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Development of molecular biological tools for the rapid determination of antibiotic susceptibility of *Mycoplasma hyopneumoniae* isolates

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ABSTRACT

Mycoplasma hyopneumoniae is the etiologic agent of porcine enzootic pneumonia, a contagious respiratory disease, causing significant economic losses worldwide. Antibiotic treatment is commonly utilised in the pig industry to control *M. hyopneumoniae* infection. Since the conventional antibiotic susceptibility test is time-consuming, taking up to weeks' period, antibiotics are usually empirically chosen.

Certain single nucleotide polymorphisms in the *parC* (C239A/T, G250A) and *gyrA* (G242C, C247 T, A260 G) genes show correlation with decreased fluoroquinolone susceptibility by the change of the target site. Furthermore, the nucleotide alteration A2059 G in the 23S rRNA sequence correlates with significantly decreased macrolide and lincosamide susceptibility of *M. hyopneumoniae*. Mismatch amplification mutation assays (MAMA) and high resolution melt (HRM) analysis, capable to detect the mentioned resistance markers, were developed in the present study, in order to provide susceptibility data in a considerably shorter time than the conventional methods. The results of the MAMA and HRM assays were congruent with the results of the conventional antibiotic susceptibility method of the tested *M. hyopneumoniae* field isolates. The sensitivity of the MAMAs was 10^3-10^4 copy numbers, while that of the HRM assay was 10^5-10^6 copy numbers.

To the best of our knowledge this was the first time that MAMA and HRM assays were developed for the rapid detection of decreased fluoroquinolone, macrolide or lincosamide susceptibility in *M. hyopneumoniae* strains.

1. Introduction

Mycoplasma hyopneumoniae is the causative agent of enzootic pneumonia, a contagious respiratory disease causing significant economic losses worldwide (Silva et al., 2019). Beside vaccination, which is not capable to completely prevent colonisation of the respiratory tract (Thacker et al., 1998; Maes et al., 2017), antibiotic treatment is frequently used in the disease control. Several antibiotics (*e.g.* pleuromutilines or tetracyclines) are effective against *M. hyopneumoniae* (Maes et al., 2008, 2017), however mycoplasmas are naturally resistant against certain agents (*e.g.* ß-lactams, glycopeptides) (Gautier-Bouchardon, 2018). Since isolation and *in vitro* antibiotic susceptibility testing of *M. hyopneumoniae* is time-consuming and fastidious, the antibiotics are rather empirically chosen. However, the inappropriate use of the agents may contribute to the emergence of resistance nor is it cost-effective. Acquired resistance of *M. hyopneumoniae* was observed against fluoroquinolones, macrolides and lincosamides in the past decades (Stakenborg et al., 2005; Le Carrou et al., 2006; Vicca et al., 2007; Gautier-Bouchardon, 2018).

Single nucleotide polymorphisms (SNPs) can cause conformation changes of the target regions of certain antibiotics, leading to resistance against the agent (Pirmohamed and Park, 2001). SNPs resulting in amino acid alterations in the *parC* and *gyrA* genes show correlation with decreased fluoroquinolone susceptibility of *M. hyopneumoniae* (Felde et al., 2018). Furthermore, substitutions in the 23S rRNA sequence play an important role in decreased susceptibility to macrolides and lincosamides in mycoplasmas (Stakenborg et al., 2005; Felde et al., 2018). Molecular biological methods like mismatch amplification mutation assay (MAMA) or high resolution melt (HRM) analysis are able to detect the SNPs correlating with the decreased antibiotic susceptibility (Sulyok et al., 2018). The aim of the study was to design molecular biological assays for the rapid detection of nucleotide substitutions showing relation with decreased antibiotic susceptibility in *M. hyopneumoniae*.

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2. Materials and methods

2.1. Samples

Porcine lung samples with typical mycoplasmal lesions were collected from Hungarian slaughterhouses with the permission of the owners. Friis broth medium was used for the isolation of *M. hyopneumoniae* (Friis, 1975) as previously described (Felde et al., 2018). DNA extraction was performed from stationary phase broth media with a maximum of three passages, using the QIAamp DNA mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. The DNA samples were used as templates for all assays described in the study.

Antibiotic susceptibility profiles of 44 Hungarian *M. hyopneumoniae* isolates and the type strain (NCTC 10,110) were defined previously by microbroth dilution method according to Hannan (2000), and decreased susceptibility to fluoroquinolones, macrolides and lincosamides was detected in certain strains (Felde et al., 2018). Since there is no official breakpoint for the antibiotic susceptibility testing of *M. hyopneumoniae,* MIC values suggested in previous publications were used to categorize the susceptibility of the strains (Felde et al., 2018). Therefore, strains, which were inhibited by ≥2 μg/ml MIC values of fluoroquinolones and/or ≥4 μg/ml MIC values of macrolides, were considered resistant, while strains inhibited by lower MIC values were considered susceptible according to Hannan et al. (1997). Both initial MIC values (defined when the growth control changed colour) and final MIC values (defined in the absence of further colour change) were recorded. The type strain with low minimum inhibitory concentration (MIC) values and field isolates with significantly increased MIC values of fluoroquinolones (MycSu1; 15; 18; 20; 39; 50), macrolides and lincosamides (MycSu18) were involved in the development of the molecular assays. The initial and final MIC values of fluoroquinolones, macrolides and lincosamides are summarised in Supplementary Table 1.

2.2. MAMA and HRM design

Previously determined genetic markers, correlating with decreased fluoroquinolone (C239 T/A and G250A in the *parC* gene; G242C, C247 T and A260 G in the *gyrA* gene, according to numbering of *Escherichia coli* strain K-12 substrain MG1655, GenBank accession number CP014225), macrolide and lincosamide (A2059 G in the 23S rRNA; Supplementary Table 1) susceptibility in *M. hyopneumoniae* (Felde et al., 2018) were targeted by mismatch amplification mutation assay (MAMA) and high resolution melt (HRM) assay.

In the present study, MAMAs were designed and tested for the detection of SNPs related to fluoroquinolone, macrolide and lincosamide resistance using competing primers (Birdsell et al., 2012; Sulyok et al., 2018). The assays were designed to show more than 2 °C difference of the melting temperatures of the different genotypes, containing either the original nucleotide sequence or the SNP. PCR mixture of the assays composed of 2 μl 5X Colour-less GoTaq Flexi Buffer (Promega Inc., Madison, WI), 1.5 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, Table 1), 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 the following for the MAMAs, 95 °C for 10 min, followed by 30 cycles (*gyrA* 242; 260, 23S rRNA) or 34 cycles (*parC* 239;250, *gyrA* 247) of 95 °C for 15 s and 45 s of 60 °C (*gyrA*247), or 60 s of 55 °C (*parC*239;250)/ 56 °C (*gyrA*242;260)/ 60 °C (23S rRNA). The PCR products were subjected to melt analysis using a dissociation protocol comprising the steps 95 °C for 15 s, followed by incremental temperature ramping of 0.3 °C from 60 °C to 95 °C. EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature. The nucleotide sequences of the primers and the utilised primer ratios are summarised in Table 1. Melt-MAMAs were carried out on an Applied Biosystems Step-One Plus real-time PCR system with StepOne Software™ v2.2.2.

Beside individual SNPs, a "hot-spot" region with two possible mutations in the *parC* gene was identified in strains with declined fluoroquinolone susceptibility (Le Carrou et al., 2006). This region of the *parC* gene (containing the nucleotide positions 239 and 250) was targeted by HRM assay (Palais et al., 2005; Sulyok et al., 2018). The primers used during an HRM assay are summarised in Table 1. PCR mixture of the assays composed of 2 μl 5X Colour-less GoTaq Flexi Buffer (Promega Inc., Madison, WI), 1.5 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, Table 1), 0.08 μl GoTaq G2 Flexi

Table 1
Characteristics of the primers and products used in the developed MAMA and HRM assays.

Assay type	Antibiotic group	Primer name with target gene and SNP[a]	Primer sequence (5'-3')	Primer[b] (μl)	T_a (°C)	Type	T_m (°C)	Amplicon (bp)
MAMA	Fluoroquinolones	gyrA_242C	TTGAGCCATTCGCACCA	0.15	56	S	77.5-79.2	58
		gyrA_242S	GAAAATACCATCCTCACGG	0.15		R	82.9-83.1	73
		gyrA_242R	ggggcggggcggggcGAAAATACCATCCTCAAGC	0.6				
		gyrA_247C	TCCGCTAGAATTGTTGGTGA	0.15	60	S	77.1-78.1	73
		gyrA_247S	ACCATCGATTCATAGACAGCAG	0.3		R	81.0-81.7	88
		gyrA_247R	ggggcggggcggggcACCATCGATTCATAGACAGCAA	0.15				
		gyrA_260C	TTGTTGGTGATGTTCTTGG	0.15	56	S	77.9-79.2	70
		gyrA_260S	CATTCGCACCATCGCTT	0.6		R	82.6-82.8	85
		gyrA_260R	ggggcggggcggggcCATTCGCACCATCGTTC	0.15				
		parC_239C	AATCTGCTAGAGTTGTCGGTG	0.15	55	S	75.0-75.5	74
		parC_239S	CAAGAGCATC/TATAGATTGgAG	0.15		R	79.8-80.1	86
		parC_239R	ggggcggggcggggcCAAGAGCATC/TATAGATTGcAA/T	0.15				
		parC_250C	AATCTGCTAGAGTTGTCGGTG	0.15	55	S	77.0-77.9	84
		parC_250S	GCAAGTCTGACAAGAGCgTC	0.15		R	80.7-81.1	96
		parC_250R	ggggcggggcggggcGCAAGTCTGACAAGAGCcTt	0.15				
	Macrolides, Lincosamides	23S_2059C	CCACCTATCCTACACATAATAAACC	0.15	60	S	76.9-77.2	85
		23S_2059S	GTTA/TCCCGCATCAAGACaAA	0.15		R	79.0	97
		23S_2059R	ggggcggggcggggcGTTA/TCCCGCATCAAGACtAg	0.6				
HRM	Fluoroquinolones	parC_239-250_R	CATTCCTGGGCAAGTCTG	0.25	55	S	78.6-78.7	93
		parC_239-250_F	AATCTGCTAGAGTTGTCGGTG	0.25		R	78.1-78.5	

Nucleotide sequences, annealing temperatures and ratios of the primers used for the assays and results including melting temperatures, sizes of the amplicons. Artificial GC-tail of the primers, enabling the differentiation of the resistant genotypes are presented with lowercased letters.
Abbreviations: S-sensitive; R-resistant; C-consensus; T_a-annealing temperature; T_m-melting temperature.
a-nucleotide position according to *E. coli* numbering; b-primer amounts per sample in melt-MAMAs and HRM.

DNA polymerase (5 U/µl; Promega Inc.) and 1 µl DNA template with a final volume of 10 µl. Thermocycling parameters were the following for the HRM assay, 95 °C for 10 min followed 34 cycles of 95 °C for 15 s and 55 °C for 60 s. The PCR products were subjected to melt analysis, with an incremental temperature ramping of 0.1 °C from 60 °C to 95 °C. HRM profiles were analysed using High Resolution Melt software, version 3.0.1 (Thermo Fisher Scientific Inc.). Fluorescent values were normalised according to user-adjustable pre- and post-melting temperature intervals.

2.3. Validation of the assays

In order to test the sensitivity of the MAMA and HRM assays, tenfold dilutions of the type strain and strains showing increased MIC values (MycSu1, 15, 18, 20, 39, and MycSu50) were used in the range of 10^6-10^1 copy number/µl. The template copy number was calculated with the help of an online tool (Staroscik, 2004) based on the length of the whole genome sequence and concentration of DNA of pure *M. hyopneumoniae* cultures measured by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.). The lowest DNA concentrations (template copy number) yielding melting temperature (T_m) specific to the genotype were considered the detection limits of the assays. The specificity of the assays was tested by including pathogen and non-pathogen porcine *Mycoplasma* species in the analysis: *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare*. The developed assays were tested on *M. hyopneumoniae* isolates with previously accomplished microbroth dilution method also. Genotypes identified by the MAMA and HRM assays were compared with MIC values of the 38 *M. hyopneumoniae* isolates.

3. Results

All of the developed MAMA and HRM assays successfully differentiated the antibiotic sensitive and resistant genotypes (Fig. 1), and correlated with the results of the previously accomplished microbroth dilution method. Melting temperatures and cycle threshold (ct) values of the assays are presented in Supplementary Table 1.

Five melt-MAMAs (targeting SNPs in the *gyrA* and *parC* genes) and an HRM assay (targeting "hot-spot" region in the *parC* gene (Fig. 2) were developed for the rapid detection of decreased fluoroquinolone susceptibility of *M. hyopneumoniae* isolates and one melt-MAMA (targeting SNP in the 23S rRNA region) was established for the detection of macrolide and lincosamide susceptibility. Since the *gyrA* gene contained several silent mutations beside the mentioned sense mutations, this region was not suitable for the development of an HRM assay.

The sensitivity of the assays was 10^3 copy numbers/reaction for both the sensitive and resistant genotypes in case of MAMAs targeting the nucleotide substitution C247 T in the *gyrA* and G250A in the *parC* gene. However, the sensitivity of the MAMAs targeting the alterations A260 G in the *gyrA* gene and C239 T/A in the *parC* gene was 10^3 copies/reaction for the sensitive genotype and 10^4 for the resistant genotype. Furthermore, the sensitivity was 10^3 copies/reaction for the resistant genotype and 10^4 for the sensitive genotype in case of MAMA targeting the nucleotide change G242C in the *gyrA* gene. The sensitivity of the MAMA differentiating sensitive and resistant genotypes according to the nucleotide alteration A2059 G in the 23S rRNA sequence was 10^3 copies/reaction for both genotypes. Normalization interval of 74.0–74.5 °C and 81.0–81.5 °C were used in the HRM analysis of the amplicons. The sensitivity of the HRM assay was 10^5 copies/reaction for the sensitive genotype and for most types of the resistant genotype (C239A or G250A), while the sensitivity of the system was 10^6 copies per reaction in case of C239 T alteration of the *parC* gene (resistant genotype). Melting temperature ranges and amplicon sizes are summarised in Table 1. Cross-reactions were only observed using the MAMAs targeting the nucleotide substitutions G250A in the *parC* gene (*M. flocculare*) and A2059 G in the 23S rRNA sequence (*M. flocculare*, *M. hyorhinis*), none of the other assays showed false positivity.

4. Discussion

Since vaccination does not provide complete protection against *M. hyopneumoniae* infection (Meyns et al., 2004, 2006; Stakenborg et al., 2006; Villarreal et al., 2009), targeted antimicrobial therapy plays an important role in the control of enzootic pneumonia in the pig industry. Antimicrobials are able to reduce clinical symptoms, moderate the mortality rate and increase weight-gain of the animals (Maes et al., 1996; Pallarés et al., 2015). However, conventional antibiotic susceptibility testing is usually not accomplished before the therapy, because it is extremely time-consuming, could take even up to months (Hannan, 2000). Therefore, the choice of the antimicrobial is usually based on earlier experiences.

In the present study, six MAMAs and one HRM assay were successfully designed for the fast detection of SNPs correlating with fluoroquinolone, macrolide and lincosamide resistance in *M. hyopneumoniae*. Although the mentioned agents are discouraged to be used in veterinary praxis, these agents showed decreased effectiveness against *M. hyopneumoniae* before (Gautier-Bouchardon, 2018; Felde et al., 2018) and SNPs showing correlation with decreased antibiotic susceptibility of *M. hyopneumoniae* were described previously only in case of the mentioned antibiotics (Gautier-Bouchardon, 2018). The results of the MAMA and HRM assays were congruent with that of the conventional microbroth dilution test. MAMA systems are cost-effective and widely available methods for diagnostic purposes. The developed HRM assay serves as confirmation option in well-equipped laboratories. Both MAMA and HRM are standard methods providing results in considerably shorter time (hours after isolation) compared to the conventional antibiotic susceptibility tests (requiring months to perform and special conditions which still need to be standardized).Furthermore, these MAMA and HRM systems can serve as examples for further assay developments. Since HRM assays are designed to examine "hot spot" regions, containing closely localised SNPs within a sequence; and only one SNP showed correlation with decreased macrolide and lincosamide susceptibility of the Hungarian isolates, therefore no HRM test was developed for the investigation of the mentioned antibiotics.

According to the number of the observed amino acid alterations, two subgroups of the fluoroquinolone resistant genotype were defined. The presence of SNP in the *parC* gene correlated with moderately increased MIC values of fluoroquinolones, while the increase of MIC values was more pronounced in case of double substitutions in the *parC* and *gyrA* genes. Since both the single and double substitutions show correlation with increased MIC values, the use of all five MAMAs is suggested for the exclusion of decreased fluoroquinolone susceptibility. The target positions in the *parC* gene can be investigated either by HRM assay (detecting both C239A/T and G250A substitutions in a single investigation; although it requires special laboratory equipment), or by the two melt-MAMAs using the same temperature profile. Furthermore, two of the three nucleotide substitutions in the *gyrA* gene can be detected simultaneously, thus application of the developed assays can provide results within 10–12 hours. According to their sensitivity, the assays are suggested to be utilised for *M. hyopneumoniae* isolates instead of clinical material, therefore the observed cross-reactions can be ignored.

Although certain mutations in the target regions of the antibiotics seem to correlate with increased *in vitro* MIC values (Stakenborg et al., 2005; Le Carrou et al., 2006; Vicca et al., 2007; Gautier-Bouchardon, 2018), PCR-based susceptibility testing of *M. hyopneumoniae* has not been published before. Due to the limited number of publications and sequence data of *M. hyopneumoniae* strains showing decreased antibiotic susceptibility, the development of the MAMA and HRM assays is also limited and challenging. The MAMA and HRM assays described in the present study offer reliable guidance for antibiotic therapy against *M. hyopneumoniae*. The application of the developed assays for the determination of antibiotic susceptibility of circulating *M. hyopneumoniae* strains would improve the targeted treatment of enzootic

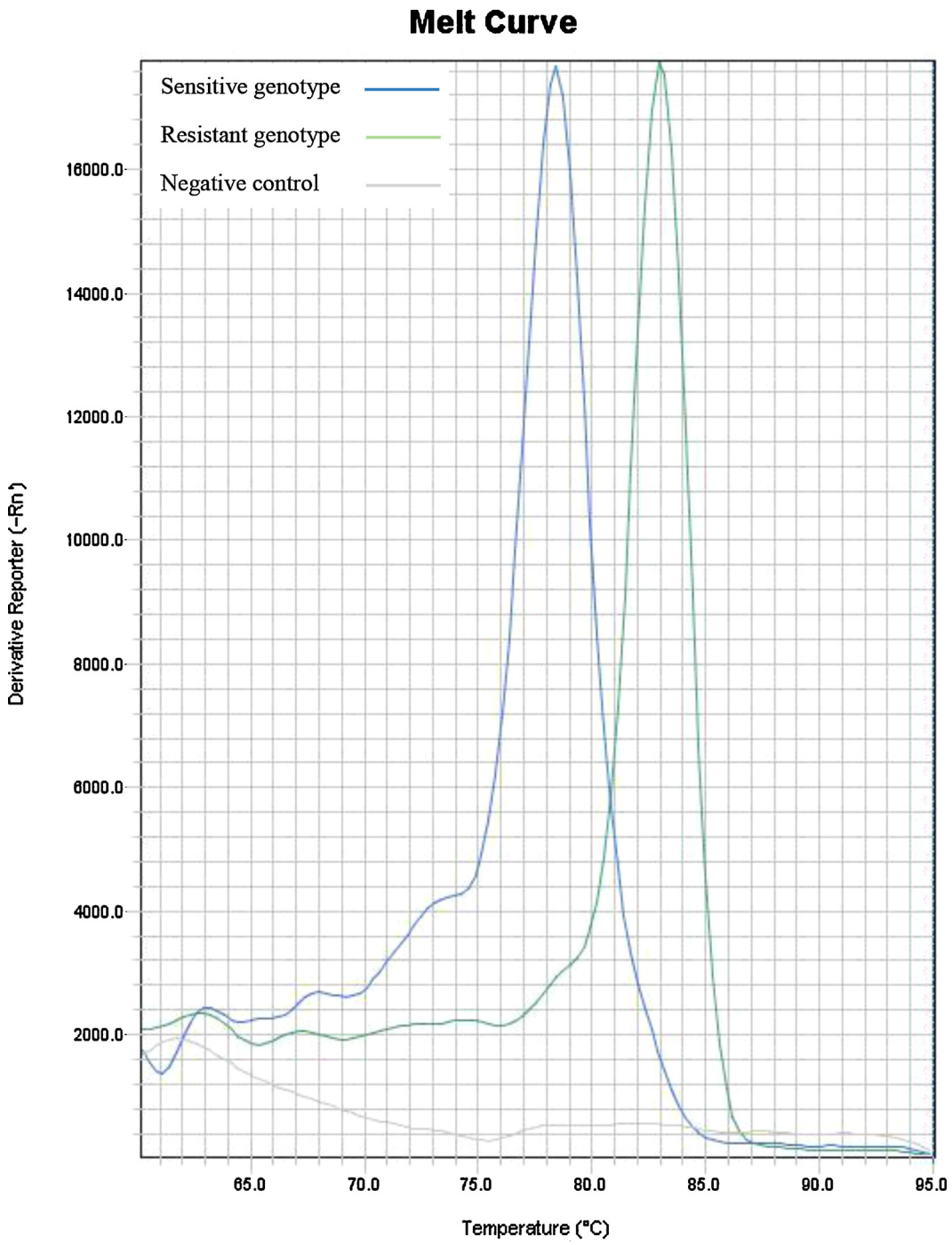

Fig. 1. Mismatch amplification mutation assay targeting the nucleotide substitution G242C in the *gyrA* gene of fluoroquinolone sensitive and resistant *M. hyopneumoniae* strains.
Fluoroquinolone sensitive type (blue lines) (Tm = 78.1–78.4 °C), fluoroquinolone resistant type (green lines) (Tm = 82.9–83.1 °C) and negative control (grey line)

pneumonia.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

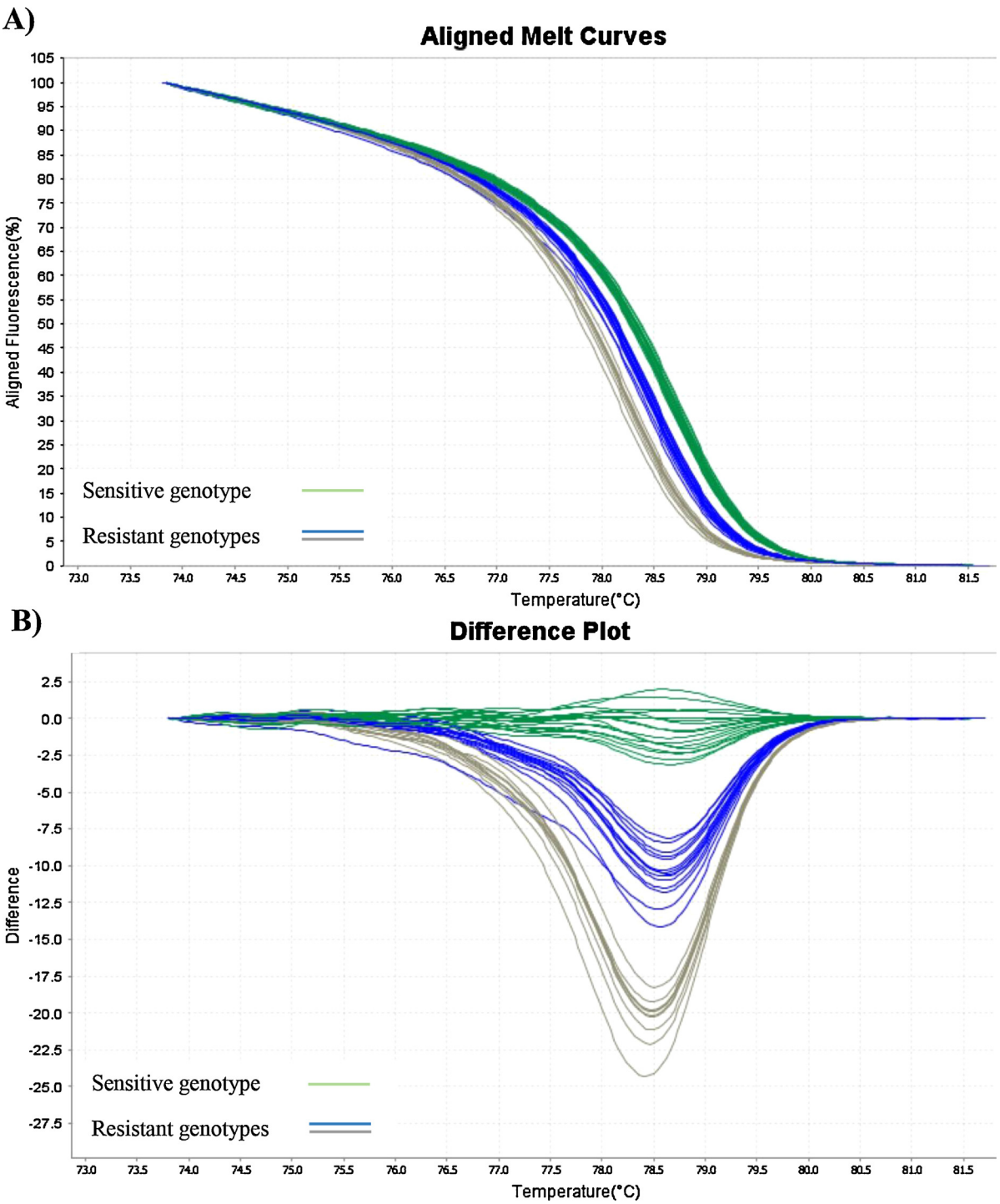

Fig. 2. High resolution melt analyses of *M. hyopneumoniae* strains for the detection of fluoroquinolone resistance.
A - Aligned melt curves and B - difference plots of the assay *parC* (hot spot region 239–250 of *parC*). Fluoroquinolone sensitive type (green lines) (Tm = 78.6–78.7 °C) and fluoroquinolone resistant type C239 T (blue lines) (Tm = 78.4–78.5 °C), C239A or G250A (grey lines) (Tm = 78.1–78.2 °C)

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