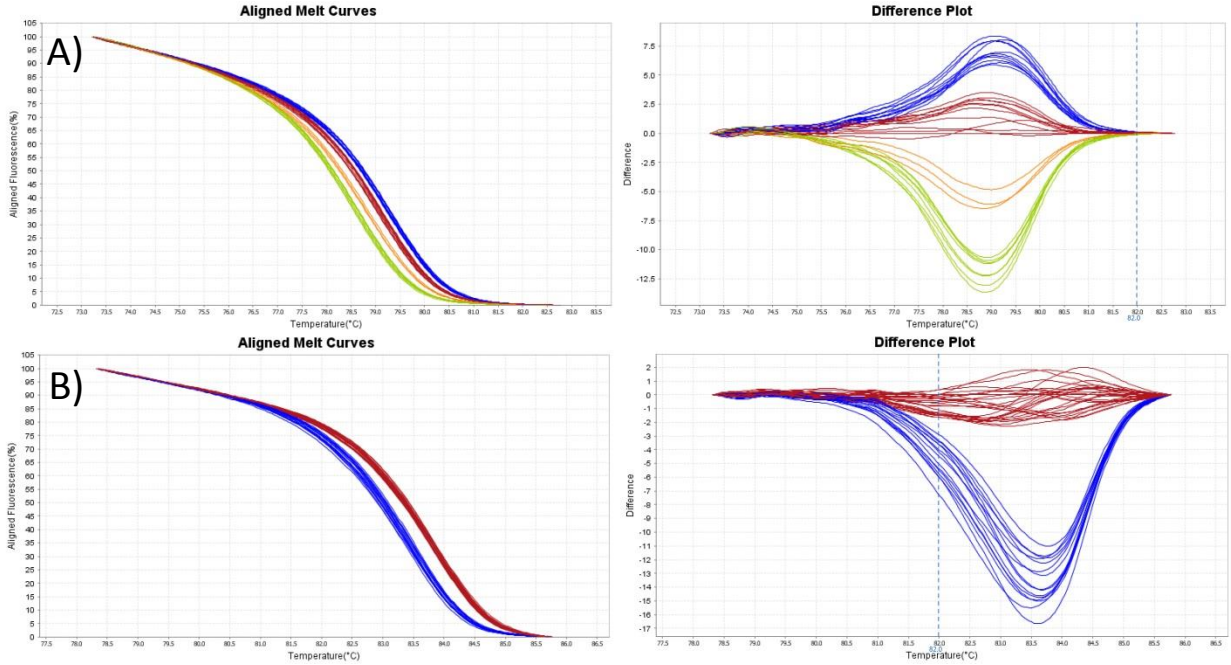

Figure 14. HRM analyses for the detection of resistance to 30S inhibitors.
Aligned melt curves and difference plots of:

A) assay rrs1/rrs2-A (872-1000 of *rrs1*/*rrs2*): Red: S_{wt}: T_m=79.08°C; Blue: R_F: T_m=79.24°C, A965T + A967T; C248T; Green: R_{M1} T_m=78.60°C, A965T+G966T / G966T; Orange: R_{M2} T_m=78.80°C, TGA deletion;

B) assay rrs1/rrs2-B (1139-1251 of *rrs1*/*rrs2*): Red: S_{wt}: T_m=83.80°C; Blue: R_F: T_m=83.43°C, C1192A / C1192T / C1195T / C1195CC+A1196T.

Abbreviations: S_{wt}: antibiotic sensitive genotypes, wild type; R_F: genotype of antibiotic resistant field isolates; R_{M1-M2}: genotypes of antibiotic resistant mutants selected *in vitro*.

5.4.4. Detection of resistance to 50S inhibitors

Two substitutions (G748A and A2059G) and two regions (672-778 and 2005-2101) of the 23S rRNA genes associated with elevated MICs to macrolides and lincomycin in *M. bovis* were targeted by MAMA and HRM systems. Two additional HRM systems (nucleotide regions 2473-2524 and 2557-2636 of *rrl1*/*rrl2* genes) were designed for the detection of SNPs found in mutant strains and related to elevated MICs to 50S inhibitors (Tables 14-15). The presented MAMA and HRM assays were able to detect 10 out of 13 mutations of the genes encoding 23S rRNA occurring in *M. bovis* (Table 13), including both SNPs

characteristic to strains with high MICs to 50S inhibitors developed under field conditions (Table S6).

Three genotypes could be distinguished by rrl1/2-A HRM assay (nucleotide region 672-778 of *rrl1*/*rrl2*): S_{wt} – wild type; R_F – G748A; and R_{M1} – with GTG insertion in this region. Only the mutant strain MYC52 with MIC ≥128 µg/ml to tilmicosin harbouring mutation G748C (T_m=82.08°C; CI95: 81.99-82.15°C) was undistinguishable from the wild type (T_m=82.14°C; CI95: 82.11-82.17°C) by this HRM system (Figure 15A).

The HRM assay rrl1/2-B (nucleotide region 2005-2101 of *rrl1*/*rrl2*) determined the following genotypes: S_{wt} – wild type, R_F – A2059G or A2060G; R_{M1} – A2063T and C2067T or G2062T. Strains with the double mutations at C2035A and A2060G could not be detected for their reverse effects on melting temperatures. The assay covering the nucleotide region 2005-2101 (HRM assay rrl1/2-B) is unable to discriminate uncommon double substitutions (e.g. C2035A and A2060G) or point mutations (e.g. A2059T or A2063T) detected in laboratory selected mutant strains due to the nucleotides thermodynamic characteristics. (Figure 15B).

The HRM assay rrl1/2-C (nucleotide region 2473-2524 of *rrl1*/*rrl2*) revealed two genotypes: S_{wt} – wild type; and R_{M1} – C2500A or G2506A (Figure 15C). Another two genotypes were obtained by the rrl1/2-D HRM (nucleotide region 2557-2636 of *rrl1*/*rrl2*): S_{wt} – wild type; and R_{M1} – C2611T or C2612T (Figure 15D).

Different normalization intervals 77.9-78.3 and 84.0-84.4°C, 74.7-75.1 and 84.3-84.7°C, 76.2-76.6 and 83.5-83.9°C and finally 77.3-77.6 and 84.4-84.8°C were used in the analysis of amplicons of rrl1/2-A, rrl1/2-B, rrl1/2-C and rrl1/2-D assays, respectively.

The sensitivity of melt-MAMA and HRM assays was 10^2-10^4 copy numbers/reaction, while the agarose-MAMAs showed 10^4 copies/reaction sensitivity. False result rate of the assays which were performed on clinical samples varied between 0% and 40.0% for 50S inhibitors (Tables 14-15).

All 20 isolates originating from the 30 clinical samples had elevated MICs to macrolides (≥32 µg/ml to tylosin and ≥128 µg/ml to tilmicosin) and genotypes R and R_F were detected with the rrl1/2-G748A MAMA and also with the HRM rrl1/2-A in all cases (Table S5). Fifteen isolates showed MIC ≥64 µg/ml to lincomycin with microbroth dilution method and were classified as genotype R with the MAMA assay targeting the point mutation A2059G of *rrl1/2* genes and with the rrl1/2B-HRM assay as well (Table S5).

As opposed to the mutant strains, none of the Hungarian field isolates harboured mutations in regions 2473-2524 and 2557-2636 of *rrl3*/*4* genes, which is in accordance with the results of microbroth dilution method (Table S5).

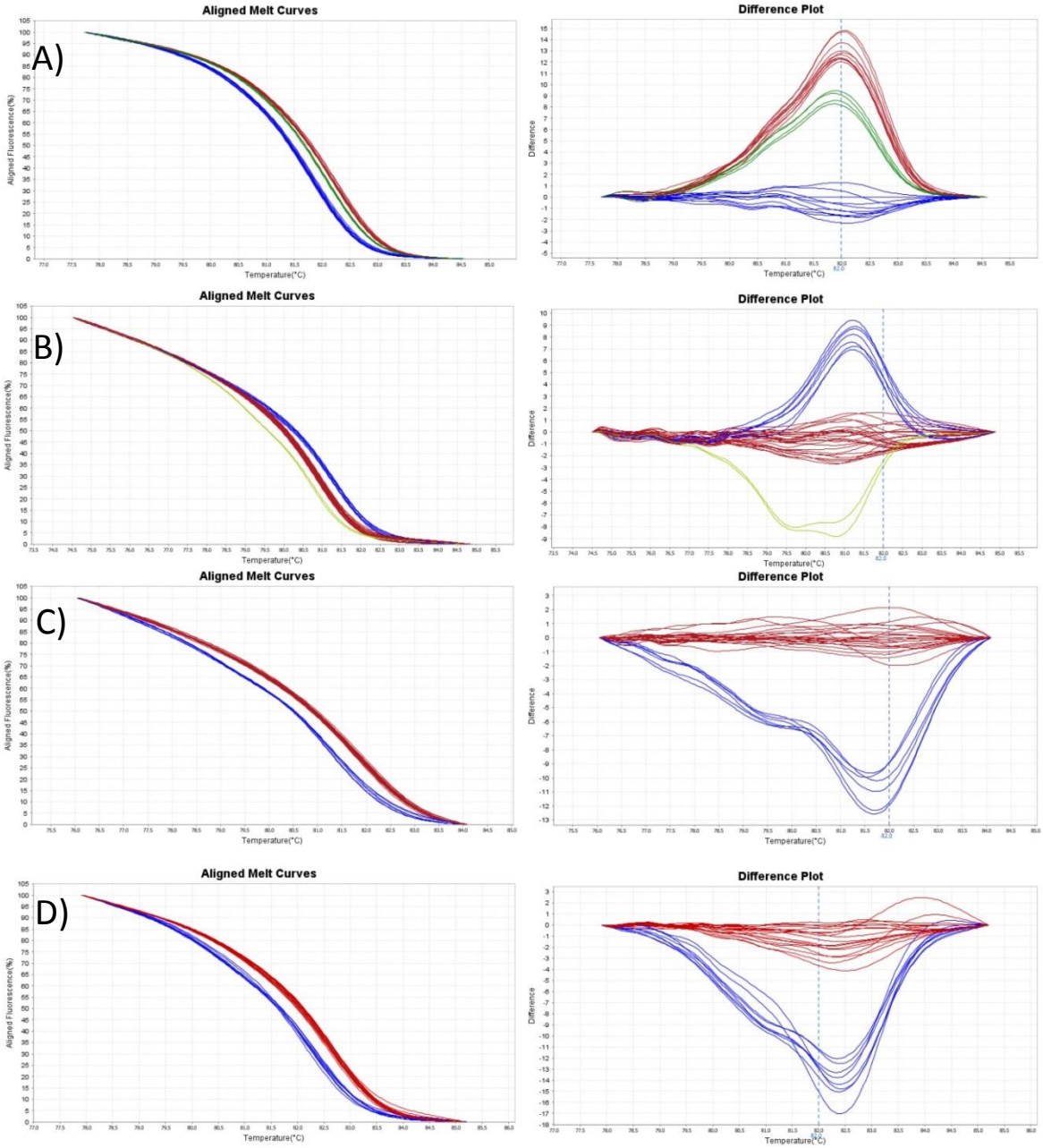

Figure 15. HRM analyses for the detection of resistance to 50S inhibitors.
Aligned melt curves and difference plots of:

A) assay rrl1/rrl2-A (672-778 of *rrl1*/*rrl2*): Red: S_{wt}: T_m=82.14°C; Blue: R_F: T_m=81.75°C, G748A; C248T; Green: R_{M1} T_m=82.0°C, GTG insertion;

B) assay rrl1/rrl2-B (2005-2101 of *rrl1*/*rrl2*): Red: S_{wt}: T_m=80.85°C; Blue: R_F: T_m=81.19°C, A2059G / A2060G; Green: R_{M1}: T_m=80.4°C, A2063T + C2067T / G2062T;

C) assay rrl1/rrl2-C (2473-2524 of *rrl1*/*rrl2*): Red: S_{wt}: T_m=81.79°C; Blue: R_{M1}: T_m=81.23°C, C2500A / G2506A;

D) assay rrl1/rrl2-D (2557-2636 of *rrl1*/*rrl2* genes): Red: S_{wt}: T_m=82.38°C; Blue: R_{M1}: T_m=82.02°C, C2611T / C2612T.

Abbreviations: S_{wt}: antibiotic sensitive genotypes, wild type; R_F: genotype of antibiotic resistant field isolates; R_{M1}: genotypes of antibiotic resistant mutants selected *in vitro*.

6. Discussion

6.1. Genetic diversity of the Hungarian *Mycoplasma bovis* isolates

M. bovis is primarily transmitted by direct contact and it is spread by the movement of infected animals; therefore efficient genotyping tools are essential for its monitoring, control and epidemiological investigation. MLST and MLVA of *M. bovis* had been developed and they proved to be useful in previous studies (Pinho et al. 2012; Manso-Silván et al. 2012; Spergser et al. 2013; Amram et al. 2013). The two methods differ considerably in their resolution. MLST is dedicated to discover the mid-term evolutionary events while MLVA is a suitable method to perform short-scale epidemiological studies. In the present work, we tested these methods on a *M. bovis* isolate collection isolated between 2010 and 2013 in Hungary in order to reveal the genetic structure of the Hungarian *M. bovis* population.

The MLST analyses distuingished 6 genetic groups among the 31 *M. bovis* isolates originating from 15 farms, showing genetic homology within the herds. Multiple genotypes within a herd were detected in only one case (Csengersima, MYC65-68). Occurrence of various genotypes in the same herd was probably due to the isolates' distinct origins (e.g. *M. bovis* might have been introduced to the herd by more than one animal of different origin). It is remarkable that the majority of the Hungarian isolates had different STs than the previously examined French, Belgian and German isolates which possessed the same sequence as the reference strain PG45 (Manso-Silván et al. 2012). However, it should be mentioned, that only one isolate per country was examined in that particular study. The high genetic variation detected among the Hungarian isolates is similar to the results of more recent examinations of total redesigned MLST schemes which were performed on large number of isolates originating from a wide range of geographic regions (Rosales et al. 2015; Register et al. 2015). The loss of diversity of French isolates has been reported as an exception from the generally described genetic heterogeneity of *M. bovis* due to the spread of a multiresistant clone (Becker et al. 2015). At least partially, the high genetic diversity of *M. bovis* observed in the Hungarian population is possibly due to the intensive national and international cattle trade.

The present study confirmed that MLVA has high discriminatory power and is able to distinguish closely related strains. This was best demonstrated during the typing of isolates from the herd from the town of Komárom (MYC69-76). These isolates formed one branch with three subgroups. Spergser et al. (2013) modified the MLVA typing scheme described by Pinho et al. (2012). With their modification Spergser et al. (2013) increased the

discriminatory power (Simpson's index of diversity) of the original MLVA system from 0.952 to 0.981 based on the analysis of their samples. In the present study, the samples were analysed according to the original Pinho et al (2012) system and resulted in a 0.970 Simpson's index of diversity. This value provides further proof for the high genetic diversity of the Hungarian *M. bovis* population. This finding is consistent with previous data published by Amram et al. (2013) who discovered eight different VNTR patterns among the eleven *M. bovis* strains isolated from calves imported to Israel from Hungary. Unfortunately, we were unable to compare our isolates with the strains analysed by MLVA in the previous studies (Pinho et al. 2012; Spergser et al. 2013; Amram et al. 2013), as the VNTR patterns of the individual isolates from these studies were not published. It would be worthwhile to establish an online database of *M. bovis* VNTR patterns or providing these data in the supplementary material of the publications, because it would enable the monitoring of the disease and to perform epidemiological investigations by allowing the comparison of isolates originating from different parts of the world. The latest study on the MLVA of *M. bovis* isolates was performed on French strains collected over the last 35 years using 4 MLVA loci (Becker et al. 2015). As the MLVA profiles of the limited 4 VNTR loci are available, these were compared with the Hungarian profiles in the present study. The comparison revealed similarities between the Hungarian isolates and one of the main cluster comprising French strains collected before 2000.

The availability of a large dataset should also improve the methods for data analysis. Although in our opinion MLST is a more appropriate method than MLVA for the genetic comparison of weakly related isolates (strains with distant geographic origin, e.g. on a country or continental level), it may also be possible to use the latter method for this purpose with certain modifications. As MLVA is primarily a fine scale typing tool (i.e. useful in following local epidemics), the different TRs are recommended to be weighted when the method is applied on weakly related isolates in order to avoid homoplasy (Al Dahouk et al. 2007). However, in order to establish an efficient TR weighting system, it is essential to have access to VNTR profile data of many isolates from different countries.

Our study has compared and evaluated two different molecular epidemiological typing techniques for *M. bovis* characterization. MLST is a robust and reproducible intermediate scale typing method which proved to be discriminating among the Hungarian *M. bovis* isolates. MLVA is an appropriate fine-scale typing tool for *M. bovis* strains, as it even allowed the differentiation of certain strains originating from the same farm. Based on these results the combined use of the two typing methods is recommended (Figure 16). The isolates first have to be typed by MLST and then the subset of strains sharing the same STs has to be further differentiated by MLVA. In this way information will be gained about both the mid-term evolutionary and the short-term epidemiological relationships of the analysed isolates.

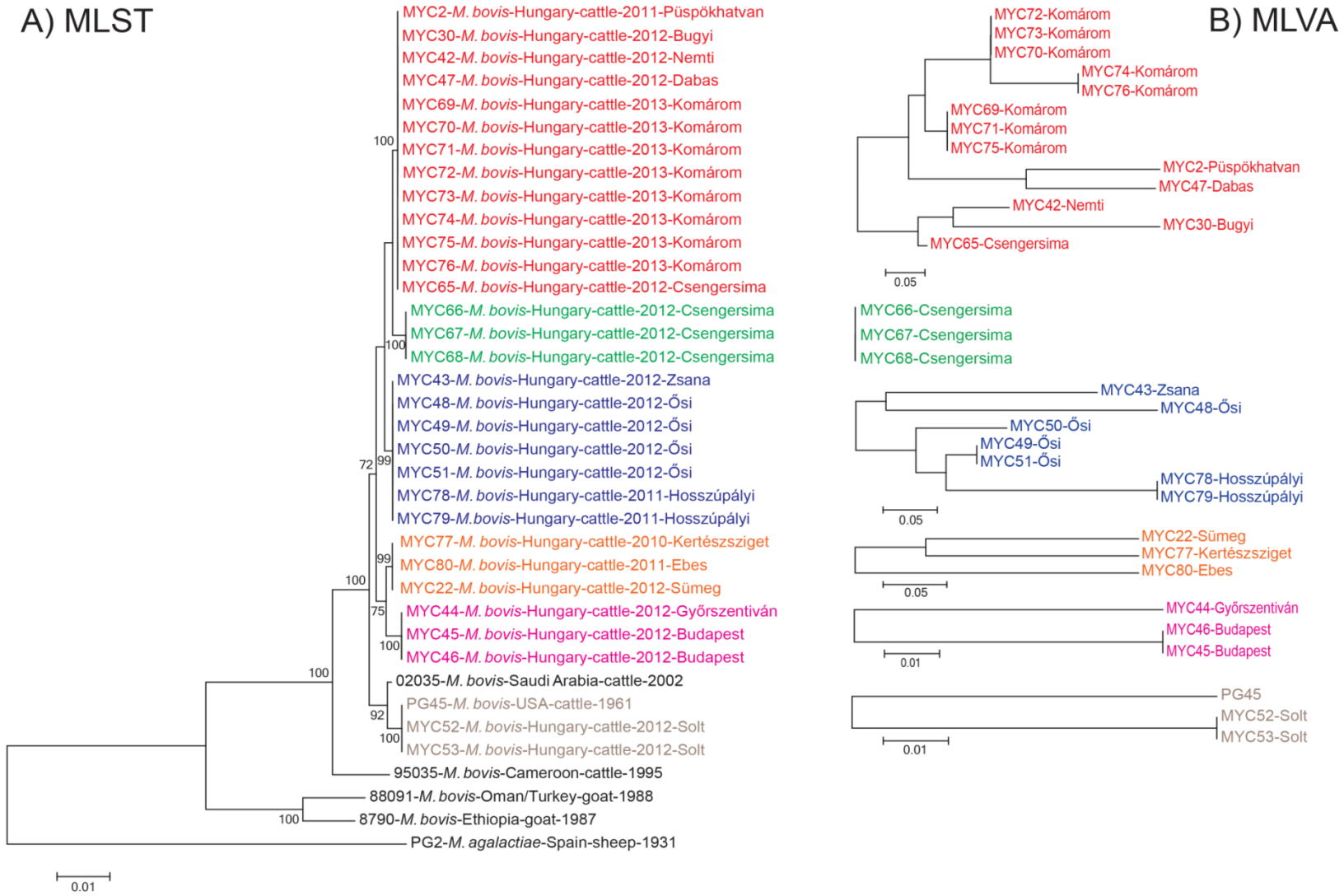

Figure 16. Genetic relationships between *M. bovis* isolates based on the combined use of MLST (A) and MLVA (B).
Dendrograms were constructed with neighbour-joining methods. Scale bars represent the average number of substitutions per site. Colours indicate the subclades determined by MLST.

6.2. Antibiotic susceptibility of the Hungarian *Mycoplasma bovis* isolates

Gerchman et al. (2009) studied 11 *M. bovis* strains isolated from cattle, which were imported from Hungary to Israel between 2005 and 2007. The most active compounds found during *in vitro* examinations were fluoroquinolones (danofloxacin, enrofloxacin and marbofloxacin), which is in accordance with the results of the present study. However, the MIC values described in the previous publication were higher than the ones detected in the current study (MIC_{90} 1.25 µg/ml, 1.25 µg/ml and 5 µg/ml versus 0.312 µg/ml, 0.312 µg/ml and 0.625 µg/ml). Decreased susceptibility to spectinomycin was detected in the strains originating from the imported animals (MIC_{90} >1024 µg/ml obtained with E-test method), which is consistent with our results (MIC_{90} ≥256 µg/ml). The MIC_{90} values of oxytetracycline (≥64 µg/ml), tilmicosin (≥128 µg/ml) and tylosin (≥128 µg/ml) yielded in the present study indicate limited susceptibility to these antibiotics, which is in contrast to the results obtained by Gerchman et al. (2009) (MIC_{90} values of 4 µg/ml, 8 µg/ml and 1 µg/ml, respectively). The comparison of the results of the previous publication and the present study emphasizes the importance of the systematic monitoring of antibiotic susceptibility of *M. bovis* strains in the region (Gerchman et al. 2009, Gautier-Bouchardon et al. 2014).

Fluoroquinolones inhibited the growth of the majority of the Hungarian *M. bovis* isolates at low MIC values (with only 3 exceptions), confirming previous observations that this group of antimicrobial agents is effective against *M. bovis* (Ayling et al. 2000, Rosenbusch et al. 2005, Francoz et al. 2005, Gerchman et al. 2009, Uemura et al. 2010, Soehnlen et al. 2011, Kroemer et al. 2012, Gautier-Bouchardon et al. 2014, Heuvelink et al. 2016). MIC values of marbofloxacin were remarkably higher than that of danofloxacin and enrofloxacin. The observed difference, first noted by Gerchman et al. (2009) is probably due to the increased use of marbofloxacin during the past years. Extremely high MIC values for fluoroquinolones (≥10 µg/ml) were found in isolates MYC44-46. The similarity in the resistance profile of these three isolates is consistent with the results of the genetic study, where these isolates clustered into a separate subclade by MLST.

Most Hungarian *M. bovis* isolates included in the present examination showed moderate susceptibility to gentamicin, with MIC values (MIC_{90} 8 µg/ml) being similar or lower than the MIC values observed against isolates from Belgium and Israel (MIC_{90} 6 µg/ml, 32 µg/ml) (Thomas et al. 2003a, Gerchman et al. 2009). Spectinomycin, the examined member of the aminocyclitols, was used traditionally as an active compound against *M. bovis* and it is still considered effective in Japan (Ter Laak et al. 1993, Rosenbusch et al. 2005, Uemura et al. 2010). However, high MIC values of spectinomycin (≥256 µg/ml) were observed in more than

half of the studied Hungarian isolates, which is in agreement with recent reports from other countries (Ayling et al. 2000, Thomas et al. 2003a, Francoz et al. 2005, Gerchman et al. 2009, Soehnlen et al. 2011, Gautier-Bouchardon et al. 2014), confirming a globally emerging resistance to spectinomycin.

Heterogeneous profiles of *M. bovis* susceptibility to tetracyclines are reported from all over the world (Ayling et al. 2000, Thomas et al. 2003a, Rosenbusch et al. 2005, Gerchman et al. 2009, Uemura et al. 2010, Gautier-Bouchardon et al. 2014). Only two Hungarian isolates (MYC52-53) showed low MIC value to oxytetracycline and tetracycline, demonstrating the high level of resistance to tetracyclines among the isolates. In accordance with our results, increasing resistance to oxytetracycline was reported previously in Great Britain, Belgium, Japan and France (Ayling et al. 2000, Thomas et al. 2003, Uemura et al. 2010, Gautier-Bouchardon et al. 2014).

All but two (MYC52-53) of the Hungarian *M. bovis* isolates showed high level of resistance to macrolides, with MIC_{90} values (≥128 µg/ml) consistent with previously published data, suggesting that macrolides are losing their efficacy on mycoplasmas (Ayling et al. 2000, Rosenbusch et al. 2005, Gerchman et al. 2009, Uemura et al. 2010, Gautier-Bouchardon et al. 2014). MIC values of tylosin were lower or similar (with gradual distribution from 0.5 µg/ml to ≥128 µg/ml) to MICs of tilmicosin (grouped around two distinct values 0.5 µg/ml and ≥128 µg/ml). Similar observations were reported in the case of *M. bovis* strains by Gerchman et al. (2009) and in the case of *M. gallisepticum* isolates by Jordan and Horrocks (1996). The slower development of tylosin resistance is supposed to be the cause of the difference between the MIC values of these antibiotics (Wu et al. 2005), and our results provide further evidence for this phenomenon. High MIC_{90} values of gamithromycin and tulathromycin were detected in the present study in concordance with results of other European countries (Godinho 2008, Ayling et al. 2014, Gautier-Bouchardon et al. 2014, Heuvelink et al. 2016) but the MIC_{50} values of the Hungarian isolates were remarkably higher. Godinho (2008) also described the trend of increasing MIC_{50} values (from 0.25 to 4 µg/ml) without changing of the MIC_{90} values (>64 µg/ml) in European *M. bovis* respiratory isolates.

Outstandingly low MIC values of all tetracyclines and macrolides were observed in two Hungarian isolates originating from the same herd (MYC52-53) and in the case of the reference PG45 strain. These three strains were closely related forming a separate genetic clade in the MLST analysis (chapters 5.1. and 6.1.).

MIC_{90} values of lincomycin (≥64 µg/ml) against the isolates from cattle in Hungary were higher than the ones (1 µg/ml - The Netherlands, 8 µg/ml - Japan, 64 µg/ml - Belgium) described elsewhere (Ter Laak et al. 1993, Thomas et al. 2003a, Uemura et al. 2010); and more than half of the isolates demonstrated high MIC values to this member of lincosamides.

The Hungarian isolates showed high MIC values to florfenicol. The MIC_{90} values (8 µg/ml) were similar to values obtained earlier in the United Kingdom (16 µg/ml), USA (4 µg/ml) and France (16 µg/ml) (Ayling et al. 2000, Rosenbusch et al. 2005, Gautier-Bouchardon et al. 2014).

Pleuromutilins are used mainly in veterinary practice especially in the treatment of swine and poultry (Van Duijkeren et al. 2014). The limited number of MIC studies on tiamulin and valnemulin showed low MIC_{90} values (4 µg/ml to tiamulin and <0.03 µg/ml to valnemulin) for both antimicrobial agents (Thomas et al. 2003a, Gautier-Bouchardon et al. 2014) in accordance with the results of the present study (MIC_{90} 0.312 µg/ml to tiamulin and 0.039 µg/ml to valnemulin). The effectiveness of pleuromutilins in the *in vitro* examinations suggests the potential of this antibiotic group in the therapy of *M. bovis* infections.

The results of the current study confirmed the increasing resistance to antibiotics commonly used for the treatment of mycoplasma infections (primarily to tetracyclines and macrolides) and highlighted the importance of regular testing of antibiotic susceptibility in the region. The *in vitro* antibiotic susceptibility tests can only predict the expected *in vivo* efficacy of the antibiotics, thus they only indicate the potential usefulness of a certain antimicrobial agent in the therapy. Standard breakpoints (susceptible, intermediate, resistant categories) have not yet been defined for the interpretation of *M. bovis* susceptibility to antibiotics (NCCLS 2013), but several authors derived breakpoints for mycoplasmas from breakpoints of other bovine pathogens, and in some cases values were adopted from other host species (Rosenbusch et al. 2005, Francoz et al. 2005, Gerchman et al. 2009, Uemura et al. 2010, Soehnlen et al. 2011, Gautier-Bouchardon et al. 2014). Taking into account all these criteria, fluoroquinolones seem to be the most active compounds *in vivo* against the *M. bovis* isolates examined in Hungary. Although the *in vitro* antibiotic susceptibility tests are promising, the use of fluoroquinolones against *M. bovis* could be controversial *in vivo* (Nicholas and Ayling 2003). Moreover, the identification of three fluoroquinolone resistant isolates lends support for the EU recommendation that prudent antimicrobial use policies have to be strictly observed when members of this antibiotic group are applied (European Medicines Agency 2010). In order to avoid the development of resistance fluoroquinolones should only be used based on the results of susceptibility testing and in cases of severe infections when treatment failed with other classes of antimicrobials.

6.3. Mutations responsible for the high MICs to seven antimicrobial families

Antibiotics are among the most important therapeutic tools in the veterinary and human medicine, but their use is limited as resistance tends to evolve in pathogenic bacteria. The microorganisms are exposed to selective pressure by the use of antimicrobials in medicine and agriculture, favouring the development, survival and spread of resistant clones (Perron et al. 2015). Antibiotic resistance is fast evolving in *M. bovis* strains all over the world (Lysnyansky and Ayling 2016).

The acquisition of resistance in *M. bovis* is generally due to the emergence and spread of a single clone decreasing the genetic heterogeneity of antimicrobial resistant *M. bovis* isolates (Becker et al. 2015, Lysnyansky and Ayling 2016). In previous studies, the mechanisms involved in resistance of human pathogenic mycoplasmas were mainly examined while information about the acquisition and the mechanisms of antibiotic resistance in mycoplasmas with veterinary relevance, including *M. bovis* is scarce. Previously, the mechanisms based on genetic point mutations altering the targets of the antibiotics were described in the case of *M. bovis* (Lysnyansky et al. 2009, Sato et al. 2013, Lerner et al. 2014, Amram et al. 2015, Schnee et al. 2014, Kong et al. 2016). Plasmid related resistance, or efflux mechanisms present in some mycoplasma species have not been described in *M. bovis* (Lysnyansky and Ayling 2016).

Previously, in nature the spread of a certain *M. bovis* clone which achieved resistance has been described (Becker et al. 2015), while laboratory selected mutants can achieve their resistance in multiple ways. This could explain the differences between mutations identified in field isolates and mutant strains with highly elevated MICs in the present study.

The obtained high MICs of mutants to some antibiotics (e.g. tetracycline, valnemulin) tended to decrease rapidly in the absence of the selector agent in this study. The reason for this rapid regaining of susceptibility has not been clarified, but mutations resulting resistance could be reverted or neutralized by additional mutations during passages.

The *in vitro* development of fluoroquinolone resistance in *M. bovis* in the current study and in other mycoplasma species in previous studies showed a gradual pattern suggesting multi-step mutation events (Gautier-Bouchardon et al. 2002, Gruson et al. 2005, Sato et al. 2013). For the increase of MICs to fluoroquinolones substitutions in GyrA are sufficient and resistance to fluoroquinolones is achieved by an additional change in ParC (Lysnyansky et al. 2009, Sato et al. 2013). All *M. bovis* isolates of the present study with high MICs (≥10 µg/ml) to fluoroquinolones contained at least one substitution both in *gyrA* and *parC* genes. Earlier described mutation hotspots in GyrA and ParC (Lysnyansky et al. 2009, Sato et al.

2013, Mustafa et al. 2013, Khalil et al. 2015) of the Hungarian field isolates with high MICs and artificially selected resistant *M. bovis* strains have been identified between the amino acid positions 81-87 and 78-84, respectively. However, the SNP (G244A) causing increased MIC values to fluoroquinolones at position 82 (Asp→Asn) in the GyrA protein of *M. bovis* is described for the first time.

An alteration in GyrB at amino acid position 320 was observed in all three field isolates (MYC44-46) with MICs of ≥10 µg/ml which was likely associated with the isolates' genetic relatedness instead of fluoroquinolone resistance given the absence of the mutation in the mutant strains with identical MICs. The mutations at the positions, which were not related to the hot spots in QRDRs (Asp680His of GyrA, His78Asn and Asn529Lys of GyrB) and occurred in only one of the *in vitro* selected isolates, have unknown influence on fluoroquinolone resistance (Table 11).

The number of mutations in region Tet-1 correlated with the increase of MICs to tetracycline in *M. bovis* strains before (Amram et al. 2015). The Tet-1 binding site is located in a pocket formed by segments 1054-1056 and 1196-1200 of helix 34 and 964-967 of helix 31 in the 30S ribosomal subunit of *Thermus thermophilus*. In helix 34, the bases 1196 and 1054 form a clamp that holds tetracycline in hydrophobic interactions (Pioletti et al. 2001). Similarly to previously examined mycoplasma species (Gautier-Bouchardon et al. 2002, Dégrange et al. 2008b), in the present study resistance to tetracyclines developed gradually in *M. bovis* suggesting the accumulation of several mutations in the genome. Mutations associated with tetracycline resistance were identified in Tet-1 site, in accordance with previous works (Dégrange et al. 2008b, Amram et al. 2015). Such mutations of helix 34 in positions 1195-1199 were described in field and mutant strains with high MICs (≥64 µg/ml), while the substitution G1058C was observed in only one case (tetracycline resistant MYC52). Mutations of helix 31 were noted between nucleotides 962-967.

The effect of the SNPs G1012A, A1153G and A1268G of *rrs2* allele described outside of the regions Tet-1 and other tetracycline binding sites identified in all *in vivo* and *in vitro* developed isolates, and even in tylosin resistant MYC52 and PG45 isolates has not been clarified (Table 12).

Cross-resistance was observed between tetracycline and oxytetracycline resistant mutants and only slightly elevated MICs to spectinomycin were detected. This finding is in agreement with a previous work suggesting the regional effect of tetracycline, which is caused by its separated binding sites both in secondary and tertiary structures of the 16S rRNA (Noah et al. 1999).

Early studies confirmed the role of 1192 position of 16S rRNA in the antibiotic susceptibility of *E. coli*: substitutions C to A, G or T results in spectinomycin resistance (Makosky and Dahlberg 1987, Noah et al. 1999). The same observations were described in *M. bovis* strains

(Schnee et al. 2014). It is important to note that susceptibility to spectinomycin of *E. coli* can be restored in C1192 mutants by an additional substitution at the position T1351C of helix 43 (Dragon et al. 1996). In contrast with the observations of the development of resistance to tetracyclines, resistance to spectinomycin evolved rapidly. This finding is in harmony with the detected spectinomycin susceptibility profile of the 35 Hungarian isolates, which either showed low (≤4 µg/ml) or high MICs (≥256 µg/ml) to this antibiotic. Whole-genome analysis distinguished two main groups among the isolates according to their MIC values to spectinomycin also. Mutation was found in only one position of the genome: in *rrs1* gene, helix 34 the substitution C1192A occurred in field isolates (with MICs of ≥256 µg/ml) and C1192T in laboratory selected mutant strains (≥256 µg/ml).

In the current study, the rapid evolution of macrolide and lincomycin resistance was observed in the *in vitro* selected *M. bovis* mutants similarly to avian mycoplasma species examined before (Gautier-Bouchardon et al. 2002), presuming the small number of mutation events needed for the change of antibiotic susceptibility in these cases. Mutations (n=1-2/isolate) in domain II of 23S rRNA genes (position 748 and/or insertion after nucleotide C752) were necessary to achieve MICs of ≥128 µg/ml and 8-32 µg/ml to tilmicosin and tylosin, respectively, while an additional mutation in domain V (position 2059, 2060, 2063 or 2067) was needed to reach highly elevated MICs to tylosin (MIC ≥128 µg/ml) and lincomycin (MIC ≥64 µg/ml). The mutation at position 2058 (the most common point mutation in other mycoplasma species) was not detected in the Hungarian isolates (Matsuoka et al. 2004, Li et al. 2010, Lerner et al. 2014, Lysnyansky et al. 2015, Kong et al. 2016). The resistant isolates occurring in Hungary harboured the alteration of a neighbouring position (2059) in *rrl* genes, an important position in macrolide resistance also (Kobayashi et al. 2005, Li et al. 2010, Lerner et al. 2014, Lysnyansky et al. 2015).

The distribution of the isolates' MIC values for tylosin and tilmicosin were in consistence with the distribution of MICs for new generation macrolides suggesting similar resistance mechanisms. The alterations found in all lincomycin resistant mutants emphasize the importance of positions 2059 and 2060 of domain V in the development of lincomycin resistance. Mutation of L4 ribosomal protein (Ala64Val) was detected in one mutant strain, but its importance has not been clarified yet.

In the present study, slow evolution of valnemulin resistance was observed in the mutant MYC52 and MYC53 isolates, while PG45 obtained high MIC (≥10 µg/ml) within 3 passages. Florfenicol and tiamulin resistant mutant strains evolved resistance rapidly also (in 2-5 steps). The number of mutations correlated with the number of passages needed for the evolution of resistance (e.g. 5 mutations in MYC53 and 1 mutation in PG45 in the case of valnemulin resistance).

In the absence of field isolates with elevated MICs to pleuromutilins or florfenicol mutant strains were examined only. Mutants harboured alterations at nucleotide positions 2035, 2060, 2062, 2063, 2448, 2500, 2506 and 2611 of the 23 rRNA genes in close association with previously described resistance related positions, which assumes that these mutations have an effect on antibiotic susceptibility (Table 13) (Davidovich et al. 2007, Long et al. 2009, Hidalgo et al. 2011, Hillen et al. 2014, Liu et al. 2015). A mutation at the position 2032 (closely associated to position 2035 of valnemulin resistant MYC53) was previously reported in *Brachyspira hyodysenteriae* isolates with high MICs to tiamulin and lincosamides also (Hidalgo et al. 2011).

In the present study, isolates with mutation G2062T of *rrl1* gene had highly elevated MICs to florfenicol, tiamulin and valnemulin, and increased MICs to lincomycin and tilmicosin with the exception of the florfenicol resistant PG45. Mutants harbouring A2063T substitution showed eight times higher MIC values to florfenicol than their parental isolates, suggesting the impact of this position in florfenicol resistance. Mutations at positions 2500 and 2506 found in the present study are closely related to the ribosomal binding sites of phenicols and pleuromutilins (Li et al. 2010, Van Duijkeren et al. 2014). In *M. gallisepticum* mutation A2503U leads to decreased susceptibility to phenicols and lincomycin beside pleuromutilins (Li et al. 2010) and alteration of nucleotides 2498, 2499 2503 and 2504 causes resistance to pleuromutilins or florfenicol in different bacteria species (Kehrenberg et al. 2005, Van Duijkeren et al. 2014). Alteration of position 2448 in tiamulin resistant MYC53 lead to decreased susceptibility to florfenicol and pleuromutilins, and the importance of position 2447 in pleuromutilin resistance was previously described in *M. gallisepticum*. Previous studies have described the substitution C2611G in domain V of *M. pneumoniae* and G2597U and C2611U mutations of *M. hyorhinis* confirming their effect on 50S inhibitor resistance (Matsuoka et al. 2004, Kobayashi et al. 2005). The effects of polymorphisms at positions 171 and 316 have not been clarified yet.

Cross-resistance between macrolides and lincomycin has long been described (Nitu et al. 1974, Lysnyansky et al. 2015) and cross-resistance was reported between macrolides, pleuromutilins, lincomycin and florfenicol also (Li et al. 2010, Van Duijkeren et al. 2014). In the present study, the valnemulin resistant MYC53 harbouring mutations at positions 2035, 2060, 2500 and 2611 had elevated MICs to all of the tested 50S inhibitors. Mutation C2611T was detected in valnemulin resistant MYC53, closely related to C2612T of lincomycin resistant MYC52 both with elevated MICs to several 50S inhibitors. In two cases (valnemulin resistant PG45 and MYC53) the rapid development of high level cross-resistance to the tested 50S inhibitors was detected. These results emphasize the importance of responsible antibiotic usage.

The present study described the emergence of *M. bovis* isolates with high MIC values to several antimicrobial families and showed that mutations of clinical isolates and laboratory-derived mutants in GyrA and ParC proteins, and in 16S rRNA and 23S rRNA genes were responsible for resistance to fluoroquinolones, and to 30S and 50S inhibitory antibiotics, respectively. Some of these mutations led to cross-resistance to other classes of antibiotics with similar mechanism of action. The knowledge of the genetic background of antibiotic resistance is essential for the development of genetic-based diagnostic assays and also for the prevention of the spread of antibiotic resistant clones.

6.4. Rapid detection of antibiotic susceptibility of *Mycoplasma bovis*

Determining the antibiotic susceptibility of *M. bovis* isolates is essential for effective treatment. Antibiotic susceptibility examination of mycoplasmas is technically demanding, time-consuming and rarely performed in diagnostic laboratories. Thereby, empirical treatment is often introduced, which can lead to therapeutic failure and to the development of resistance to critically important antimicrobials. The rapid detection of resistance-associated mutations may help to accelerate the appropriate decision for antimicrobial treatment. Up to now, only a limited number of rapid genetic assays have been described for the screening of key mutations especially for *M. bovis* (Lysnyansky et al. 2009, Ben Shabat et al. 2010).

In the current study, 9 MAMA and 7 HRM tests were designed for the simultaneous detection of markers responsible for high MICs to certain antibiotics in *M. bovis* (Tables 6-7). The low genetic heterogeneity of *M. bovis* isolates with decreased antibiotic susceptibility is explained by the spread of a single resistant clone (Lysnyansky et al. 2009, Becker et al. 2015). For this reason the mutations of the field isolates (R_F) had particular importance in the development of the diagnostic assays. HRM tests presented here distinguished several genotypes but those occurring only in *in vitro* selected mutants (R_{M1}-R_{M3}) have minor importance in routine diagnostics (Table 15).

In the present study mutations in the *gyrA* (C248T) or both in the *gyrA* (C248T) and *parC* (G239T or G250A) genes were detected by the presented molecular methods within the Hungarian *M. bovis* population (Table S4). These results were in accordance with the results of the MIC determination tests, as slightly elevated MICs (0.625 µg/ml - 1.25 µg/ml) were observed when mutations were detected in the *gyrA* gene only, and MICs of at least 5 µg/ml to fluoroquinolones were determined in the second case. This finding provides further evidence to the observation of previous studies that a change in GyrA is sufficient to achieve elevated MICs to fluoroquinolones, but an additional change in the ParC protein is required

for resistance (Lysnyansky et al. 2009, Sato et al. 2013). The application of the three novel MAMA assays (one for *gyrA*, two for *parC*) and the HRM analysis of the hot-spot region of the *gyrA* gene (Tables 14-15) together was suitable for the discrimination of the fluoroquinolone susceptible *M. bovis* isolates, the samples with elevated MIC (≥0.625-1.25 µg/ml) and the specimens with high level resistance (≥5 µg/ml) to fluoroquinolones as well.

In *M. bovis*, point mutations A965T, A967T and G1058C and nucleotide regions 962-967 and 1195-1199 of the genes encoding the 16S rRNA have particular diagnostic importance and the number of mutations in Tet-1 binding site correlates with the increase of MICs to tetracycline in *M. bovis* (Amram et al. 2015). In the present examinations, with the exception of the substitution G1058C the above mutations were all identified in the *M. bovis* isolates, but the number of the detected mutations altered among the isolates. The results of the conventional antibiotic susceptibility tests and the novel molecular methods developed in the present study were in harmony both in the case of the 35 *M. bovis* pure cultures and of the 20 *M. bovis* isolates originated from the tested clinical samples.

The molecular marker for spectinomycin resistance in *M. bovis* field isolates is a C to A transversion at position 1192 either in *rrs1* or in both *rrs* alleles (Schnee et al. 2014). Thirty-four of the field isolates (n=55) characterized in this study showed resistant genotype by the MAMA test (rrs1/2-C1192A) and all 55 isolates obtained high MICs (≥256 µg/ml) by the microbroth dilution test (Table S5). The reliability of this MAMA performed on clinical samples was extremely low (53.3%), which could be explained by the low sensitivity (10^5 copies/reaction) of the assay, thus its application is recommended on *M. bovis* pure cultures.

In *M. bovis* field isolates, alterations in the macrolide binding site on the 23S rRNA at the nucleotide positions 748 and 752 of domain II and at the positions 2058 and 2059 of domain V have important role in the evolvement of tylosin, tilmicosin or lincomycin resistance (Lerner et al. 2014, Kong et al. 2016). In laboratory-derived mutants alterations of the nucleotide regions 748-752, 2058-2067, 2500-2506 and 2611-2612 of the *rrl* genes increase the MIC values to 50S inhibitory antibiotics (macrolides, lincomycin, florfenicol and pleuromutilins) (Li et al. 2010). In the Hungarian *M. bovis* population mutation G748A was identified in 53/55 isolates, while A2059G was detected in the case of 24 isolates with the described molecular methods (HRM rrl1/2-A and B; MAMA rrl1/2-G748A and A2059G). These results were in harmony with the results of MIC determination as 53 isolates showed high MICs to macrolides (≥8 µg/ml to tylosin and ≥128 µg/ml to tilmicosin) out of which 24 isolates yielded ≥128 µg/ml MICs to tylosin and ≥64 µg/ml to lincomycin (Table S6). Previous studies found the substitution A2058G more abundant among field isolates than A2059G (Lerner et al. 2014, Kong et al. 2016); however, it did not occur in the Hungarian isolates, and in the lack of positive control no MAMA was designed for this substitution. Nevertheless, the HRM assay

rrl1/2-B analysing the nucleotide region 2005-2101 of the *rrl1*/*2* genes can be suitable for the discrimination of A2058G point mutation as it reliably discriminates the A2059G substitution.
Two additional HRM assays (rrl1/2-C and rrl1/2-D) were designed in the present study to cover the nucleotide regions 2473-2524 and 2557-2636 as the mutations found in these regions (C2500A, G2506A and C2611T, C2612T) in mutant strains selected *in vitro* were associated with high MICs to some 50S inhibitors.
Rapidity, robustness and cost effectiveness are the powerful advantages of genetic-based antibiotic resistance determination. As these methods are applicable directly on clinical samples, technical problems of isolation and time-consuming traditional sensitivity tests could be avoided which is particularly important in the case of mycoplasmas. The reduced detection time helps in the choice of adequate antibiotics and as a consequence increases the therapeutic efficiency (Sundsfjord et al. 2004). Although the use of genetic methods in the determination of antibiotic susceptibility provides excellent guidance for antibiotic therapy, the presence of factors which can complicate the genetic detection of certain mechanisms should be considered as well (e.g. novel mutations, variable mutation events on single nucleotides, undetectable efflux systems, high mutation rate of mycoplasmas (Citti and Blanchard 2013).
In the current study all systems (MAMAs and HRMs) were designed with the same thermal profile in the current study allowing simultaneous application. The advantages of MAMA are that it can be performed on basic real-time PCR machines (without HRM function) and on conventional PCR equipment coupled with agarose gel electrophoresis. Although the HRM technique requires special equipment it is able to detect different kinds of mutations and distinguish genotypes among resistant strains based on the analysis of whole regions (Table 15). Assays developed in the present study reliably discriminate the resistant or sensitive genotypes to the examined antibiotics when examining pure *M. bovis* isolates. However, the limitation of the assays in the case of the examination of the DNA of clinical samples should be considered: degenerated oligos or samples containing low amount of *M. bovis* DNA (eg. BM354, BM356, BM368, BM373) generally worked with lower reliability in assays targeting universal regions (e.g. 16S rRNA) (Tables S4-6). In these cases, the combined use of melt-MAMA (with the melting profile of HRM) and HRM tests and/or the isolation of *M. bovis* are recommended for the reliable identification of resistant *M. bovis*. It should be noted that samples containing high amount of *M. bovis* DNA worked well regardless the presence of other *Mycoplasma* spp. (eg. BM121, BM122, BM124, BM375, Tables S4-6).
Traditionally, the detection of antibiotic susceptibility of *M. bovis* strains is time-consuming, might take up to 4 weeks starting with the isolation from the clinical sample followed by viable counting and microbroth dilution methods. Real-time based genetic methods described in the present study are able to identify resistant *M. bovis* DNA within a day applied directly on the

DNA of clinical samples and using pre-mixed plates, while approximately 3-4 days are needed when the DNA is extraxted for the examination after isolation of *M. bovis*. For the most reliable antibiotic resistance determination, particularly in the case of clinical samples, the regions examined by the MAMA and HRM tests are overlapping. Although the assays were designed based on the genetic analyses of *M. bovis* isolates of limited geographic origin, the isolates showed high genetic variability by MLST and MLVA and the targeted genomic regions involve mutations detected in field isolates (which developed resistance under field conditions). Also, additional assays (HRMs rrl1/2-C and rrl1/2-D) were designed to detect other possible mutations (developed in resistant mutant strains) and the correlation between the examined regions and resistance to antibiotics was confirmed in the case of other mycoplasmas and bacteria species. Hence the developed assays are assumed to be suitable for the evaluation of *M. bovis* strains from a wider geographical area as well.

As the World Health Organization (WHO) intends to decrease the usage of critically important antimicrobials for human purpose (3rd and 4th generation cephalosporins, macrolides, colistin and fluoroquinolones) in the veterinary field, there is a growing need for a reliable and quick method to detect antimicrobial susceptibility of *M. bovis*. As macrolides and fluoroquinolones are frequently used as an empirical choice for the management of the disease in Europe (Maunsell et al. 2011), the individual susceptibility profile provided by the presented methods can promote the use of less important antibacterials (spectinomycin, lincomycin, florfenicol, tetracyclines and pleuromutilins) for this purpose, and might contribute to the preservation of these critically important antibiotics. The use of the developed methods also significantly reduces the detection time of antibiotic susceptibility and enables early prediction of antibiotic efficacy, thus can contribute to quick therapeutic success, prudent antibiotic usage and decrease public health concerns related to bacterial resistance.

This is the first report describing molecular assays for the simultaneous detection of multiple mutations responsible for elevated MICs to fluoroquinolones, tetracyclines, spectinomycin, macrolides, lincomycin, pleuromutilins and florfenicol in *M. bovis*, the data provided by the assays allow clinicians to promptly select the appropriate treatment options. Also, the methods represent convenient tools for the conduction of surveillance of genetic mutations associated with antibiotic resistance.

7. Overview of the new scientific results

Ad 1. High genetic heterogeneity was described of the *M. bovis* isolates originating from Hungary with both MLVA and MLST methods. The combined use of the two molecular epidemiological typing techniques is recommended in the case of *M. bovis* isolates. Strains have to be characterized first by MLST, as an intermediate scale typing, and it should be followed by the fine scale typing of identical STs with MLVA.

Ad 2. Based on our *in vitro* examinations of fifteen antibiotics of eight antimicrobial families, fluoroquinolones proved to be the most effective drugs for the therapy of *M. bovis* infections in Hungary. Increasing resistance to antibiotics commonly used in the therapy of mycoplasma infections, primarily to tetracyclines and macrolides was described in Hungary. The results emphasize the necessity of periodic testing for antibiotic susceptibility in *M. bovis*. The effectiveness of pleuromutilins in the *in vitro* examinations suggests the potential of this antibiotic group in the therapy of *M. bovis* infections.

Ad 3. The whole genomes of 35 Hungarian *M. bovis* isolates were sequenced for the first time. Molecular markers responsible for high MICs to 3rd generation fluoroquinolones, tetracyclines, spectinomycin, 16-membered macrolides and to lincomycin were identified within the Hungarian *M. bovis* population.
In vitro development of resistance to tetracyclines, spectinomycin, macrolides, lincomycin, florfenicol and pleuromutilins in *M. bovis* was described for the first time. In laboratory derived *M. bovis* mutants novel molecular markers of high MICs to fluoroquinolones, tetracyclines, spectinomycin and macrolides were described. Molecular mechanism of acquired resistance to florfenicol and pleuromutilins in *M. bovis* were investigated for the first time identifying mutations in the 23S rRNA genes.

Ad 4. Nine MAMA and seven HRM assays were designed and evaluated for the simultaneous detection of multiple mutations responsible for high MICs to fluoroquinolones, tetracyclines, spectinomycin, macrolides, lincomycin, florfenicol and pleuromutilins in *M. bovis*. The presented method is highly cost-effective and can provide an antibiogram to 12 antibiotics of 7 antimicrobial groups within a day performing directly on the clinical samples, while approximately 3-4 days are needed when previous isolation of *M. bovis* is applied. For the most reliable antibiotic resistance determination, especially in the case of clinical samples, the combined use of MAMA and HRM is recommended.

8. References

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10. Supplements

Table S1. Susceptibility of *M. bovis* strains before and after *in vitro* antibiotic resistance selection to the 12 examined antibiotics.

Drug used for selection	Strain	Initial MIC (µg/ml)	No. of passages	MIC values (µg/ml)											
				D	E	M	Sc	O	Tet	Til	Ty	F	L	Tia	V
	MYC52	0.156	10	>10	>10	>10	8	2	0.5	≤0.5	≤0.5	4	1	0.078	≤0.039
D	MYC53	0.156	9	>10	>10	>10	4	1	≤0.25	≤0.5	≤0.5	4	≤0.25	0.039	≤0.039
	PG45	0.156	7	>10	>10	>10	4	2	0.5	≤0.5	≤0.5	4	0.5	0.078	≤0.039
	MYC52	0.156	4	>10	>10	>10	8	2	0.5	≤0.5	≤0.5	4	1	0.078	≤0.039
E	MYC53	0.156	8	>10	>10	>10	8	2	0.5	≤0.5	≤0.5	4	1	0.078	≤0.039
	PG45	0.156	6	>10	>10	>10	8	2	0.5	≤0.5	≤0.5	2	0.5	0.039	≤0.039
	MYC52	0.312	5	>10	>10	>10	8	2	0.5	≤0.5	≤0.5	4	1	0.156	≤0.039
M	MYC53	0.625	5	>10	>10	>10	8	2	0.5	≤0.5	≤0.5	4	1	0.078	≤0.039
	PG45	0.625	7	>10	>10	>10	8	4	0.5	≤0.5	≤0.5	2	0.5	0.039	≤0.039
	MYC52	4	2	0.156	0.156	0.625	>256	4	1	≤0.5	≤0.5	4	0.5	0.156	≤0.039
Sc	MYC53	4	2	0.156	0.156	0.625	>256	4	1	≤0.5	≤0.5	4	1	0.156	≤0.039
	PG45	4	3	0.156	0.156	0.312	>256	4	1	≤0.5	≤0.5	2	0.5	0.039	≤0.039
	MYC52	2	9	0.156	0.156	0.625	8	>64	>64	≤0.5	≤0.5	2	≤0.25	≤0.039	≤0.039
O	MYC53	2	10	0.156	0.156	0.625	8	>64	>64	≤0.5	≤0.5	2	≤0.25	0.039	≤0.039
	PG45	2	15	0.312	0.312	0.625	8	32	8	1	≤0.5	4	1	0.078	≤0.039
	MYC52	≤0.25	13	0.156	0.156	0.312	16	>64	>64	≤0.5	≤0.5	4	1	0.156	≤0.039
Tet	MYC53	≤0.25	13	0.156	0.156	0.625	16	>64	>64	≤0.5	≤0.5	2	0.5	0.039	≤0.039
	PG45	≤0.25	18	0.156	0.156	0.625	16	>64	32	≤0.5	≤0.5	4	1	0.078	≤0.039
	MYC52	≤0.5	5	0.156	0.156	0.625	4	2	0.5	>128	>128	8	>64	0.625	≤0.039
Til	MYC53	≤0.5	2	0.156	0.156	0.625	8	2	0.5	>128	>128	8	>64	1.25	≤0.039
	PG45	≤0.5	3	0.156	0.156	0.625	8	4	1	>128	64	8	1	0.078	≤0.039
	MYC52	≤0.5	6	0.156	0.156	0.625	4	2	0.5	>128	>128	4	1	0.312	≤0.039
Ty	MYC53	≤0.5	3	0.156	0.156	0.625	8	2	0.5	>128	>128	>32	2	0.312	≤0.039
	PG45	≤0.5	6	0.156	0.156	0.625	4	4	1	64	>128	>32	1	0.156	≤0.039
	MYC52	≤0.5	4	0.156	0.156	0.312	4	2	≤0.25	>128	>128	8	>64	1.25	≤0.039
L	MYC53	1	2	0.156	0.156	0.625	8	2	0.5	>128	>128	8	>64	5	0.039
	PG45	1	5	0.156	0.156	0.312	4	2	0.5	>128	16	4	16	0.625	≤0.039
	MYC52	4	2	0.156	0.156	0.312	4	1	≤0.25	8	2	>32	8	>10	>10
F	MYC53	4	2	0.156	0.156	0.312	4	1	≤0.25	8	4	>32	8	0.625	0.039
	PG45	4	5	0.156	0.312	0.625	4	4	≤0.25	16	>128	>32	2	0.156	≤0.039
	MYC52	0.078	2	0.156	0.156	0.625	4	2	≤0.25	1	≤0.5	>32	4	>10	>10
Tia	MYC53	0.156	2	0.156	0.156	0.312	4	1	≤0.25	1	1	>32	4	>10	0.156
	PG45	0.078	3	0.156	0.156	0.625	4	4	≤0.25	32	1	>32	16	>10	>10
	MYC52	≤0.039	14	0.156	0.312	0.625	8	4	1	4	8	>32	1	>10	0.078
V	MYC53	≤0.039	10	0.156	0.156	0.625	8	1	≤0.25	>128	>128	16	>64	>10	>10
	PG45	≤0.039	3	0.156	0.156	0.625	4	2	0.5	64	≤0.5	>32	16	>10	>10

Abbreviations are: D: danofloxacin; E: enrofloxacin; M: marbofloxacin; Sc: spectinomycin; O: oxytetracycline; Tet: tetracycline; Til: tilmicosin; Ty: tylosin; L: lincomycin; F: florfenicol; Tia: tiamulin; V: valnemulin.

Table S2. MLVA profiles of the thirty-one *M. bovis* strains examined.

Sample ID	Copy numbers of tandem repeats								
	TR14	TR29	TR30	TR31	TR35	TR40-41	TR49-51	TR52	TR59
PG45	2.2	4.8	2.3	5.8	4.0	4.0	2.2	4.0	2.4
MYC2	-	-	3.3	-	3.0	-	1.2	-	-
MYC22	-	0.8	3.3	-	3.0	8.0	1.2	3.0	2.4
MYC30	-	0.8	2.3	5.8	3.0	8.0	1.2	3.0	2.4
MYC42	-	12.8	3.3	5.8	3.0	8.0	1.2	4.0	2.4
MYC43	-	2.8	3.3	-	3.0	11.0	1.2	3.0	2.4
MYC44	-	2.8	3.3	-	3.0	-	0.6	3.0	1.4
MYC45	-	2.8	3.3	-	3.0	11.0	0.6	3.0	1.4
MYC46	-	2.8	3.3	-	3.0	11.0	0.6	3.0	1.4
MYC47	-	2.8	3.3	-	3.0	11.0	1.2	3.0	-
MYC48	-	-	3.3	-	3.0	-	1.2	-	-
MYC49	-	12.8	3.3	-	3.0	8.0	1.2	4.0	2.4
MYC50	-	12.8	3.3	-	3.0	8.0	1.2	4.0	-
MYC51	-	12.8	3.3	-	3.0	8.0	1.2	4.0	2.4
MYC52	2.2	4.8	2.3	7.8	4.0	4.0	2.2	4.0	2.4
MYC53	2.2	4.8	2.3	7.8	4.0	4.0	2.2	4.0	2.4
MYC65	-	1.8	3.3	5.8	3.0	8.0	1.2	4.0	2.4
MYC66	-	-	3.3	5.8	3.0	8.0	1.2	6.0	2.4
MYC67	-	-	3.3	5.8	3.0	8.0	1.2	6.0	2.4
MYC68	-	-	3.3	5.8	3.0	8.0	1.2	6.0	2.4
MYC69	-	1.8	3.3	-	3.0	8.0	1.2	4.0	-
MYC70	-	1.8	3.3	4.8	3.0	8.0	1.2	4.0	-
MYC71	-	1.8	3.3	-	3.0	8.0	1.2	4.0	-
MYC72	-	1.8	3.3	4.8	3.0	8.0	1.2	4.0	-
MYC73	-	1.8	3.3	4.8	3.0	8.0	1.2	4.0	-
MYC74	-	1.8	3.3	4.8	-	8.0	1.2	4.0	-
MYC75	-	1.8	3.3	-	3.0	8.0	1.2	4.0	-
MYC76	-	1.8	3.3	4.8	-	8.0	1.2	4.0	-
MYC77	2.2	0.8	3.3	-	3.0	11.0	0.6	3.0	2.4
MYC78	-	0.8	3.3	5.8	3.0	8.0	1.2	4.0	2.4
MYC79	-	0.8	3.3	5.8	3.0	8.0	1.2	4.0	2.4
MYC80	2.2	0.8	3.3	-	-	25.0	1.2	4.0	2.4

Table S3. *In vitro* activity of 15 antibiotics against 35 Hungarian *M. bovis* strains.

Strain ID	MIC values (µg/ml)														
	D	E	M	G	Sc	O	Tet	Til	Ty	Gh	Tu	F	L	Tia	V
PG45	0.156	0.156	0.625	4	4	2	0.25	0.5	0.5	8	4	4	1	0.078	0.039>
MYC2	0.156	0.156	0.625	2	2	16	4	128	16	16	16	4	1	0.156	0.039>
MYC22	0.156	0.312	0.625	4	256	64	16	128	128	128	128	8	64	0.312	0.039>
MYC30	0.156	0.156	0.625	4	256	32	8	128	128	16	128	4	64	0.156	0.039>
MYC42	0.156	0.156	0.625	8	4	64	8	128	32	16	16	8	1	0.156	0.039>
MYC43	0.156	0.156	0.312	4	256	64	16	128	128	16	128	8	64	0.312	0.039>
MYC44	10	10	10	2	256	64	8	128	128	128	128	8	64	0.312	0.039>
MYC45	10	10	10	2	256	64	8	128	128	128	128	4	64	0.312	0.039>
MYC46	10	10	10	4	256	64	8	128	128	128	128	8	64	0.625	0.039>
MYC47	0.156	0.156	0.625	8	256	64	8	128	128	128	128	8	64	0.312	0.039>
MYC48	0.156	0.156	0.625	8	256	64	16	128	128	128	128	4	64	0.312	0.039>
MYC49	0.156	0.156	0.625	8	256	64	16	128	128	32	128	4	64	0.312	0.039>
MYC50	0.156	0.156	0.625	4	256	64	8	128	128	16	128	4	64	0.312	0.039>
MYC51	0.156	0.08	0.312	4	256	64	8	128	128	64	128	4	64	0.156	0.039>
MYC52	0.156	0.156	0.312	8	4	2	0.25	0.5	0.5	8	2	4	0.5	0.078	0.039>
MYC53	0.156	0.156	0.625	16	4	2	0.25	0.5	0.5	8	8	4	1	0.156	0.039>
MYC65	0.156	0.156	0.625	2	2	64	16	128	8	8	4	4	0.5	0.039	0.039>
MYC66	0.156	0.156	0.625	8	4	64	8	128	16	16	16	8	1	0.156	0.039>
MYC67	0.08	0.08	0.312	4	4	16	4	128	16	16	64	8	2	0.625	0.039>
MYC68	0.156	0.156	0.625	4	4	32	4	128	16	8	8	4	0.5	0.039	0.039>
MYC69	0.156	0.156	0.625	2	4	32	8	128	32	16	16	8	1	0.156	0.039>
MYC70	0.156	0.156	0.625	4	2	32	4	128	32	16	16	4	1	0.156	0.039>
MYC71	0.156	0.156	0.625	4	2	32	4	128	32	16	16	4	1	0.078	0.039>
MYC72	0.156	0.156	0.625	4	4	32	4	128	32	16	16	4	1	0.078	0.039>
MYC73	0.156	0.156	0.625	4	4	32	8	128	32	16	16	4	1	0.078	0.039>
MYC74	0.156	0.156	0.625	4	4	32	8	128	16	8	8	4	1	0.156	0.039>
MYC75	0.156	0.08	0.312	2	2	32	4	128	32	16	16	4	1	0.156	0.039>
MYC76	0.156	0.156	0.625	4	4	64	8	128	16	8	8	8	2	0.312	0.039>
MYC77	0.312	0.156	0.625	2	256	64	8	128	128	128	128	4	64	0.312	0.039>
MYC78	0.156	0.156	0.625	4	256	64	8	128	128	64	128	4	64	0.312	0.039>
MYC79	0.156	0.156	0.625	8	256	64	16	128	128	128	128	8	64	0.312	0.039>
MYC80	0.156	0.156	0.625	4	256	32	4	128	128	128	128	4	64	0.312	0.039>
MYC81	0.156	0.156	0.625	8	256	64	8	128	128	64	128	4	64	0.312	0.039>
MYC82	0.156	0.156	0.625	4	256	64	8	128	128	128	128	8	64	0.156	0.039>
MYC83	0.312	0.156	0.625	4	256	64	8	128	128	128	128	8	64	0.156	0.039>
MYC84	0.156	0.156	0.625	4	256	64	8	128	128	16	128	4	64	0.156	0.039>

Abbreviations are: D: danofloxacin; E: enrofloxacin; M: marbofloxacin; G: gentamycin; Sc: spectinomycin; O: oxytetracycline; Tet: tetracycline; Til: tilmicosin; Ty: tylosin; Gh: gamithromycin; Tu: tulathromycin; F: florfenicol; L: lincomycin; Tia: tiamulin; V: valnemulin.

Table S4. MIC values and genotype calls of fluoroquinolone resistance for the 30 clinical samples with the 20 isolated *M. bovis* strains included in the study.

Sample ID	*M. bovis* state of clinical DNA sample[a]	MIC (µg/ml)			gyrA-C248T		gyrA-HRM		parC-G239T		parC-G250A	
		D	E	M	Strain	Clinical sample	Strain	Clinical sample	Strain	Clinical sample	Strain	Clinical sample
BM121	+++	0.312	0.625	0.312	S	S	S	S	S	S	S	S
BM122	+++	0.312	0.625	0.156	S	S	S	S	S	S	S	S
BM124	+++	0.625	1.25	0.625	R	R	R_F	R_F	S	S	S	S
BM218	+++	0.312	0.625	0.312	S	S	S	S	S	S	S	S
BM239	+++	5	10	5	R	R	R_F	R_F	S	S	R	-
BM274	+++	0.625	1.25	0.625	R	R	R_F	R_F	S	S	S	S
BM338	+++	0.312	0.625	0.312	S	S	S	S	S	S	S	S
BM343	+++	0.156	0.625	0.156	S	S	S	amb	S	S	S	S
BM367	+++	0.156	0.625	0.156	S	S	S	amb	S	S	S	S
BM375	+++	0.312	0.625	0.312	S	S	S	S	S	S	S	S
BM381	+++	0.625	0.625	0.312	S	S	S	S	S	S	S	S
BM385	+++	10	10	10	R	R	R_F	R_F	R	R	S	S
BM388	+++	10	10	10	R	R	R_F	R_F	R	R	S	S
BM396	+++	0.625	0.625	0.625	S	S	S	S	S	S	S	S
BM237	++	0.312	0.625	0.312	S	S	S	S	S	S	S	S
BM272	++	nd	nd	nd	nd	S	nd	S	nd	S	nd	S
BM394	++	0.625	0.625	0.625	S	S	S	-	S	-	S	S
BM270	+	0.312	0.625	0.312	S	-	S	S	S	-	S	-
BM354	+	0.312	0.625	0.312	S	S	S	S	S	-	S	S
BM356	+	0.156	0.625	0.156	S	S	S	R_F	S	-	S	S
BM140	-	nd	nd	nd	nd	-	nd	-	nd	-	nd	-
BM302	-	nd	nd	nd	nd	-	nd	-	nd	-	nd	-
BM323	-	nd	nd	nd	nd	-	nd	-	nd	-	nd	-
BM346	-	nd	nd	nd	nd	-	nd	-	nd	-	nd	-
BM348	-	nd	nd	nd	nd	-	nd	amb	nd	-	nd	-
BM357	-	0.156	0.625	0.312	S	-	S	-	S	-	S	-
BM368	-	nd	nd	nd	nd	-	nd	-	nd	-	nd	-
BM369	-	nd	nd	nd	nd	-	nd	-	nd	-	nd	-
BM373	-	nd	nd	nd	nd	-	nd	-	nd	-	nd	-
BM416	-	nd	nd	nd	nd	-	nd	-	nd	-	nd	-

[a] According to Subramaniam et al. (1998). Weak positive „+" results were distinguished from medium „++" and strong positive „+++" samples. D=danofloxacin, E=enrofloxacin, M=marbofloxacin; S=sensitive, R=resistant, R_F = genotype of resistant strains developed under field conditions, R_{M2}= resistant genotype found in mutant strain, nd=not determined, amb=ambiguous result, - =negative

Table S5. MIC values and genotype calls of tetracycline and spectinomycin resistance for the 30 clinical samples with the 20 isolated *M. bovis* strains included in the study.

Sample ID	*M. bovis* state of clinical DNA sample[a]	MIC (µg/ml)		rrs1/2-A965T		rrs1/2-A967T		rrs1/2-A-HRM		rrs1/2-G1058C		rrs1/2-B-HRM		MIC (µg/ml)	rrs1/2-C1192A	
		Tet	O	Strain	Clinical sample	Strain	Clinical sample	Strain	Clinical sample	Strain	Clinical sample	Strain	Clinical sample	Sc	Strain	Clinical sample
BM122	+++	8	32	R	R	R	R	R_F	R_F	S	S	S	S	8	S	-
BM124	+++	32	64	R	R	R	R	R_F	R_F	S	S	R_F	R_F	256	R	R
BM218	+++	16	64	R	R	R	R	R_F	R_F	S	S	S	S	8	S	-
BM239	+++	16	64	R	R	R	R	R_F	R_F	S	S	S	S	256	R	-
BM274	+++	16	64	R	amb	R	R	R_F	R_F	S	S	R_F	R_F	256	R	-
BM338	+++	16	64	R	R	R	R	R_F	R_F	S	-	R_F	R_F	256	R	amb
BM343	+++	16	64	R	amb	R	R	R_F	R_F	S	S	S	S	256	R	-
BM367	+++	8	64	R	R	R	R	R_F	amb	S	S	S	R_F	256	R	-
BM375	+++	16	64	R	R	R	R	R_F	R_F	S	S	S	S	256	R	R
BM381	+++	4	16	R	R	R	R	R_F	R_F	S	S	S	S	4	S	-
BM385	+++	16	64	R	amb	R	R	R_F	amb	S	amb	R_F	R_F	256	R	-
BM388	+++	16	64	R	R	R	R	R_F	R_F	S	S	R_F	R_F	256	R	-
BM396	+++	8	32	R	R	R	R	R_F	R_F	S	S	R_F	R_F	256	R	-
BM237	++	16	64	R	R	R	R	R_F	R_F	S	-	R_F	R_F	256	R	-
BM272	++	nd	nd	nd	R	nd	R	nd	amb	nd	S	nd	S	nd	nd	S
BM394	++	8	32	R	amb	R	R	R_F	amb	S	amb	R_F	R_F	256	R	R
BM270	+	32	64	R	R	R	R	R_F	amb	S	-	S	S	256	R	-
BM354	+	16	64	R	R	R	R	R_F	R_F	S	S	S	amb	256	R	amb
BM356	+	8	64	R	R	R	R	R_F	R_F	S	S	S	amb	4	S	S
BM140	-	nd	nd	nd	-	nd	-	nd	-	nd	-	nd	-	nd	nd	-
BM302	-	nd	nd	nd	-	nd	-	nd	-	nd	-	nd	-	nd	nd	-
BM323	-	nd	nd	nd	-	nd	-	nd	-	nd	-	nd	amb	nd	nd	-
BM346	-	nd	nd	nd	-	nd	-	nd	-	nd	-	nd	-	nd	nd	-
BM348	-	nd	nd	nd	-	nd	amb	nd	-	nd	amb	nd	-	nd	nd	-
BM357	-	16	64	R	-	R	amb	R_F	R_F	S	S	R_F	amb	256	R	-
BM368	-	nd	nd	nd	S	nd	-	nd	-	nd	-	nd	-	nd	nd	-
BM369	-	nd	nd	nd	R	nd	-	nd	-	nd	-	nd	-	nd	nd	-
BM373	-	nd	nd	nd	S	nd	-	nd	-	nd	-	nd	amb	nd	nd	-
BM416	-	nd	nd	nd	-	nd	-	nd	-	nd	-	nd	amb	nd	nd	-

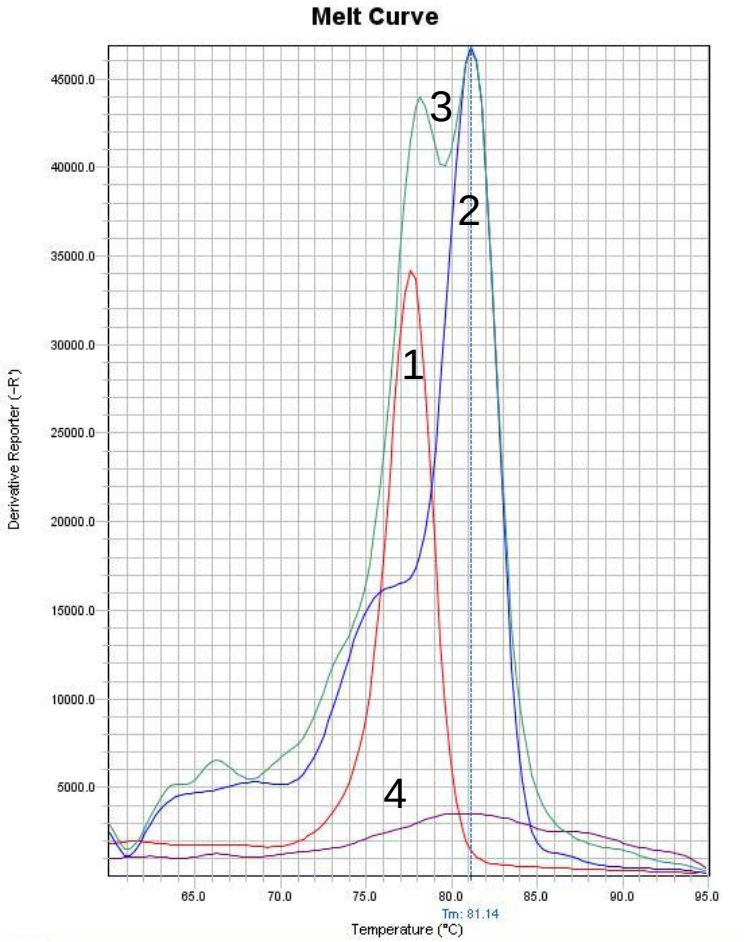
[a] according to Subramaniam et al. (1998). Weak positive „+" results were distinguished from medium „++" and strong positive „+++" samples. Tet=tetracycline, O=oxytetracycline, Sc=spectinomycin; S=sensitive, R=resistant, R_F = genotype of resistant strains developed under field conditions, nd=not determined, amb=ambiguous result, - =negative

Table S6. MIC values and genotype calls of macrolide, lincomycin, florfenicol and pleuromutilin resistance for the 30 clinical samples with the 20 isolated *M. bovis* strains included in the study.

Sample ID	*M. bovis* state of clinical DNA sample[a]	MIC (µg/ml)	rrl1/2-G748A		rrl1/2-A-HRM		MIC (µg/ml)		rrl1/2-A2059G		rrl1/2-B-HRM		MIC (µg/ml)			rrl1/2-C-HRM		rrl1/2-D-HRM	
		Til	Strain	Clinical sample	Strain	Clinical sample	Ty	L	Strain	Clinical sample	Strain	Clinical sample	F	Tia	V	Strain	Clinical sample	Strain	Clinical sample
BM121	+++	128	R	R	R_F	R_F	32	2	S	S	S	S	8	0.312	<0.039	S	S	S	S
BM122	+++	128	R	R	R_F	R_F	32	1	S	S	S	S	8	0.078	<0.039	S	S	S	S
BM124	+++	128	R	R	R_F	R_F	128	64	R	R	R_F	R_F	8	0.625	<0.039	S	S	S	S
BM218	+++	128	R	R	R_F	R_F	64	4	S	S	S	S	16	1.25	<0.039	S	S	S	S
BM239	+++	128	R	R	R_F	R_F	128	64	R	R	R_F	R_F	8	1.25	<0.039	S	S	S	S
BM274	+++	128	R	R	R_F	R_F	128	64	R	R	R_F	R_F	8	0.625	<0.039	S	amb	S	S
BM338	+++	128	R	R	R_F	R_F	128	64	R	S	R_F	R_F	8	0.625	<0.039	S	S	S	S
BM343	+++	128	R	R	R_F	R_F	128	64	R	S	R_F	R_F	8	0.312	<0.039	S	S	S	S
BM367	+++	128	R	R	R_F	R_F	128	64	R	S	R_F	amb	16	5	0.039	S	S	S	amb
BM375	+++	128	R	R	R_F	R_F	128	64	R	R	R_F	R_F	8	0.312	<0.039	S	S	S	S
BM381	+++	128	R	R	R_F	R_F	64	2	S	S	S	S	8	0.312	<0.039	S	S	S	S
BM385	+++	128	R	R	R_F	R_F	128	64	R	S	R_F	R_F	16	1.25	<0.039	S	S	S	S
BM388	+++	128	R	R	R_F	R_F	128	64	R	S	R_F	amb	16	1.25	<0.039	S	S	S	S
BM396	+++	128	R	R	R_F	R_F	128	64	R	R	R_F	R_F	8	2.5	<0.039	S	S	S	S
BM237	++	128	R	R	R_F	R_F	128	64	R	amb	R_F	R_F	8	0.625	<0.039	S	S	S	S
BM272	++	nd	nd	R	nd	R_F	nd	nd	nd	R	nd	R_F	nd	nd	nd	nd	S	nd	S
BM394	++	128	R	R	R_F	R_F	128	64	R	amb	R_F	R_F	8	2.5	0.039	S	S	S	S
BM270	+	128	R	R	R_F	R_F	128	64	R	amb	R_F	R_F	16	1.25	0.039	S	S	S	amb
BM354	+	128	R	R	R_F	R_F	128	64	R	S	R_F	amb	8	0.625	<0.039	S	S	S	S
BM356	+	128	R	R	R_F	R_F	64	2	S	S	S	amb	8	0.312	<0.039	S	S	S	S
BM140	-	nd	nd	nd	nd	-	nd	nd	nd	-	nd	-	nd	nd	nd	nd	-	nd	-
BM302	-	nd	nd	-	nd	-	nd	nd	nd	-	nd	-	nd	nd	nd	nd	-	nd	-
BM323	-	nd	nd	-	nd	-	nd	nd	nd	-	nd	-	nd	nd	nd	nd	-	nd	-
BM346	-	nd	nd	-	nd	-	nd	nd	nd	-	nd	-	nd	nd	nd	nd	-	nd	-
BM348	-	nd	nd	-	nd	-	nd	nd	nd	S	nd	-	nd	nd	nd	nd	-	nd	-
BM357	-	128	R	R	R_F	amb	128	64	R	S	R_F	amb	8	1.25	<0.039	S	S	S	S
BM368	-	nd	nd	-	nd	-	nd	nd	nd	S	nd	-	nd	nd	nd	nd	-	nd	amb
BM369	-	nd	nd	-	nd	-	nd	nd	nd	-	nd	-	nd	nd	nd	nd	amb	nd	-
BM373	-	nd	nd	-	nd	-	nd	nd	nd	-	nd	amb	nd	nd	nd	nd	-	nd	amb
BM416	-	nd	nd	-	nd	-	nd	nd	nd	-	nd	-	nd	nd	nd	nd	-	nd	-

[a] according to Subramaniam et al. (1998). Weak positive „+" results were distinguished from medium „++" and strong positive „+++" samples. Til=tilmicosin, Ty=tylosin, L=lincomycin, F=florfenicol, Tia=tiamulin, V=valnemulin; S=sensitive, R=resistant, R_F = genotype of resistant strains developed under field conditions, nd=not determined, amb=ambiguous result, - =negative

Figure S1. Ambiguous result of melt-MAMA assay (rrs1/2-A965T) due to bimodal shape of the melt curve performing directly on the DNA isolated from clinical sample.



Curve 1: tetracycline sensitive *M. bovis* type strain (PG45, NCTC 10131), T_m=77.6°C; Curve 2: tetracycline resistant MYC44 (DNA of purified *M. bovis* isolate), T_m=81.0°C; Curve 3: BM394, ambiguous result (DNA of of clinical sample); Curve 4: negative control.

11. Acknowledgements

First I would like to express my honest gratitude to my supervisor, Miklós Gyuranecz for all of his help, support, patience and inspiration throughout the years.

I am grateful to my consultants, Ádám Dán, Béla Dénes and Sándor Hornok for their wise advices during my PhD work. A special thank has to be granted to Enikő Wehmann for her helpfulness and useful consultance at all time.

It is my honour to acknowledge László Makrai, Szilárd Jánosi, Zsuzsanna Rónai, Levente Szeredi, Ibolya Turcsányi and Csaba Nemes for their help in the sample collection.

I am grateful to Krisztián Bányai and Szilvia Marton for their contribution in the whole genome sequencing. I thank Inna Lysnyansky for her help in the interpretation of the results of antibiotic resistance associated mutations.

I would also like to express my special thanks to my colleagues, Veronika Hrivnák, Orsolya Felde, Dénes Grózner and Katinka Bekő for their helpfulness and kindness. A special thank is granted to Zsuzsa Kreizinger for her reviews and advices.

I thank my family and friends for their support and encouragement. Last, but not least I would like to thank my husband, Tamás Görföl for all his endless love and support in research and at home as well.

The study was founded by the Lendület (Momentum) program (LP2012-22) of the Hungarian Academy of Sciences.