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RESEARCH ARTICLE

Development of a molecular biological assay for the detection of markers related to decreased susceptibility to macrolides and lincomycin in *Mycoplasma hyorhinis*

DOROTTYA FÖLDI[1], ZSUZSA KREIZINGER[1], KATINKA BEKŐ[1], NIKOLETT BELECZ[1], KRISZTIÁN BÁNYAI[1], KRISZTIÁN KISS[2], IMRE BIKSI[3] and MIKLÓS GYURANECZ[1]*

[1] Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungária krt. 21, H-1143, Budapest, Hungary

[2] SCG Diagnostics Ltd., Délegyháza, Hungary

[3] Department of Pathology, University of Veterinary Medicine, Budapest, Hungary

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ABSTRACT

The control of *Mycoplasma hyorhinis* infection relies mainly on antimicrobial therapy. However, the antibiotic susceptibility testing of the bacteria is usually not performed before applying the treatment, and thus therapeutic failures are not uncommon. In the case of *M. hyorhinis*, several antibiotic-resistance-related single nucleotide polymorphisms (SNPs) are known but assays for their detection have not been described yet. The aims of the present study were to investigate macrolide- and lincomycin-resistance-related SNPs in Hungarian *M. hyorhinis* isolates and to develop mismatch amplification mutation assays (MAMA) to detect the identified resistance markers. Minimal inhibitory concentrations (MIC) of different drugs and whole genome sequences of 37 *M. hyorhinis* isolates were used to find the resistance-related mutations. One MAMA assay was designed to detect the mutation of the 23S rRNA gene at nucleotide position 2058 (*Escherichia coli* numbering). For further evaluation, the assay was challenged with 17 additional isolates with available MIC data and 15 DNA samples from clinical specimens. The genotypes of the samples were in line with the MIC test results. The developed assay supports the practice of targeted antibiotic usage; hence it may indirectly reduce some bacterial resistance-related public health concerns.

KEYWORDS

Mycoplasma hyorhinis, antibiotic susceptibility, macrolides, rapid detection, MAMA, SNP

INTRODUCTION

Mycoplasma hyorhinis is a facultative pathogenic *Mycoplasma* species causing polyserositis and arthritis in weaners and finishers from 3 to 10 weeks of age (Rovira et al., 2010). It can also induce more severe lung lesions in animals infected with *Mycoplasma hyopneumoniae* or porcine reproductive and respiratory syndrome virus (Lee et al., 2016; Luehrs et al., 2017). The infection results in significant economic losses worldwide (Hansen et al., 2010). A vaccine is available against *M. hyorhinis* infection (Ingelvac MycoMAX™, Boehringer Ingelheim Animal Health USA Inc., Duluth, USA) in the United States, but the reduction of incidence still relies mainly on prevention by providing optimal housing conditions and the protection against other, viral pathogens (Palzer et al., 2015). A proper antibiotic treatment

*Corresponding author.
E-mail: m.gyuranecz@gmail.com

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can decrease the severity of clinical signs of the infection and, thus, reduce the economic losses. The *in vitro* antibiotic susceptibility of *Mycoplasma* species is tested by the broth or agar microdilution method. Both methods are labour-intensive and time-consuming techniques which require the prior isolation of the pathogen. Therefore, antibiotic susceptibility testing prior to treatment is often not possible and antimicrobials are chosen empirically, which can lead to the development of antimicrobial resistance (Kobayashi et al., 2005). Further difficulties are the lack of standard breakpoints and international standards which complicate the interpretation and comparison of the results (Hannan, 2000; Kreizinger et al., 2017; Bekő et al., 2019).

Macrolide and lincosamide antimicrobials inhibit the bacterial protein synthesis by binding to the 50S subunit of the ribosome. Resistances to both of these groups are often linked because of the overlap of the binding sites at the 23S rRNA of the 50S subunit (Pyörälä et al., 2014). *Mycoplasma hyorhinis* is intrinsically resistant to 14-membered macrolides (Ter Laak et al., 1991; Wu et al., 2000) and a decreased susceptibility to 15- and 16-membered macrolides has also been reported (Kobayashi et al., 1996, 2005; Jang et al., 2016; Bekő et al., 2019; Rosales et al., 2020). *In vitro* selected resistant mutants and Japanese field isolates have been investigated for resistance-related point mutations (SNPs) in the 23S rRNA gene and the L4, L22 protein genes of the 50S ribosome by Kobayashi et al. (2005). A point mutation A2059G (according to *Escherichia coli* numbering) at the V domain of the 23S rRNA was found in *in vitro* mutants and field isolates as well. In addition, point mutations between nucleotide positions 2058 and 2062 (*E. coli* numbering) were connected to macrolide and lincosamide resistance in several *Mycoplasma* species (Sulyok et al., 2017; Chernov et al., 2018; Gautier-Bouchardon, 2018; Bekő et al., 2020a; Felde et al., 2020).

The aim of the present study was to investigate Hungarian *M. hyorhinis* isolates for macrolide and lincomycin resistance-related SNPs and to develop rapid and cost-effective molecular biological assays for their detection.

MATERIALS AND METHODS

Mycoplasma hyorhinis strains, clinical samples and susceptibility testing

In this study, 55 *M. hyorhinis* isolates from previous studies (Bekő et al., 2019; Földi et al., 2020) or collected in 2019–2020 and the type strain NCTC 10130 (GenBank Accession number: LS991950.1) were used. DNA extracted from the original clinical specimens of 15 clinical isolates was also examined (*Supplementary Table 1*). All samples originated from Hungary except for two isolates, MycSu60 and MycSu213 which originated from Slovakia. Ethical approval was not required for the study as the samples were taken either during routine diagnostic examinations with the consent of the owners or in slaughterhouses. The isolates collected in 2019–2020 were cultured as described before (Bekő et al., 2019). DNA extraction from the clinical specimens and the field isolates was performed using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions for Gram-negative bacteria. All isolates were identified by polymerase chain reaction (PCR) targeting the *p37* gene of *M. hyorhinis* (Assunção et al., 2005). The presence of other *Mycoplasma* species was excluded by a universal *Mycoplasma* PCR system targeting the 16S/23S rRNA intergenic spacer region in Mollicutes (Lauerman et al., 1995) followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, USA), then sequence analysis and BLASTN search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The DNA samples were tested for the presence of *M. hyopneumoniae*, *Mycoplasma hyosynoviae* and *Mycoplasma flocculare* as well by a species-specific PCR according to Assunção et al. (2005). Detailed information about the *M. hyorhinis* isolates used in the study can be found in *Supplementary Table 1*.

The antibiotic susceptibility of 38 isolates to macrolides (tylosin, tilmicosin, tylvalosin, tulathromycin, gamithromycin) and lincomycin has been previously determined by the broth microdilution method (Bekő et al., 2019) according to Hannan (2000). The susceptibility of further 17 isolates to at least one macrolide and in some cases to lincomycin was determined in this study as described before (Bekő et al., 2019). Briefly, 10^4 colour changing units/mL (CCU/mL) of the isolates were tested in duplicates. The antibiotics originated from VETRANAL (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) except for tylvalosin (Aivlosin), which was purchased from ECO Animal Health Ltd. (London, UK). Stock solutions of 1 mg/mL were prepared in sterile distilled water, and stored at −70 °C, according to the recommendations of Hannan (2000). For each test, freshly prepared twofold dilutions of the antibiotics were made in the concentration range of 0.25–64 µg/mL. The *M. hyorhinis* type strain (NCTC 10130) was included in each plate to confirm the validity of the results. The initial minimal inhibitory concentration (MIC) of each strain was defined as the lowest concentration of the antibiotic that completely inhibited the growth at the time of colour change in the growth control. Since there are no official breakpoints available for the antibiotic susceptibility testing of *M. hyorhinis,* previously published breakpoints for *M. hyopneumoniae* or other porcine respiratory pathogens were used (Hannan et al., 1997; Felde et al., 2020; Vilaró et al., 2020). MIC values ≥4 µg/mL of tylosin, tylvalosin, gamithromycin and lincomycin were considered high (Hannan et al., 1997; Felde et al., 2020), while for tilmicosin and tulathromycin higher breakpoints were suggested, thus MIC values ≥16 µg/mL were considered high (Vilaró et al., 2020).

Development of the assay

Single nucleotide polymorphisms associated with high (genotype H) or low (genotype L) MIC values were identified by analysing the whole genome sequences of 37 *M. hyorhinis*

field isolates and the type strain (NCTC 10130). The sequencing was performed as described before (Földi et al., 2020).

The 23S rRNA and 50S ribosomal protein L4 and L22 coding genes were previously connected to macrolide and lincomycin resistance in *Mycoplasma* species (Kobayashi et al., 2005; Sulyok et al., 2017; Bekő et al., 2020a; Felde et al., 2020). These genes were aligned and analysed in Geneious Prime software version 2019.2.1 (Kearse et al., 2012) to detect SNPs. In the protein-coding genes only SNPs resulting in amino acid change were analysed, but in the 23S rRNA gene all SNPs were investigated. The numbering of nucleotide position according to *E. coli* strain K-12 substrain MG1655 (GenBank accession number: NC_000913) is indicated in parentheses throughout the text to enable the comparison of our results with data of the literature. Nevertheless, nucleotide positions are discussed in the text based on the numbering of individual genes of the *M. hyorhinis* type strain (NCTC 10130) in order to avoid misunderstandings due to gaps in the alignment. To identify potentially resistance-related SNPs, the correlation between the MIC values and the occurrence of mutations were analysed. Mutations were considered resistance-related when they occurred more frequently with increasing MIC values or exclusively in isolates with high MIC values.

To detect the resistance-related SNPs, mismatch amplification mutation assays (MAMA) were designed. In brief, MAMA is a molecular biological tool to discriminate SNP in bacteria. The assay is based on competing allele-specific primers, which are SNP specific at the 3′ end and contain a single destabilising mismatch at the antepenultimate position of the 3′ end to enhance the discriminatory power. One of the allele-specific primers is marked with an additional 15- to 20-bp-long GC clamp that increases the melting temperature and the size of the amplicon as well. The temperature shift is detected on a real-time PCR platform in the presence of an intercalating fluorescent DNA dye (melt-MAMA) and the size difference is revealed in agarose gel electrophoresis (agarose-MAMA) (Birdsell et al., 2012; Sulyok et al., 2018; Bekő et al., 2020b; Felde et al., 2020). Primers were designed based on the whole genome sequences of *M. hyorhinis* field isolates and the type strain (NCTC 10130) in the Geneious Prime software version 2019.2.1 (Kearse et al., 2012). Melting temperatures and suitability were calculated using the NetPrimer software (Premier Biosoft International, Palo Alto, CA, USA) and the specificity of the primers was checked with BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The agarose-MAMA PCR mixture consisted of 5 µL 5 × Green GoTaq Flexi buffer (Promega Inc., Madison, WI, USA), 2.5 µL $MgCl_2$ (25 mM; Promega), 0.5 µL dNTP (10 mM; Qiagen), 0.2 µL GoTaq Polymerase (5 U/µL, Promega), 1 µL DNA template and primers according to Table 1 with a total volume of 25 µL. The PCR was performed on a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The thermocycling parameters were 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The final elongation step was performed at 72 °C for 5 min. After amplification, 5 µL of each reaction mixture were submitted to electrophoresis in 3% agarose gel (MetaPhor Agarose, Lonza Inc., Rockland, ME, USA) using a 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific, Waltham, USA) as a molecular weight marker. The amplified PCR products were visualised with ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics Inc., Torrance, CA, USA), under UV light. The melt-MAMA PCR mixture contained 4.5 µL 2x HSTaq Mix (PCR Biosystems Ltd., London, UK), 0.5 µL EvaGreen (20X, Biotium Inc., Hayward, USA), 1 µL target DNA and primers according to Table 1 in a final volume of 10 µL. The thermocycling parameters were 95 °C for 1 min, followed by 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 5 s. The PCR products were subjected to melt analysis using a dissociation protocol comprising 95 °C for 15 s, followed by 0.3 °C incremental temperature ramping from 55 °C to 95 °C. The real-time PCRs were performed using Bio-Rad C1000 Touch™ Thermal Cycler, CFX96™ Real-Time System (Bio-Rad Laboratories Inc.). The EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature.

Validation of the assay

In order to validate the developed assays, PCRs on 37 field isolates with already established MIC values (Bekő et al., 2019; Földi et al., 2020) were performed. The resulting genotypes (genotypes H and L) were compared with the examined gene sequence.

In order to test the sensitivity of the developed assays, tenfold dilutions of each genotype (*M. hyorhinis* type strain (NCTC 10130) for genotype L and MycSu24 for genotype

Table 1. Primer sequences, volumes per reaction and results of the designed mismatch amplification mutation assay

Primer name	Primer sequence (5′-3′)[a]	Primer volume (µL)[b]		Genotype	Amplicon size (bp)	Melting temperature range of amplicons (°C)
		melt-MAMA	agarose-MAMA			
23S-L	TTACCCGCATCAAGACtAA	1.6	4	L	119	78.3–78.9
23S-H	gggcgggcgggcgggcgggcTTACCCGCATCAAGACaAg	0.4	1	H	139	81.3–82.2
23S-C	TCCACTAGAACTAGCGTCCC	0.4	1			

[a] The GC-clamp, the targeted point mutation and the destabilising point mutation are in lower case; [b] Primer concentration: 10 pmol/µL.

H) were used in the range of 10^8–10^0 copy number/µL. The template copy number was calculated with the help of an online tool (Staroscik, 2004) by measuring the concentration of the DNA of pure *M. hyorhinis* culture by Nanodrop (2000) Spectrophotometer (Thermo Fisher Scientific). The lowest DNA concentrations detectable with agarose gel electrophoresis or yielding melting temperature specific for the genotype were considered the detection limit of the assays. The specificity was tested by including *M. hyopneumoniae*, *M. hyosynoviae* and *M. flocculare* in the analyses.

For further evaluation, an additional set of 17 strains was tested and the assay was also challenged with 15 DNA samples from clinical specimens. The genotypes resulting from the DNAs of the clinical specimens were compared to the genotypes of the isolates derived from these samples. The tests were considered to give false results in those cases, when the resulting genotype was not in line with the tested MIC data. For the clinical specimens the tests were considered to give false results when the resulting genotype was not in line with the genotype of the isolate derived from the sample.

RESULTS

After aligning the corresponding genes (23S rRNA and L4, L22 50S ribosomal protein genes) of the *M. hyorhinis* type strain (NCTC 10130) and the 37 Hungarian field isolates, only one SNP, at nucleotide position A2066G (A2058G according to *E. coli* numbering), in the 23S rRNA gene (GenBank accession number: MW498281–MW498317), was found to be related to macrolide and lincomycin resistance. The primer sequences, fragment lengths and melting temperatures of the developed MAMA, specific for the SNP, are listed in Table 1. The developed assay successfully distinguished genotypes H and L (Fig. 1) in the 55 clinical isolates, the type strain (NCTC 10130) and the 15 DNA samples from the clinical materials. The sensitivity of the assay was 10^3 copy numbers/reaction for both genotypes in the melt and the agarose assay as well. No cross reactions were detected in the agarose-MAMA; however, *M. flocculare* showed a genotype L specific melting temperature profile in the melt-MAMA.

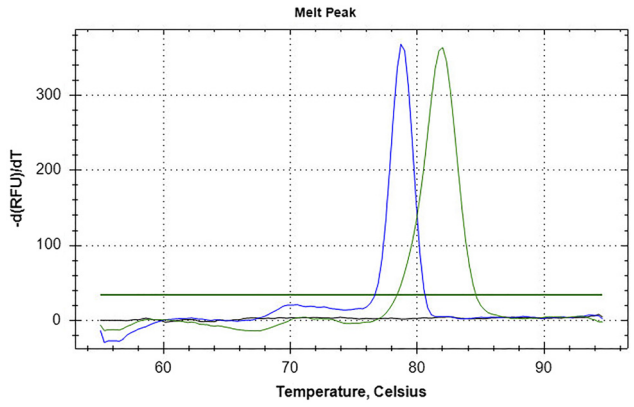

Fig. 1. Determination of macrolide and lincomycin susceptibility by the developed MAMA assay. Melting temperatures of the amplicons for genotype L (*Mycoplasma hyorhinis* type strain NCTC 10130, blue line, Tm 78.6 °C) and genotype H (MycSu24, green line, Tm 81.6 °C) were unique. The negative control (black line) was not amplified. X axis: melting temperature (°C), Y axis: negative value of the change in relative fluorescence units (RFU) over the change in temperature

In the case of the clinical isolates, both the agarose- and the melt-MAMA gave the same results. No false reactions were detected for tylosin, tulathromycin and lincomycin. False results were detected in one case for gamithromycin (MycSu128) and tilmicosin (MycSu94) and in two cases for tylvalosin (MycSu141, MycSu161). In MycSu128, MycSu141 and MycSu161, genotype H was detected; the isolates showed elevated MIC values against five of the examined antimicrobials, but were susceptible to the remaining one. While MycSu94 was inhibited at an elevated MIC value of tilmicosin, the isolate was susceptible to all the other examined antimicrobials and presented genotype L in the assays (*Supplementary Table 1*).

For the clinical specimens, the test of one DNA sample (Su21) showed a mixed genotype by the agarose-MAMA, while genotype H was determined in the corresponding clinical isolate (MycSu101). The microdilution test showed elevated MIC values of the tested antibiotics against of MycSu101. At position 2066 in the whole genome sequence of this isolate, a guanine nucleotide was found, and in line with that, the assays of the pure culture resulted in genotype H. The test of the clinical specimen Su21, however, resulted in genotype L in the case of the melt-MAMA and double amplicons in the size of both genotypes appeared by the agarose-MAMA.

DISCUSSION

The control of *M. hyorhinis* infections depends mainly on the use of antimicrobial agents. However, a targeted antibiotic treatment is usually inaccessible. Elevated MICs of macrolides or lincomycin to *M. hyorhinis* isolates have been reported from Japan, Korea, Hungary, Spain, Italy and Portugal since 1996 (Kobayashi et al., 1996, 2005; Jang et al., 2016; Bekő et al., 2019; Rosales et al., 2020), and point mutations associated with elevated MIC values were described by Kobayashi et al. (2005). In the present study, among Hungarian *M. hyorhinis* field isolates a transversion substitution in the previously examined region, at position 2066 (2058 *E. coli* numbering) of the 23S rRNA was found, an adenine was replaced by a guanine in the isolates with elevated MIC values. A mismatch amplification mutation assay was designed to detect this SNP in *M. hyorhinis*. The developed MAMA is suitable to detect the SNP in agarose gel based on template size differences, and in real-time PCR based on template melting temperature differences. All isolates showed uniformly high or low MIC results for all examined macrolides and lincomycin in the broth microdilution tests except for four (MycSu94, MycSu128,

MycSu141, MycSu161) where the MIC of one antibiotic differed (*Supplementary Table 1*, highlighted with orange). Consequently, the results of the developed MAMA corresponded to the MIC values of the majority of the antibiotics in these samples. In the case of MycSu94 we assume that an evolving resistance is indicated by the elevated MIC value of tilmicosin, which did not show up in a nucleotide change at the examined position. Other SNPs or resistance mechanisms can induce the elevated MIC value, but neither can be determined based on one isolate. Against MycSu128, MycSu141 and MycSu161, we would not advise the use of macrolides, because besides the susceptibility to one of the examined macrolides, highly elevated MIC values (≥64 µg/mL) were observed for the majority of the antimicrobials. Therefore, we believe that the result of the assay (genotype H) supports the proper antibiotic treatment.

The assay is suitable for the examination of field isolates and DNA extracted directly from clinical specimens. In one case the detected genotype of the clinical specimen (in Su21 both genotypes were observed with the agarose-MAMA) differed from the genotype of the isolate that derived from it (in MycSu101 only genotype H was present). This can be explained by the filter cloning step of the isolation: *in vitro* testing and sequencing were made from the same one-colony broth, but based on our observation, in the clinical specimen probably more populations with different antibiotic susceptibility profiles were present.

In order to avoid the misinterpretation of the results due to the presence of *M. flocculare* in the melt-MAMA, we advise to submit the samples to a species-specific PCR first, and in the presence of *M. flocculare* only the agarose-MAMA is recommended. Clinical specimens with low specific DNA concentration (below 10^3 copy numbers/reaction) may show false negative results, therefore in these cases isolation prior to the test is suggested.

The developed assay is convenient, cost-effective and provides data on the susceptibility of *M. hyorhinis* to critically important antimicrobials in a significantly shorter time than the conventional MIC tests. The presented MAMA can be performed directly on the clinical specimens using standard laboratory equipment and methods, therefore it is suitable for routine diagnostic purposes.

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SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1556/004.2021.00026.

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