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Antimicrobial susceptibility and resistance mechanisms in Mannheimia haemolytica isolates from sheep at slaughter

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Simple Summary: *Mannheimia haemolytica* is a key bacterial pathogen contributing to important ruminant veterinary diseases and leading to a large proportion of the overall antimicrobial usage in cattle and sheep. The recent emergence of ovine strains with reduced susceptibility to antimicrobials, which could lead to treatment failures, increased costs of animal production and the dissemination of antimicrobial-resistant genes to other bacteria and possibly the environment, is of concern. This study investigates the levels of antimicrobial resistance of *M. haemolytica* isolated from clinical healthy sheep at slaughter and their genetic resistance mechanisms. Low levels of phenotypic resistance were detected for most of the antimicrobial tested but tetracycline (4.3%) and tylosin (89.1%). Few antimicrobial resistance determinants were found in the genome of six isolates, consisting in genes conferring resistance to tetracyclines (*tetH)*, aminoglycosides (*strA*) and sulfonamides (*sul2*), sometimes linked to the presence of plasmids but not always leading to resistance phenotypes. Our results suggest there is limited resistance in *M. haemolytica* strains of veterinary origin, but the presence of several resistance genes, some of which were present in mobile genetic elements that are known to play a major role in the dissemination of antimicrobial resistance in members of the *Pasteurellaceae* family, deserves further consideration.

Abstract: *Mannheimia haemolytica* is the main pathogen contributing to pneumonic pasteurellosis in sheep. The aim of this study was to investigate the antimicrobial resistance levels in *M. haemolytica* from the lungs of slaughtered sheep and the genetic resistance mechanisms involved. A total of 256 *M. haemolytica* isolates, 169 from lungs with pneumonic lesions and 87 without, were analyzed by the disk diffusion method for 12 antimicrobials, and the whole genome of 14 isolates sequenced to identify resistance determinants. Levels of phenotypic resistance ranged from <2 % for 10 antimicrobials (amoxicillin, amoxicillin-clavulanic, ceftiofur, cefquinome, lincomycin/spectinomycin, gentamicin, erythromycin, florfenicol, enrofloxacin, and doxycycline) to 4.3% for tetracycline and 89.1% for tylosin. Six isolates carried *tetH* genes and four carried, in addition, *strA* and *sul2* genes in putative plasmid sequences. No mutations associated with macrolide resistance were identified in 23 rDNA sequences suggesting that *M. haemolytica* phenotypic results for tylosin should be interpreted with care in the absence of well-established epidemiological and clinical breakpoints. The identification of strains phenotypically resistant to tetracycline and of several resistance genes, some of which were present in plasmids, highlight the need for continuous monitoring of susceptibility patterns in *Pasteurellaceae* isolates from livestock.

Keywords: *Mannheimia haemolytica*; antimicrobial resistance; mobile genetic elements; *tetH*; *strA*; *sul2*; sheep

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1. Introduction

Mannheimia haemolytica is an important ruminant pathogen contributing to bovine respiratory disease (BRD) in cattle and pneumonic pasteurellosis in sheep and leading to a large proportion of the overall antimicrobial usage in both cattle and sheep [1]. *M. haemolytica* is a commensal in the nasopharynx that can lead to clinical disease usually when animals are exposed to predisposing factors, which include stress induced by change in the environment or by other bacterial and viral infections [2]. In sheep, pneumonic pasteurellosis can course as acute disease, but subclinical or chronic cases are frequent, and brochopneumonic lesions derived from these presentations are common findings at the slaughterhouse [3]. *M. haemolytica* can be isolated from pneumonic lungs and from the lung of healthy lambs, with isolates retrieved from both kind of clinical presentations showing a similar genetic background consistent with the idea that individual cases of pneumonia are due to commensal *M. haemolytica* strains reaching lungs from the upper respiratory in the presence of predisposing factors [3]. Optimized control strategies for pneumonic pasteurellosis in sheep require information about the antimicrobial susceptibility of *M. haemoytica* respiratory isolates. However, only a limited number of studies have evaluated the antimicrobial susceptibility in vitro of ovine *M. haemolytica* isolates, and most of these included a limited number of strains [4, 5]. Even though most clinical ovine isolates are usually phenotypically susceptible to antibiotics, the emergence of ovine strains with reduced susceptibility to antimicrobials has been reported [4, 5]. These potentially resistant bacteria may lead to treatment failures, increased costs of animal production and the dissemination of antimicrobial-resistant genes to other bacteria and possibly the environment [6]. Nevertheless, due to the absence of clinical efficacy studies considering the dosing and route of administration of antimicrobial agents used in veterinary medicine to treat animal diseases for multiple pathogen-drug combination, the clinical significance of in-vitro resistance (or reduced susceptibility) in terms of risk of potential therapeutic failures is difficult to ascertain [7]. *M. haemolytica* associated with bovine respiratory disease (BRD) harbouring multiresistance-mediating integrative and conjugative elements (ICEs) have been identified in North America and in European countries [6, 8]. However, very little knowledge exists about the genetic mechanisms conferring antimicrobial resistance in *M. haemolytica* isolates from sheep in spite of its emerging nature. In this context, it is necessary to generate more information regarding the frequency of antimicrobial resistance in isolates belonging to this pathogen originating from different livestock animals and countries [1]. Thus, the current study aims to investigate the levels of antimicrobial resistance of *M. haemolytica* isolated from the lungs of slaughtered sheep and the genetic resistant mechanism involved.

2. Materials and Methods

2.1. M. haemolytica isolates

A total of 256 *M. haemolytica* isolates retrieved from lung samples of 139 apparently healthy lambs collected at slaughter were included in this study. Of them, 169 were isolates recovered from lungs with pneumonic lesions (n = 92 sheep with pneumonic lungs) while 87 were isolates obtained from lungs without pneumonic lesions (n = 47 animals with non-pneumonic lungs). Between one and five isolates per lung (i.e., per animal) were included (one isolate from 56 lungs, two isolates from 55 lungs, three isolates from 23 lungs, four from four lungs and five isolates from one lung). Lung samples were collected at slaughter in three different abattoirs of Spain over 22 months [3] and cultured onto Columbia blood agar plates (bioMérieux), which were incubated at 37°C for 24 h. Isolates were biochemically identified by the commercial identification system Diatabs Diagnostic Tablets (Rosco Diagnostica, Taastrup, Denmark). Biochemical identification was further confirmed by a species-specific PCR assay [9]. After identification, bacterial isolates were frozen and stored at −80ºC until they were used.

2.2. Antimicrobial susceptibility testing

The resistance phenotype of all 256 *M. haemolytica* isolates to 12 antimicrobials was investigated by the disk diffusion method as described in the Clinical and Laboratory Standards Institute document M31-A3 [10] using Müeller-Hinton plates (Oxoid, ltd.). Inocula were prepared from a 24 h Columbia blood agar plate by resuspending four colonies in 5 ml of Müeller-Hinton broth and adjusted to a 0.5 McFarland standard. The following commercial antimicrobial disks (A/S Rosco Diagnostica, Taastrup, Denmark UK) were used: amoxicillin (AMOX, 30 µg), amoxicillin/clavulanic acid (AMC, 30/15 µg), ceftiofur (XNL, 30 µg), cefquinome (CFQUI, 30 µg), lincomycin/spectinomycin (LI+SP, 15+200 µg), gentamicin (GEN, 10 µg), erythromycin (ERY, 78 µg), tylosin (TYLO, 150 µg), florfenicol (FFC, 30 µg), enrofloxacin (ENRO, 10 µg), doxycycline (DOXYC, 80 µg) and tetracycline (TET, 30 µg). The agar plates were examined after 24 h of incubation at 37ºC. Breakpoints used for qualitative interpretation of results and classification of isolates into wild-type

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(susceptible) and non-wild type (resistant) categories were those recommended by the Clinical and Laboratory Standards Institute (CLSI) [11, 12] and, for antimicrobials with no CLSI recommended breakpoint (AMOX, AMC, CEFQ, LI+SP, ERY, TYLO and ENRO, DOXYC), those recommended by Rosco Diagnostica [13] (Table 1). *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were included as quality controls with each batch of organisms tested. Multidrug-resistance (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. A bacterial isolate was considered resistant to an antimicrobial category when it was resistant to at least one agent in that category.

The resistance phenotype of a selection of six isolates, that yielded inhibition zone diameters (IZDs) for tylosin ranging from 10 to 21 mm and were subjected to whole genome sequencing (see below) was further investigated using the microdilution method. Briefly, isolates were inoculated into Mueller-Hinton broth supplemented with 3% lysed horse blood and their minimal inhibitory concentrations (MICs) for the antibiotics included in panel EUVSEC3 were determined using the two-fold broth microdilution reference method according to ISO 20776-1:2021.

2.3. Whole Genome Sequencing

The whole genome of nine isolates representative of those phenotypically resistant to tylosin (with IZDs ranging from 10 to 22 mm) and five isolates representative of those phenotypically susceptible to tylosin (with IZDs ranging from 23 to 30 mm) based on the disk diffusion method (see results) were sequenced using Illumina technology to identify any resistance mechanisms associated with their resistance phenotype. DNA was purified from axenic cultures with the Qiagen DNA blood & tissue kit, following the manufacturer's instructions and quantification of the DNA concentration and further libraries was done using a Qubit® fluorometer (Invitrogen). WGS libraries were prepared from 1 ng of bacterial DNA by using the Nextera XT DNA Library Preparation Kit. The concentrations of each library were adjusted to 4 nM to obtain equimolar DNA concentrations in an in a single pool of libraries to be sequence in a MiSeq device (Illumina).

Illumina reads were processed using in-house pipelines [14, 15]. Briefly, adaptors in the raw reads were removed and low-quality raw reads filtered out with Trimmomatic. The reads that passed the quality control with FastQC were assembled by SPAdes and the quality of the assemblies was evaluated with QUAST. Assemblies were then screened for the presence of antimicrobial resistance genes using Resfinder with default parameters, and for the presence of plasmid replicons with PlasmidFinder using an identity threshold of >80%. Plasmid identities were further confirmed by MOB-suite [16]. The presence of additional resistance markers [*macA* and *macB* genes [17] bla_{ROB-2} gen [18] was investigated in the annotated assemblies using PROKKA and by aligning the raw reads from the isolates to the sequence of the bla_{ROB-2} gene [18], respectively.

Finally, in order to identify point mutations associated with resistance to macrolides (23S rDNA sequence) [19] and fluoroquinolones (quinolone resistance determinant regions, QRDR) [20], reads were aligned to the reference *M. haemolytica* genome CP005383.1 using bwa with defaults parameters. The resulting SAM files were sorted and compressed into BAM files by SAMtools. The variant calling was performed by BCFtools using 'mpileup' and 'call' commands options, excluding SNPs with a base quality lower than 30 and mapping quality lower than 30. Then, consensus sequences for each strain were created using BCFtools 'consensus' command. The six copies of the 23S rRNA subunit were located in the annotation of the reference genome, and the consensus sequences of each strain were annotated with PROKKA in order to locate the 23S rRNA operons for each strain. The 23S operons from all strains were then extracted and aligned to the 23S reference sequence NR103087.1. Variant calling was performed for each operon of each isolate as described above. SNPs were outputted in vcf files to identify the presence of mutations associated with macrolide resistance in the 23S rRNA. The raw reads generated in this study were deposited in the European Nucleotide Archive under project PRJEB61140.

2.4. Statistical analysis

The association between the clinical origin of the isolates and their antimicrobial susceptibility was determined using the Chi-square test, with $P < 0.05$ considered significant. In order to account for the lack of independence between isolates coming from the same animal (i.e., lung), the significance of the association between the presence of lesions and the antimicrobial susceptibility to a given antimicrobial (or their resistotype) was also tested using a mixed logistic regression model including the animal as a random effect. Data were analysed using the Epi InfoTM 7 program of the Centers for Disease Control and Prevention (CDC).

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3. Results

3.1. Phenotypic susceptibility to 12 antimicrobials

The results of in vitro susceptibility testing for the 256 ovine *M. haemolytica* isolates by disk diffusion method are shown in Table 1. Using the breakpoints indicated in the table, less than 2% of the isolates were classified as resistant for 10 of the 12 antimicrobials tested, ranging between 0 % for LI+SP, ERY and FFC to 1.2 % for AMOX and CEFQ (Table1). A higher level of resistance was detected for TET (4.3%), and the highest level was found for TYLO (89.1 %; Table1). No statistically significant differences ($P < 0.05$) were detected between the proportions of resistant isolates in pneumonic and non-pneumonic lungs for all antimicrobials tested (Table1). Eleven resistotypes were identified in this study, with most of the isolates (83.6 %) being resistant only to the macrolide TYLO, whereas 9.8 % of isolates were susceptible to all antimicrobials (Table2). No significant differences in the resistotype frequencies among isolates from lungs with and without pneumonic lesions were observed.

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Table 1. *In vitro* susceptibility by disk diffusion method for the 256 ovine *M. haemolytica* isolates. 1

Antimicrobial agent	Units	IZD Breakpoints (mm)	No. of isolates showed IZD (mm) of:[a]																Resistant isolates (%) [e]		
			7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	>36	from lungs with pneumonic lesions	from lungs without pneumonic lesions	Total
AMOX	30 µg	≤16[b]	1		2					3	8	14	15	41	73	39	38	22	0,6	2,3	1,2
AMC	30/15 µg	≤16[b]			1			2			8	10	14	51	59	48	40	23	0,6	0,0	0,4
CEFT	30 µg	≤17[c]	1			1			3	1	5	6	9	35	57	39	37	62	1,2	0,0	0,8
CEFQ	30 U	≤19[b]	2				1				7	18	18	46	54	36	38	36	1,2	1,1	1,2
LI+SP	15/200 µg	≤16[b]							9	12	54	77	67	19	10	2	2	4	0,0	0,0	0,0
GEN	10 µg	≤12[d]	1			10	54	101	73	3	2	2	5	1	4				0,6	0,0	0,4
ERY	78 µg	≤18[b]							22	32	62	65	45	16	8	2	3	1	0,0	0,0	0,0
TYLO	150 µg	≤22[b]		1		3	8	32	117	67	20	1	3	1	1		1	1	87.6	91.9	89,1
FFC	30 µg	≤14[c]							1		5	11	20	50	64	42	49	14	0,0	0,0	0,0
ENRO	10 µg	≤16[b]				1	1				5	16	17	54	63	31	34	34	0,0	2,3	0,8
DOXYC	80 µg	≤18[b]						1	4	3	1	17	19	57	80	38	24	12	0,6	0,0	0,4
TET	30 µg	≤14[d]	5	3		3	3		4	4	36	72	60	43	16	3	2	2	5,9	1,1	4,3

Abreviations: AMOX, amoxicillin; AMC, amoxicillin/clavulanic acid; CEFT, ceftiofur; CEFQ, cefquinome; LI+SP, lincomycin/spectinomycin; GEN, gentamicin; ERY, erythromycin; TYLOS, tylosin; FFC, florfenicol; ENRO, enrofloxacin; DOXYC, doxycycline and TET, tetracycline. aIn order to present clearer data, inhibition zone diameters (IZD) have been grouped into 2 mm intervals. bBreakpoints recommended by Rosco Diagnostica (Neo-Sensitabs user's guide; A/S Rosco Diagnostica). cBreakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) for M. haemolytica (2018;VET08). dBreakpoints recommended by the CLSI for bacteria isolated from animals (2002; M31-A2).e No statistical significant differences were detected among lungs with and without pneumonic lesions. 2 3 4 5 6

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Table 2 Antimicrobial resistance patterns (resistotypes), clinical origin and capsular types of the *M. haemolytica* isolates.

Resistotype	Antimicrobial family[a]					*M. haemolytica* isolates from lungs[b]		Total
						with pneumonic lesions	without pneumonic lesions	
	MAC	LAC	TET	QUI	AMI	n = 169	n = 87	n=256
TIL[c]	■					139 (82.2)	75 (86.2)	214 (83.6)
TIL. TET	■		■			6 (3.6)	1 (1.1)	7 (2.7)
TIL. AMOX	■	■				0	2 (2.3)	2 (0.8)
TIL. CEFQ	■	■				0	1 (1.1)	1 (0.4)
TIL. AMOX. AMC. CEFT	■	■				1 (0.6)	0	1 (0.4)
TIL. CEFQ. TET	■	■	■			1 (0.6)	0	1 (0.4)
TIL. TET. DOX	■		■			1 (0.6)	0	1 (0.4)
TIL. ENRO	■			■		0	1 (1.1)	1 (0.4)
ENRO.				■		0	1 (1.1)	1 (0.4)
CEFT. CEFQ. TET. GEN		■	■		■	1 (0.6)	0	1 (0.4)
TET			■			1 (0.6)	0	1 (0.4)
None						19 (11.2)	6 (6.7)	25 (9.8)

[a] Abbreviations: MAC, Macrolides; LAC, β-Lactams; TET, Tetracyclines; QUI, Quinolones; AMI, Aminoglycosides.
[b] Statistically significant differences were not detected among percentages of isolates from lungs with and without pneumonic-lesions (P>0.05).
[c] See footnote in table1 for antimicrobial abbreviations

3.2. *WGS analysis*

Three of the isolates with large IZD and one with small IZD for TYLO were discarded due to the low quality of the reads. Among the remaining 10 isolates (six and four with IZDs above and below the selected TYLO breakpoint, respectively), antimicrobial resistance determinants were found in six: two carried genes conferring resistance to tetracyclines (tetH), aminogylcosides (strA) and sulfonamides (sul2), while the remaining four isolates all carried the tetH gene (Table 3). Putative plasmid sequences were found in five strains (plasmid replicons rep21 and IncP according to PlasmidFinder, present in three different strains, and a putative plasmid sequence in two strains found by MOB-suite) (Table 3). The sequences identified as plasmid-associated by MOB-suite, of 5,488 bp, found in two different strains were identical and also carried the strA and sul2 genes. These putative plasmids were very similar (>99.9% identity) to plasmid pHN06 (5,360 bp), found in a P. multocida strain retrieved from a pig with atrophic rhinitis in China and that also harbored AMR genes strA and sul2 [21]. No mutation previously associated with resistance to fluoroquinolones or macrolides were identified in the QRDR and 23 rDNA sequences, respectively, from any of the sequenced strains.

The MIC values, available in five of the six sequenced strains with IZD> the TYLO breakpoint, were identical for 10 out of the 15 antimicrobials tested, with values differing by more than one dilution for sulfametoxazol, amikacin, trimethoprim, tetracycline and gentamicin (Table 3).

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Table 3. Presence of resistance genes, plasmid sequences and resistance profile according to the microdilution method in a subset of isolates

		Resistance genes			Plasmids			Antimicrobial susceptibility phenotype (concentration ranges tested in ug/ml)													
ID	IZD - TYLO (mm)	*tetH*	*strA*	*sul2*	rep 21	Inc P	pH N06	SMX (8-512)	AMI (4-128)	TMP (0.25-16)	CIP (0.015-8)	TET (2- 32)	MERO (0.03-16)	AZI (2-64)	NAL (4-64)	CHL (8-64)	FOT (0.25-4)	TGC (0.25-8)	TAZ (0.25-8)	COL (1-16)	AMP (1- 32)	GEN (0.5-16)
M411	10	0	0	0	0	**1**	0	>512	8	≤0,25	≤0,015	≤2	0,06	≤2	≤4	≤8	≤0,25	≤0,25	≤0,25	≤1	≤1	2
M214	14	**1**	0	0	0	0	0	>512	8	≤0,25	≤0,015	≤2	≤0,03	≤2	≤4	≤8	≤0,25	≤0,25	≤0,25	≤1	≤1	2
M280	18	0	0	0	0	0	0	>512	16	≤0,25	≤0,015	≤2	≤0,03	≤2	≤4	≤8	≤0,25	≤0,25	≤0,25	≤1	≤1	2
M242	19	**1**	0	0	0	0	0	≤8	16	8	≤0,015	32	≤0,03	≤2	≤4	≤8	≤0,25	≤0,25	≤0,25	≤1	≤1	2
M273	21	**1**	**1**	**1**	0	0	**1**	32	≤4	8	≤0,015	32	≤0,03	≤2	≤4	≤8	≤0,25	≤0,25	≤0,25	≤1	≤1	≤0,5
M239	22	**1**	0	0	**1**	0	0															
M487	23	0	0	0	0	0	0															
M469	28	0	0	0	0	**1**	0															
M270	30	**1**	**1**	**1**	0	0	**1**															
M229	30	**1**	0	0	0	0	0															

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ECOFFs included in the concentration range included in the EUVSEC3 plate were only available for tetracycline; based on the ECOFF, two isolates (both carrying the tetH gene) were classified as resistant, whereas the remaining four isolates (one of which also carried the tetH gene) were considered susceptible (Table 3). For the remaining four antimicrobials in which different MICs were found higher values were not associated with the carriage of specific resistance genes (Table 3).

4. Discussion

M. haemolytica is a ruminant-specific pathogen associated with pneumonic pasteurellosis in sheep, a relevant disease due to the important economic losses that generates [2]. It is generally accepted that the mere presence of *M. haemolytica* in lungs is not sufficient to produce lesions [2] and that individual cases of pneumonia are associated with commensally *M. haemolytica* strains of the upper respiratory tract that reach lungs following predisposing factors [3]. Antimicrobial surveillances provide relevant information necessary to gain knowledge about the epidemiology and empirical treatment options. In this study, we have investigated the antimicrobial susceptibility of 256 *M. haemolytica* isolates recovered at slaughter from ovine lungs with and without pneumonic lesions. Moreover, we have also determined the genetic antimicrobial mechanisms of resistance in a selection *M. haemoytica* resistant to macrolides.

Overall, most ovine *M. haemolytica* isolates investigated in this study were classified as susceptible to most (10 of 12) antimicrobials tested, with resistance rates between 0% and 1.2% (Table 2). These results agree with the high susceptibility of ovine *M. haemolytica* isolates to most antimicrobials observed in different studies [4, 5]. Despite this overall high susceptibility to most antimicrobials, moderate resistance rates were observed for TET (Table 1). Although not statistically significant ($P > 0.05$), the frequency of resistance to TET was higher among isolates from lungs with pneumonic lesions (5.9% and 1.1%, respectively; Table 1). Tetracyclines are among the most frequently antimicrobials used in animals [22]. Therefore, these resistance rates may reflect the predominance of their use in sheep farming. The *tetH* tetracycline-resistance gene, encoding an energy-dependent membrane-associated protein that exports tetracyclines out of the cell [23], was found in six of the 10 sequenced strains, of which only two were resistant according to the disk diffusion test. When considering the results from the microdilution test (only available for six of the 10 sequenced strains), two of the three strains carrying the gene were classified as resistant (Table 3). This gene was identified as the predominant *tet* gene in *Pasteurellaceae* isolates of bovine and swine origin in North America in the nineties [23] and was more recently described in phenotypically resistant *M. haemolytica* isolates retrieved from cattle in the United States [24]. It was also the most common resistance gene found using metagenomics in nasopharyngeal samples from chronically ill feedlot cattle in Canada [25], suggesting it may play a significant role in the occurrence of tetracycline resistance in respiratory pathogens from ruminants. However, the presence of *tetH* in *M. haemolytica* isolates classified as phenotypically susceptible to certain tetracyclines (chlortetracycline) based on clinical breakpoints, as found in this study, has been also previously described, further highlighting the complexities of predicting resistance phenotypes from genetic data in this bacterial species [26].

The most striking result was the very high resistance rate to TYLO found in this study based on the breakpoint used (89.1 %; Table 1). This antimicrobial has been classified by WHO as critically important with the highest priority for human medicine [27], and therefore the spread of genetic determinants mediating to this antimicrobial may represent a serious concern. Similar levels of TYLO resistance have been observed in other respiratory pathogens such as *Pasteurella multocida* in sheep and in pigs [28, 29] in Spain. It has been suggested that this low degree *in vitro* susceptibility of these bacteria to this macrolide could be due to a long-term exposure to that agent given that it was used at subtherapeutic doses as growth promotor until its ban in 1999 in Spain [29]. The acquire resistance to macrolides in members of the family *Pasteurellaceae* have been associated with the presence of the macrolide resistance gene coding for an efflux pump (*msrE*), genes that cause methylation of the ribosomal target (*ermA, ermC and erm42*) and the gene that codes for a phosphorylase-inactivating enzyme (*mphE*) [30]. In addition, rRNA mutations that confer resistance to macrolides have been described in field isolates of *M. haemolytica* and *P. multocida* in cattle [19] and *H. parasuis* in pigs [30]. However, no genetic determinants (antimicrobial resistance genes or point mutations) conferring resistance to macrolides were identified in any of the isolates sequenced in this study regardless of their TYLO resistance phenotype (Table 3). Furthermore, no difference in treatment efficacy when using TYLO or tulathromycin in cattle from which TYLO-resistant (and tulathromycin-susceptible) *Pasteurellaceae* isolates had been retrieved was previously reported, indicating a lack of direct correlation between in-vitro resistance and therapeutic success [31]. Previous studies have also reported very high MIC values in *M. haemolytica* isolates retrieved from goats [32] and cattle [33] while also being predominantly

susceptible to other macrolides (tulathromycin, tilmicosin) and/or not harboring known genetic determinants conferring resistance mechanisms to this antimicrobial class. Altogether, these results suggest that *M. haemolytica* antimicrobial susceptibility test results for tylosin should be interpreted with care in the absence of well-established epidemiological and clinical breakpoints, since lack of correlation between genetic and phenotypic resistance data observed here and elsewhere could be derived from the presence of still unrecognized resistance mechanisms but also from inappropriate breakpoints [26].

Mobile genetic elements are known to play a major role in the dissemination of antimicrobial resistance in members of the *Pasteurellaceae* family [6]. In this study, we identified the sequence of a short (~5,300 bp) plasmid carrying both *sul2* and *strA* resistance genes (Table 3). The co-occurrence of these two genes together in plasmids carried by clinical *Pasteurellaceae* strains has been already described in isolates retrieved from cattle and swine in several countries [34, 35] including Spain [36]. The plasmid retrieved here had a high level of identity with a plasmid sequence originally described in a *P. multocida* strain from a diseased pig in China [21]. Even though the presence of the *strA* and *sul2* genes was not associated with increased MIC values to the sulfonamides and aminoglycosides included in the antimicrobial susceptibility testing performed in this study, additional studies to assess their possible role conferring resistance to members of this antimicrobial families are needed.

5. Conclusions

In conclusion, we found a very limited proportion of resistance to all antimicrobials but TYLO in *M. haemolytica* isolates retrieved from clinically healthy animals sampled at slaughterhouse. The high levels of TYLO-resistant isolates based on the breakpoint used, however, should be interpreted with care, since no resistance mechanisms associated with macrolide resistance was found in a subset of sequenced isolates and very high MICs for this antimicrobial have been described previously in clinical *M. haemolytica* strains from ruminants, thus suggesting that the cut-off used here may not be differentiating truly non-wild type genotypes. Our results agree with most evidence pointing out at a limited degree of in-vitro resistance in *M. haemolytica* strains of veterinary origin, but the identification of a subset of strains resistant to tetracycline and of the presence of several resistance genes, some of which were carried in a plasmid, further highlight the need for continuous monitoring of susceptibility patterns in *Pasteurellaceae* isolates from livestock in order to detect newly emerging resistance mechanisms of clinical significance. The apparent lack of agreement between results from the predicted (based on presence of AMR determinants) and the observed phenotypic resistance profiles highlight the need of additional data to calibrate WGS-derived data for the in-silico assessment of the susceptibility/resistance in clinical strains from *M. haemolytica*.

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