

Research article

ANTIMICROBIAL SUSCEPTIBILITY OF *PASTEURELLA MULTOCIDA* ISOLATED FROM SHEEP WITH FIBRINOUS PNEUMONIA

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Ovine respiratory complex is a significant cause of death in sheep flocks, where *Pasteurella multocida* is the most frequent microorganisms isolated from animals with pneumonia. There is an urgent need to refine the use of different antimicrobials to avoid the problem of antimicrobial resistance and optimize the control of this disease in ovine livestock. The first step in approaching this problem is gaining an insight into the antimicrobial susceptibility of ovine pathogens. This study evaluated the *in vitro* activity of tildipirosin, gamithromycin, oxytetracycline, and danofloxacin against *Pasteurella multocida* strains isolated from sheep with fibrinous pneumonia. The strains were incubated following Clinical and Laboratory Standards Institute (CLSI) standard conditions and also with a modified method by 25% supplementation with sheep serum. Minimum inhibitory concentrations (MIC) were determined using the broth microdilution technique. The lowest MIC₉₀ under standard conditions and by supplementation with sheep serum was obtained with tildipirosin. Sheep serum significantly reduced tildipirosin, gamithromycin, and danofloxacin MIC values for *Pasteurella multocida* strains. In brief, the potency of tildipirosine, gamithromycin, and danofloxacin against *Pasteurella multocida* increases when sheep serum is added to the culture media.

Keywords: Antimicrobial susceptibility, pneumonia, sheep, *Pasteurella multocida*

INTRODUCTION

The ovine respiratory complex is a common respiratory disease in sheep, with a wide prevalence worldwide [1,2]. It represents a real problem in many sheep flocks in most countries with intensive sheep production. It is the leading cause of economic losses

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due to high morbidity, treatment costs, and negative impact on lamb weight gain and growth [3]. The main agents of this complex are *Mannheimia haemolytica* and *Pasteurella multocida* [4]. Immunosuppression of animals due to stressful handling and adverse climatic conditions predisposes to bacterial colonization of the respiratory system [5], where *Pasteurella multocida* is most frequently isolated from respiratory samples in sheep with pneumonia [6-8]. This agent has become an emerging respiratory disease in sheep, mainly in tropical and temperate climate zones [9].

This respiratory complex is usually treated with broad-spectrum antibiotics such as tetracyclines, macrolides, and fluoroquinolones, often prescribed extra-label or off-label in sheep [10]. There are few studies on this subject in sheep, with some results achieved in different species, like pigs or cattle, that have been extrapolated for use in sheep [4]. Antimicrobial resistance is currently a worldwide concern, therapeutic options may be challenging, and the knowledge of antimicrobial susceptibility is essential when implementing rational and effective therapy [11,12]. *Antimicrobial resistance* is a prominent issue requiring an interdisciplinary approach to provide and assure effective treatments to the animal and human population to prevent the spreading of antimicrobial drug resistance to other animals, people, and the environment. Currently, one of the approaches to reduce inappropriate antimicrobial use for treating animals or humans is to apply pharmacokinetic–pharmacodynamic principles to calculate therapeutic regimens where the exact determination of the minimum inhibitory concentration is essential for this approach [13]. Therefore, studies that describe antibacterial susceptibility in veterinary medicine are valuable because they allow early recognition of the appearance of antibiotic resistance as well as information for PK-PD integration studies.

The pharmacokinetic properties of new macrolides (tildipirosin and gamithromycin), such as rapid absorption, extensive distribution in pulmonary tissues, and long half-lives after parenteral administrations can make these antimicrobials a suitable option as antibiotic therapy against *Pasteurella multocida* in sheep [12,14,15]. Moreover, some experiments suggest that the antimicrobial activity of some macrolides may be undervalued if we only consider the obtained MIC by CLSI methods. For example, a study showed that MIC values of *Actinobacillus pleuropneumoniae* against tildipirosin incubated according to standard methods was higher than those obtained when adding serum in different proportions to the culture medium (5%, 10%, 25%, and 50%) [15]. In another study, the potency of tildipirosin and gamithromycin against susceptible caprine mastitis pathogens (*M. agalactiae*, *E. coli*, *S. aureus*, *Streptococcus* spp., and coagulase-negative *Staphylococci*) increased when serum (25%) was present in culture media [16]. Therefore, determining macrolide dosages for therapeutic use should be derived from pharmacodynamic data obtained from biological fluids because *in vitro* measurement of MIC in broth, performed following international recommended methods, may be misleading for estimating the *in vivo* potency of these antibiotics. Thus, the aim of the study was to evaluate the *in vitro* activity of tildipirosin, gamithromycin, oxytetracycline, and danofloxacin against *Pasteurella multocida* incubated on CLSI conditions and with a deviation from CLSI methods by 25% supplementation with sheep serum.

MATERIAL AND METHODS

Animals and sampling procedure

The animals used for this study were sheep slaughtered at Municipal abattoirs in the Cartagena Region of Murcia in Spain. Lungs with lesions suggestive of fibrinous pneumonia, such as pulmonary congestion, fibrin deposits on the serosa, pulmonary edema, or pleural effusion, were selected to obtain swabs tissues. A total of 50 lungs with pneumonic lesions were sampled through an aseptic incision in the trachea to introduce the swab for bronchial mucosa sampling. The samples were deposited in Stuart medium and maintained at 4 °C until transferred at the Microbiology laboratory.

Isolation and identification of *Pasteurella multocida*

Bacteria were isolated from swab lung samples on Columbia blood agar plates (bioMérieux España, Madrid, Spain) and incubated at 37 °C for 24 h. After obtaining the pure culture, Gram staining was performed. Gram-negative coccobacilli were tested for oxidase. Gram-negative and oxidase-positive isolates were biochemically identified by the commercial identification system API 20NE strips (bioMérieux España, Madrid, Spain) and further confirmed by a species-specific PCR assay. Finally, thirty bacterial isolates were included after their characterization and maintained at the Microbiology laboratory strain collection until antimicrobial susceptibility analysis was performed.

Antimicrobial susceptibility testing

Tildipirosin, gamithromycin, oxytetracycline, and danofloxacin (Cymit Química, Barcelona, Spain) were selected for the antimicrobial susceptibility testing. Antibiotics were dissolved in suitable solvents to make stock solutions and then diluted in sterile distilled water following the guidelines of the Clinical and Laboratory Standards Institute [17]. Sheep serum was obtained from six healthy sheep. Blood samples were centrifuged at 1500 g for 10 min, and the freshly collected serum was pooled and divided into 1 ml portions, stored at -80°C, and thawed immediately before the experiment.

The minimum inhibitory concentrations of tildipirosin, gamithromycin, danofloxacin, and oxytetracycline were determined following standard conditions of CLSI or with a modification of the CLSI method by 25% supplementation of sheep serum [17]. Minimum inhibitory concentration tests were performed by the microdilution broth method [17]. Brief, serial two-fold dilutions of the antimicrobial agents were prepared to start from the stock solution of each antibiotic. Broth dilutions were made using cation-adjusted Mueller-Hinton broth (Merck, Madrid, Spain) with 5% of defibrinated horse blood (Thermo Fisher Scientific, Massachusetts, USA). Concentrations of all antibiotics ranging from 0.03 to 128 mg/L were used. Inoculums were prepared by diluting an overnight MHB culture in buffered saline solution to a 0.5 McFarland

Turbidity Scale and diluting again 40-fold before testing. The U-bottomed microtiter plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of antibiotic where bacterial growth was completely inhibited. The reference strains *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) were used as controls for each plate.

RESULTS

The present study provides the profile of antibacterial susceptibility of *Pasteurella multocida* strains isolated from sheep with fibrinous pneumonia. *Pasteurella multocida* was susceptible to tildipirosin, gamithromycin, and danofloxacin but not for oxytetracycline on standard conditions. The lowest MIC₉₀ values were obtained for tildipirosin on standard condition (MIC₉₀ = 0,5 µg/ml) and those deviated from CLSI method (MIC₉₀ = 0,25 µg/ml). Tildipirosin, gamithromycin and danofloxacin showed a decrease in the MIC₉₀ under deviated conditions compared to standard CLSI conditions. Oxytetracycline showed no change in MIC₉₀ between standard condition and deviation from the CLSI method. Minimal inhibitory concentration values for the antibiotics tested against *Pasteurella multocida* are summarized in Table 1.

Table 1. Frequency of minimal inhibitory concentrations for tildipirosin (TIL), gamithromycin (GAM), oxytetracycline (OXY), and danofloxacin (DAN) under standard conditions or addition of serum (+SERUM) in the culture media against *Pasteurella multocida* strains isolated from sheep (n=30), as well as MIC₅₀ and MIC₉₀.

Antibiotics	MIC (µg/ml) / Number of strains														MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)
	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128		
TIL	1		5	14	7		1	1						1	0.25	0.5
TIL + SERUM	6		8	14	1									1	0.25	0.25
GAM	2			3	13	5	6							1	0.5	2
GAM+SERUM	6	1		7	14	1								1	0.5	0,5
DAN	5	2	10	5	3	1	4								0.12	2
DAN+SERUM	3	3	14	4	3	1	1	1							0.12	0,5
OXY			2	2	7	3	1	1				3	7	4	4	> 128
OXY+SERUM				1	7	3	5					1	7	6	2	> 128

The addition of the serum to the culture medium was associated with an appreciable increase in the potency of tildipirosin, gamithromycin, and danofloxacin. However, the potency of oxytetracycline was unchanged against *Pasteurella multocida* (Figure 1).

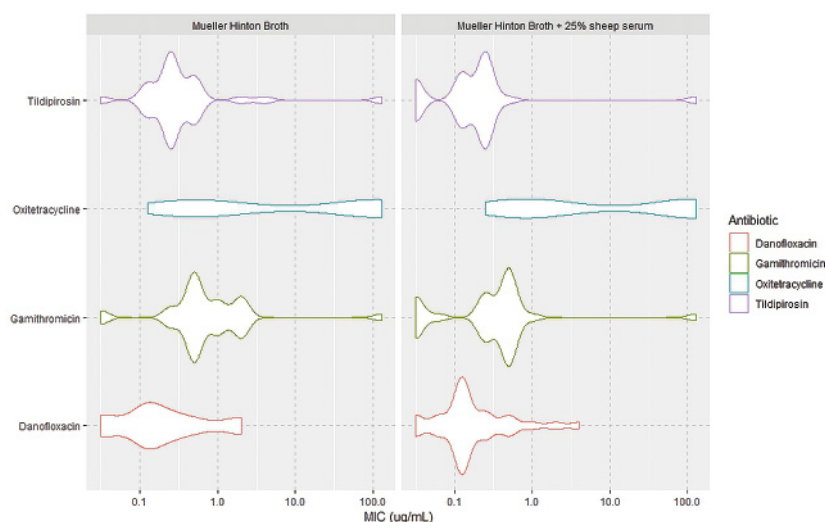


Figure 1. Minimum inhibitory concentration ($\mu\text{g}/\text{mL}$) of tildipirosin, gamithromycin, danofloxacin and oxytetracycline against *Pasteurella multocida* strains isolated from sheep inoculated on Mueller Hinton Broth and Mueller Hinton Broth supplemented with 25% sheep serum.

DISCUSSION

Extensive knowledge of antimicrobial activity for treating diseases caused by microorganisms such as *Pasteurella multocida* in sheep is required to achieve an effective and rational treatment that prevents the appearance of bacterial resistance. However, there are limited studies of bacterial susceptibility on isolated strains from sheep. The current work provides a brief description of the antimicrobial susceptibility of several antibiotics that could be used against *Pasteurella multocida*. Previous studies on sheep isolates showed lower values of MIC_{90} than those obtained in the present study for danofloxacin ($\text{MIC}_{90}=0,12 \text{ mg}/\text{L}$), oxytetracycline ($\text{MIC}_{90}=16 \text{ mg}/\text{L}$) [4] and for tetracycline and doxycycline (MIC_{90} values of 32 and 16 mg/L , respectively) [18]. The same occurs in the case of other fluoroquinolones as enrofloxacin, with MIC_{90} values of 0,5 mg/L [18]. Other studies in sheep also evidenced the susceptibility of *Pasteurella multocida* to fluoroquinolones, mainly enrofloxacin [2,19,20].

Previous studies showed higher MIC values for tetracyclines than fluoroquinolones and macrolides, as was found in the present work, although the strains studied showed extensive susceptibility to these antibiotics [18,20]. Concerning macrolides, there are no studies on tildipirosin and gamithromycin in sheep isolates. Previous studies of new macrolides such as tulathromycin in this species showed higher MIC_{90} values ($\text{MIC}_{90}=4 \text{ mg}/\text{L}$) than tildipirosin and gamithromycin [4]. For tilimicosin the values obtained are also higher in sheep than in our work [4]. Furthermore, the present study demonstrates that macrolides and danofloxacin have markedly lower MICs against different pathogens when assayed in culture media broth supplemented with serum, compared with MHB (CLSI recommendation for *in vitro* susceptibility testing studies).

Considering these variations between biological fluids and artificial growth media guided some authors to advocate for the use of physiological fluids in studies of antimicrobial activity testing when the objective is to establish optimal dosing regimens for bacterial killing *in vivo* [21,22]. It has been reported for tildipirosin, after adding serum in different proportions, that MIC values were lower than those obtained from CLSI methods [15,16].

CONCLUSION

The results obtained in this study demonstrate that macrolides and fluoroquinolones show a valuable activity against *Pasteurella multocida* isolated from sheep with respiratory disease. Nevertheless, oxytetracycline has considerably MIC higher values than those obtained for similar studies in Spain, suggesting a variation in its susceptibility that should require surveillance and monitoring programs to control and prevent antibiotic resistance.

Culture media modification with sheep serum achieved lower values of MIC₉₀ for macrolides and danofloxacin compared with standard recommended susceptibility testing *in vitro*. These observations have a potentially important expectation concerning the clinical benefits of macrolides in sheep with respiratory disease caused by *Pasteurella multocida*.

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Authors' contributions

JSG, EE, JCC and PM participated in conceptualization, methodology, microbiological validation and formal data analysis. JSG and JCC performed microbiological assays. JSG, EB, MTY and PM performed data curation, prepared original draft and figure elaboration. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ANTIMIKROBNA OSETLJIVOST NA *PASTEURELLA MULTOCIDA* KOD OVACA SA FIBRINOZNOM PNEUMONIJOM

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Respiratorni kompleks ovaca je značajan uzrok uginuća u stadima gde je *Pasteurella multocida* najčešći mikroorganizam izolovan kod životinja sa pneumonijom. Postoji urgentna potreba da se poboljša upotreba različitih antimikrobnih sredstava kako bi se izbegao problem antimikrobne rezistencije i optimizovala kontrola ove bolesti kod ovaca. Prvi korak u pristupu ovom problemu je sticanje uvida u antimikrobnu rezistenciju. Studija je procenila *in vitro* aktivnost tildipirozina, gamitromicina, oksitetraciklina i danofloksacina protiv sojeva *Pasteurella multocida* izolovanih kod ovaca sa fibrinoznom pneumonijom. Sojevi su inkubirani u skladu sa standardnim uslovima Instituta za kliničke i laboratorijske standarde (CLSI), a takođe i modifikovanom metodom uz dodatak 25% ovčijeg seruma. Minimalne inhibitorne koncentracije (MIC) su određene primenom tehnike mikrodilucije bujona. Najniži MIC₉₀ u standardnim uslovima, kao i modifikovanom suplementacijom ovčijim serumom, dobijen je za tildipirozin. Ovčiji serum je značajno smanjio vrednosti MIC tildipirozina, gamitromicina i danofloksacina za sojeve *Pasteurella multocida*. Ukratko, potentnost tildipirozina i gamitromicina i danofloksacina protiv *Pasteurella multocida* se povećava kada se ovčiji serum doda u medijum za kulturu.