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Development of molecular methods for the rapid detection of antibiotic susceptibility of *Mycoplasma bovis*

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ABSTRACT

Determining the antibiotic susceptibility profile of *Mycoplasma bovis* isolates *in vitro* provides the basis for the appropriate choice of antibiotics in the therapy. Traditionally, the antibiotic susceptibility examination of mycoplasmas is technically demanding, time-consuming and rarely performed in diagnostic laboratories. The aim of the present study was to develop rapid molecular assays to determine mutations responsible for elevated minimal inhibitory concentrations (MICs) to fluoroquinolones, tetracyclines, aminocyclitols, macrolides, lincosamides, phenicols and pleuromutilins in *M. bovis*.

The nine mismatch amplification mutation assays (MAMA) and seven high resolution melt (HRM) tests designed in the present study enable the simultaneous detection of these genetic markers. The sensitivity of the assays varied between 10^2–10^5 copy numbers/reaction. Cross-reactions with other mycoplasmas occurring in cattle were detected in assays targeting universal regions (*e.g.* 16S rRNA). Nevertheless, results of the novel method were in accordance with sequence and MICs data of the *M. bovis* pure cultures. Also, the tests of clinical samples containing high amount of *M. bovis* DNA were congruent even in the presence of other *Mycoplasma* spp. The presented method is highly cost-effective and can provide an antibiogram to 12 antibiotics in approximately 3–4 days when previous isolation of *M. bovis* is applied. In order to assure the proper identification of the genetic markers at issue, the regions examined by the MAMA and HRM tests are overlapping.

In conclusion, the developed assays have potential to be used in routine diagnostics for the detection of antibiotic susceptibility in *M. bovis*.

1. Introduction

M. bovis is a cell wall-less bacterium, the causative agent of respiratory disease, mastitis and arthritis of intensively farmed cattle (Nicholas and Ayling, 2003). *M. bovis* is primarily transmitted by direct contact and spread through animal transportation to numerous countries of the world. Since there is no effective vaccine for *M. bovis*, promptly selected antibiotic treatment is promoted in the control of the disease. Antimicrobials usually used for the treatment or prevention of *M. bovis* infections and other bacterial pathogens of bovine respiratory disease are fluoroquinolones, tetracyclines, macrolides, and florfenicol (Nicholas and Ayling, 2003; Maunsell et al., 2011; Lysnyansky and Ayling, 2016). Although antibiotics are not recommended for the treatment of *M. bovis*-associated mastitis (Nicholas et al., 2016), the antibiotic therapy of pneumonia has shown some success (Stipkovits et al., 2005; Godinho et al., 2005). Nevertheless, increasing number of isolates with elevated minimal inhibitory concentrations (MICs) for many of the commercially available antimicrobials have been reported worldwide due to extensive drug use in the therapy of bovine pneumonia (Nicholas and Ayling, 2003; Gautier-Bouchardon et al., 2014; Lysnyansky and Ayling, 2016).

In routine practice the determination of the antibiotic susceptibility of mycoplasmas may require several weeks because of the time-consuming isolation methods prior to the *in vitro* susceptibility tests. Another disadvantage is the lack of standard quality control strains and breakpoints defined for the interpretation of antibiotic susceptibility of animal pathogen mycoplasmas (Hannan, 2000; Lysnyansky and Ayling, 2016). Thereby, empirical treatment is often introduced, which can

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lead to therapeutic failure and development of resistance to critically important antimicrobials. The most suitable strategy for the treatment of *M. bovis*-associated diseases can be determined by clinical trials. However, this option is costly and time-consuming and it is rarely available in the practice, thus substitutional methods, such as the rapid detection of mutations related to high MICs can provide a feasible option to help in the selection of the appropriate antibiotics for the therapy. Up to now, only a restricted number of antibiotic resistance related markers of *M. bovis* can be identified with the methods published earlier Lysnyansky et al., 2009; Ben Shabat et al., 2010). Mutations of field strains had particular importance in designing diagnostic assays. In *M. bovis* strains with increased MICs to fluoroquinolones the mutations which have key role in the increased resistance are the SNPs C248T of *gyr*A, G239T and G250A of *parC* or the non-synonymous alterations of nucleotide region 244–260 of *gyrA* or 232–250 of *parC* (according to *Escherichia coli* numbering) (Mustafa et al., 2013; Sato et al., 2013; Khalil et al., 2015; Sulyok et al., 2017b). Mutations at the positions 965, 967, 1058 and 1199 of the genes encoding the 16S rRNA are associated with high MICs to tetracyclines (Amram et al., 2015; Sulyok et al., 2017b; Khalil et al., 2017). The molecular marker for MICs ≥256 μg/ml to spectinomycin in *M. bovis* field strains is a C to A transversion at position 1192 either in *rrs1* or in both *rrs1* and *rrs2* alleles (Schnee et al., 2014; Sulyok et al., 2017b). Nucleotide positions 748, 752, 2058 and 2059 of the 23S rRNA genes have an important role in the evolution of tylosin or tilmicosin resistance; in addition, mutation at position 2058 or 2059 was associated with high MIC to lincomycin as well. Amino acid substitution in the L4 and L22 ribosomal proteins were also described but their importance in macrolide resistance have not been clarified (Lerner et al., 2014; Kong et al., 2016; Sulyok et al., 2017b). In laboratory-derived mutant strains alterations of nucleotide regions 748–752, 2058–2067, 2500–2506 and 2611–2612 of *rrl* genes could increase the MIC values to 50S inhibitory antibiotics (macrolides, lincomycin, florfenicol and pleuromutilins) (Li et al., 2010; Sulyok et al., 2017b).

In the current study, genetic markers (single nucleotide polymorphisms (SNPs) and hot-spot regions) related to increased MICs to antibiotics potentially used in the treatment of mycoplasmosis in food producing animals (tetracyclines, aminocyclitol, macrolides, lincosamide, pleuromutilins, phenicols and fluoroquinolones) were selected and feasible diagnostic methods were designed for their identification in order to improve accessibility to adequate therapy. The present study describes the development and characterization of rapid and cost-effective real-time PCR based assays for the simultaneous discrimination of *M. bovis* strains with low or high MICs to seven antimicrobial groups.

2. Materials and methods

2.1. M. bovis strains, clinical samples and susceptibility testing

A total of 35 *M. bovis* field strains and 36 mutant strains with increased MICs derived from previous studies (Sulyok et al., 2014a, 2017a, 2017b), 30 clinical samples (lungs and a nasal swab) and 20 *M. bovis* strains isolated from the clinical samples were examined in the present study (Table 1). All *M. bovis* isolates and clinical samples originated from different parts of Hungary and were collected from cattle with respiratory diseases. The isolation and identification methods of the strains have been described previously (Sulyok et al., 2014a). In brief, clinical 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. All isolates were identified by polymerase chain reaction (PCR) targeting the *uvrC* gene of *M. bovis* (Subramaniam et al., 1998). The purity of the cultures was confirmed by a universal *Mycoplasma* PCR system (Lauerman et al., 1995) followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA), sequence analysis and BLAST search. High genetic diversity of the 35 *M. bovis* field strains was previously determined by multi-locus sequence typing and multi-locus variable number of tandem repeats analysis (Sulyok et al., 2014b).

Susceptibility of the 35 *M. bovis* isolates, the 36 *in vitro* selected mutants and the reference strain (*M. bovis* PG45, NCTC 10131) to certain fluoroquinolones (danofloxacin, enrofloxacin, marbofloxacin), an aminocyclitol (spectinomycin), tetracyclines (tetracycline, oxytetracycline), macrolides (tylosin, tilmicosin), a phenicol (florfenicol), a lincosamide (lincomycin) and pleuromutilins (tiamulin and valnemulin) were previously determined by microbroth dilution method (Sulyok et al., 2014a, 2017a, 2017b) according to the recommendation of Hannan (2000). The subsequently isolated 20 *M. bovis* strains (Table 1) were submitted for antibiotic susceptibility testing in the present study (Supplementary material, Datasets 1–3).

The final MIC value of each strain 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. The reference strain (*M. bovis* PG45, NCTC 10131) was used as quality control of MIC determination.

2.2. MAMA and HRM design

MAMA-PCR is a 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 discriminative 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 high MICs to certain antibiotics (Table 2). Besides SNPs, related regions with several mutations were identified in *M. bovis* strains with elevated MICs. These “hot-spot” regions of genes related to high MICs were targeted by HRM assays (Table 3). 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).

The presented MAMA assays differentiated the genotypes of *M. bovis* with low or with high MIC (genotype L or genotype H) to certain antibiotics, while HRM assays could distinguish more genotypes among the isolates with high MICs: H_F indicates *M. bovis* with high MICs developed under field conditions and H_{M1-3} indicate genotypes found in *in vitro* selected mutant strains.

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, Waltham, MA, USA). All MAMA and HRM primers were designed and tested on the *M. bovis* reference strain (PG45, NCTC 10131), the 35 field strains included in previous studies and on the 36 mutants selected *in vitro* with increased MIC values (Sulyok et al., 2017b). 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 2 and 3. PCR mixture of Melt-MAMAs and HRMs were identical and composed of 2 μl 5X Color-less GoTaq Flexi Buffer (Promega Inc., Madison, WI), 1 μl $MgCl_2$ (25 mM), 0.3 μl dNTP (10 mM, Qiagen Inc., Valencia, CA), 0.5 μl EvaGreen (20X, Biotium Inc., Hayward, CA), primers (10 pmol/μl, Tables 2 or 3), 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 s and 60 °C for 1 min. PCR products were subjected to melt analysis using a dissociation protocol comprising the steps 95 °C