Antimicrobial susceptibility of Mannheimia haemolytica and Pasteurella multocida isolated from ovine respiratory clinical cases in Spain and Portugal

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ARTICLE INFO
Keywords:
Ovine Respiratory pathogens MIC Ovine respiratory disease complex

ABSTRACT
Antimicrobials are used to control respiratory disorders during the ovine rearing period. There is an urgent need to optimize the use of different antimicrobial families in livestock to tackle the general problem of antimicrobial resistance. The first step to addressing this problem is gaining insight into the antimicrobial susceptibility (pharmacodynamic) parameters of ovine pathogens. In this study, the key pharmacodynamic parameter (MIC) was determined for Pasteurella multocida and Mannhaemia haemolytica isolated from ovine respiratory clinical cases using accepted laboratory methods for bacterial isolation, identification and MIC determination. In the case of Pasteurella multocida, for sulfamethoxazole/trimethoprim, MICrange, MIC50 and MIC90 values were 0.004–32 μg/mL, 0.063 μg/mL and 1 μg/mL; for tetracycline 0.016–256 μg/mL, 1 μg/mL and 32 μg/mL; for enrofloxacin 0.002–32 μg/mL, 0.016 μg/mL and 0.5 μg/mL; for doxycycline 0.063–32 μg/mL, 1 μg/mL and 16 μg/mL. In the case of Mannhaemia haemolytica for sulfamethoxazole/trimethoprim, MICrange, MIC50 and MIC90 values were 0.004–1024 μg/mL, 0.063 μg/mL and 1 μg/mL; for tetracycline 0.063–256 μg/mL, 8 μg/mL and 64 μg/mL; for enrofloxacin 0.004–32 μg/mL, 0.032 μg/mL and 16 μg/mL; for doxycycline 0.063–256 μg/mL, 2 μg/mL and 16 μg/mL. The antimicrobial pattern showed good susceptibility for ovine respiratory pathogens to various licensed antibiotics including fluoroquinolones and sulfonamides. However, the antimicrobial susceptibility of antibiotics in the tetracycline family was variable. Doxycycline showed a better antimicrobial pattern than tetracycline. Finally, antimicrobial susceptibility monitoring programs are recommended to provide evidence-based guidance for antimicrobial therapy of bacterial diseases.

1. Introduction

In Spain and Portugal, the production of lambs is based on small ewe production farms. However, the market of fattening animals needs lambs from many farms to achieve a minimum size in feedlots (González et al., 2016), such that these facilities have sufficient volume to satisfy meat distribution channels. This industry organization has advantages in terms of achieving market goals but has serious drawbacks in terms of disease management because mixing animals of different origins is a well-known risk factor for disease outbreak in many veterinary species (Thrusfield, 2018).

Respiratory disease remains one of the most challenging problems in intensive ovine production systems. The term ovine respiratory disease complex (ORDC) describes a polymicrobial syndrome that results from a combination of infectious agents, environmental stressors, population size, management strategies, age, and genetics. This syndrome causes reduced performance, and an increase in mortality rates and production costs in the ovine fattening industry worldwide (Black et al., 1997; Luzón and de las Heras, 1999; Gonzalez et al., 2001; Vilallonga, 2013). The aetiology of the ORDC has been in continuous progression due to pathogen evolution as well as in management and stressor changes in ovine feedlots (González et al., 2016). This ORDC is the consequence of impairment of the normal respiratory immune system due to pathogens that are able to damage these defences and establish infection on their own. Several pathogens have been involved in ORDC, making it a multifactorial complex. In any case, Mannhaemia haemolytica (MH), Pasteurella multocida (PM), Mycoplasma ovipneumoniae and Biberstenia thralosi are the most common etiologic agents involved (Gonzalez, 2015) but viruses like ParaInfluenza 3, Pestivirus, Adenovirus 6 and Syncytial Respiratory Virus could predispose an
animal to bacterial colonization in some cases (Brodgen et al., 1998; Martin and Cid, 2013; González et al., 2016). On the other hand, many non-infectious predisposing factors are involved in the ORDC, such as poor environmental conditions (e.g., deficient ventilation), density, stressors (weaning, feeding changes, transport and social stress), subacute acidosis, season of the year (summer in Spain and Portugal) and oxidative stress (pulmonary hypertension and damage to epithelia). Finally, coccidiosis could be one of the most important trigger factors (González et al., 2016) mainly during the first week at the feedlot.

The clinical manifestation of affected lambs can be septicemic, acute, subacute, chronic and subclinical (Gómez and Garijo, 2013; Gonzalez, 2015). Although ORDC is the most important cause of mortality in lambs older than 42 days of age (Lacasta et al., 2008), clinical signs of the syndrome may not be as evident highlighting the importance of the chronic or subclinical form. Thus, close to 33% of lambs raised in current feedlots have some degree of lesions in their lungs at the slaughterhouse (Gonzalez, 2015). Moreover, 75% of lambs were asymptomatic and had never been treated despite presenting some lung lesions at slaughter (Luzón and De las Heras, 1999).

The diagnosis, prophylaxis and treatment strategies for ovine respiratory disease complex should be adapted, in a case-by-case situation, depending on the relevance of the agents involved. As a general approach, the medical preventive programs should be based on applying measures to control diseases in a cost-effective way such as improving environmental conditions, decreasing density, mitigating stressors and controlling subacute acidosis and parasitism. Other measures include vaccinating against the major bacterial diseases and the use of antimicrobials to control bacterial diseases with a therapeutic or metaphylactic goal. Unfortunately, there are few registered vaccines and antimicrobials to apply in ovine animals. Thus, antimicrobials remain an essential tool to control ORDC under field conditions (Martin and Cid, 2013; Fernandez and Rey, 2013).

The major issues for practitioners when treating a large population of animals with antimicrobials are maximizing the likelihood of a favorable clinical outcome and minimizing the appearance and development of antimicrobial resistance. To optimize the use of these drugs, it is critical to have updated pharmacokinetic (PK) and pharmacodynamic (PD) data about antimicrobials (Mckellar et al., 2004). Unfortunately, there is a scarcity of public knowledge about the PK and PD of antimicrobials in ovine medicine. On the other hand, it has been strongly recommended that program be developed to monitor the usage of antimicrobial agents and the occurrence of antimicrobial resistance among food animals at the European level (Aarestrup et al., 2008; EMA AMEG, 2014, 2019). In Spain, a national antimicrobial resistance monitoring program has been underway since 2014 (PRAN, 2014). The bacterial species monitored, the antimicrobial agents tested, as well as the methodology used, are being homogenized to make data comparable between and across species in Spain (PRAN, 2019). In addition, this program also provides data on the consumption of antimicrobial agents in all veterinary species. Today, there is no public or updated information about antimicrobial susceptibility of ovine pathogens involved in ORDC in Spain and Portugal. Much of the information available is very old, was obtained by obsolete methodology (Diker et al., 1994) or comes from a geographical area unrelated to the European context (Marru et al., 2013).

The main objective of this work was to obtain pharmacodynamic information about Pasteurella multocida and Mannhaemia haemolytica in ovine animals following a methodology recommended by a national antimicrobial resistance control program in Spain. This information is necessary to obtain updated information to optimize the use of antimicrobials for this species following the general recommendations about prudent use of antimicrobials.

2. Material and methods

2.1. Animals and sampling

One-hundred twenty-eight respiratory clinical cases from 60 ovine feedlots were studied between February of 2015 and March of 2019 in Spain and Portugal. In these cases, a percentage of the feedlot showed respiratory symptoms (cough, fever, depression and dyspnoea) and the mortality rate significantly increased versus the baseline situation during the respiratory outbreak due mainly to respiratory causes. It was not sampled any animal that was treated with antimicrobials prior to sample collection. In each clinical case, a minimum and maximum number of 3 and 5 animals, with overt respiratory symptoms, were sampled, respectively. These animals were randomly selected if more than 5 animals showed respiratory symptoms at the same time. Unfortunately, it was not possible to have information about exact age, breed and days on feed of the animals because this data is not individually recorded in ovine fattening farms. Samples were drawn from pneumatic lesions in the lung during necropsy (64 samples) or from tracheobronchial lavages of live animals showing respiratory symptoms (63 samples). In the case of lung sample collection, a section of 5 x 5 cm of lung was collected from a lobule including healthy and pneumatic tissue. On the other hand, tracheobronchial lavage was carried out using a previous published method adapted to ovine (Hoffman, 2008). Briefly, 30–40 cm of a catheter of 2.7 mm of diameter was inserted through one of the nostrils into the trachea. Then, 20 cm³ of physiological serum were flushed and immediately aspirated with the same syringe. Afterwards, between 3 to 10 cm³ of tracheobronchial lavage were drawn. This sample was stored in a sterile container and sent immediately to the laboratory. In only one case, a nasal swab (one sample) was also collected from a sick lamb.

2.2. Bacterial isolation and identification

The surface of the lung was sterilized using surgical material at high temperature. Afterwards, an incision was performed with a scalpel and a sterile loop was used to sample inside the lung. In the case of tracheobronchial lavages, samples were uniformly mixed, and 10 μl were streaked on the blood agar plate surface with a sterile loop. Finally, swab samples were surfaced onto the blood agar. For all the samples, sterile loops were surface onto blood agar (tryptic soy agar containing 5% sheep red blood cells) (BA) plates (Oxoid PB 5039A) with an incubation at 35–37 °C in aerobic conditions for 24–48 h. Identification of isolates were carried out by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF, Bruker Daltonics, Bremen, Germany). Individual strains were stored at –70 °C in skim milk. For MIC testing, bacteria were thawed and cultured two times on the same agar media.

2.3. Antimicrobial compounds

Trimetoprim (batch J1511036) and sulfadiazine (batch P.X.1-SD-1510033) were kindly provided by Laboratorios Jaer SA whereas doxycycline hylcate (batch YD140201051) was provided by Laboratorios SP Veterinaria. Tetracycline hydrochloride (batch T8032) and enrofloxacin (batch17849) were purchased commercially from Sigma Aldrich. All the antimicrobials were reconstituted based on manufacturer’s recommendations. Fresh stock solutions or those prepared from frozen samples (–70 °C) were used. For quality control, Staphylococcus aureus ATCC 29,213 from American Type Culture Collection (ATCC) control strain was included in each susceptible assay to ensure performance of the susceptibility assays; MIC values needed to be within acceptable ranges for each organism/drug.

2.4. MIC determination

MIC testing was carried out following the recommended CLSI
procedure for ten strains, five of PM and MH (VET08, 2018) that were randomly chosen between all the available ones. Briefly, Mueller-Hinton broth (MHB) containing a two-fold concentration of drug was added to the first column of a 96-well micro-dilution tray and serially diluted concentrations with MHB solution were prepared (from 0.001 to 1024 μg/mL for all the antimicrobials). A 0.5 McFarland density, established with a calibrated nephelometer (Biosan Medical-Biological Research & Technologies, Riga, Latvia), of Pasteurella multocida and Mannheimia haemolytica was further diluted to 5 × 10^5 cfu/mL added to the microdilution tray containing drug and incubated for 18–24 h (35–37 °C) in aerobic conditions. To ensure the inoculum concentration, the counting of bacterial colonies per mL (cfu/mL) was carried out by serial dilution over the surface of a blood agar plate for 10 strains. The MIC was established as the lowest drug concentration inhibiting visible growth. This standard MIC procedure was repeated twice on separate days and the average value was accepted as the final one. In parallel, MIC for the same Pasteurella multocida and Mannheimia haemolytica strains were performed by test strip (Epsilon test or E-test) to evaluate the agreement between the two techniques. For MIC determination by test strip, tetracycline (TET) (Oxoid Limited, Hampshire, United Kingdom), doxycycline (DOX) (Lioflachem S.R.L., Roseto degli Abruzzi, Italy), trimethoprim/sulfamethoxazole (SXT) (Lioflachem S.R.L., Roseto degli Abruzzi, Italy), and enrofloxacin (ENR) (Lioflachem S.R.L., Roseto degli Abruzzi, Italy) strips were used. It was not feasible to use the E-test with sulfadiazine/trimethoprim because it is not commercially available. The inoculum was prepared as previously described. Briefly, a 0.5 McFarland density, established with a nephelometer (Biosan Medical-Biological Research & Technologies, Riga, Latvia), of Pasteurella multocida and Mannheimia haemolytica was spread on a Muller Hinton Agar plate (Oxoid Limited, Hampshire, United Kingdom) using a sterile cotton swab and the test strip was applied to the agar surface. After 18 h of incubation at 37 °C the MIC value was read from the scale in units of μg/mL where the ellipse edge intersects the side strip.

For the rest of the bacterial strains of PM and MH, minimum Inhibitory Concentrations (MIC) of SXT, TET, ENR and DOX were determined using the E-Test technique based on the results obtained using both techniques.

### 2.5. Data analysis

MIC distributions were determined for each species-antimicrobial combination. The MIC distributions were used to define MIC50, MIC90, and a tentative epidemiological breakpoint ECOFF for differentiation between susceptibility and strains with some gene of resistance. MIC50 and MIC90 were defined as MICs inhibiting 50% and 90% of the strains, respectively. ECOFFs were defined from MIC distributions for each species-drug combination, as recommended by EUCAST (Turnidge et al., 2006), with susceptible strains being the population with MIC at or below the ECOFF and strains with some gene of resistance having a MIC of > ECOFF from an epidemiological point of view.

The agreement between the microdilution and E-test technique to determine MIC was assessed using the Lin’s concordance correlation coefficient as described by Watson and Petrie, 2010.

### 3. Results

Pasteurella multocida (n = 60) and Mannheimia haemolytica (n = 68) were isolated in most cases, although Bibersteinia thelrasoi and Escherichia coli were found, but only in a few cases. These latter microorganisms were not included in the study due to the low number of available strains. As explained in the Material and Methods section, the MIC value was determined using microdilution and E-test techniques for 10 randomly selected strains whose MIC values were highly variable. The agreement between the two techniques was perfect (> 0.999) for enrofloxacin, and substantial (between 0.950 and 0.999) for tetracycline and sulfamethoxazole/trimethoprim. In the case of doxycycline, the agreement between the two techniques was moderate (0.930). Based on these results (Table 1), the E-test was used to determine the antimicrobial susceptibility for all the strains included in the study.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Lin’s concordance correlation coefficient between the MIC value obtained using microdilution techniques versus the E-test method.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin’s concordance coefficient</td>
<td>MIC Sulfadiazine/trimethoprim</td>
</tr>
<tr>
<td>E-test SXT</td>
<td>0.980</td>
</tr>
<tr>
<td>E-test TET</td>
<td>NA</td>
</tr>
<tr>
<td>E-test ENR</td>
<td>NA</td>
</tr>
<tr>
<td>E-test DOX</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: Non-applicable.

### 4. Discussion

The choice of an antimicrobial and the design of a rational dosing regimen depends on the knowledge of the microorganism that causes the disease (clinical experience or isolation), the action of the drug on the microorganism (pharmacodynamics), the action of the drug on the animal treated (toxicity) and the availability of the drug for the animal in question (pharmacokinetics). Other considerations include the appearance of antimicrobial resistance, animal welfare and the economic cost of the treatment (Fraile, 2013). In the current work, updated information was obtained about antimicrobial susceptibility (pharmacodynamic parameter) of ovine respiratory pathogens under field conditions in Spain and Portugal. Currently, little is known about antibiotic susceptibility distributions among the target pathogens of sheep and lambs in EU countries. The current study aims to address this gap by determining the minimal inhibitory concentrations (MICs) for two major respiratory tract pathogens recovered, prior to antibiotic treatment, from diseased lambs in Spain and Portugal. One of the critical points is the selection of antimicrobials to be tested. In this case, we focused on the antibiotics most frequently used through oral (premix or water) administration for lambs in Spain and Portugal. Thus, sulfadiazine and trimethoprim (SXT), tetracycline (TET), enrofloxacin (ENR) and doxycycline (DOX) were chosen following this criteria. From a strictly scientific point of view, it would have been advisable to include antimicrobials of the macrolide, fenicol and beta-lactamic...
Table 2
MIC₅₀, MIC₉₀ and tentative epidemiological breakpoint (ECOFF) with a confidence level of 99% for *Pasteurella multocida* (n = 60 strains) and *Mannhaemia haemolytica* (n = 68 strains) isolated from lung or tracheobronchial lavage of lambs with respiratory symptoms.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Bacterial species</th>
<th>MIC₅₀ (μg/mL)</th>
<th>MIC₉₀ (μg/mL)</th>
<th>Tentative Epidemiological breakpoint (ECOFF) (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethoxazole/trimethoprim (SXT)</td>
<td><em>Pasteurella multocida</em></td>
<td>0.063</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td><em>Mannhaemia haemolytica</em></td>
<td>0.063</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Tetracycline (TET)</td>
<td><em>Pasteurella multocida</em></td>
<td>1</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Mannhaemia haemolytica</em></td>
<td>8</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Enrofloxacin (ENR)</td>
<td><em>Pasteurella multocida</em></td>
<td>0.016</td>
<td>0.5</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td><em>Mannhaemia haemolytica</em></td>
<td>0.032</td>
<td>16</td>
<td>0.25</td>
</tr>
<tr>
<td>Doxycycline (DOX)</td>
<td><em>Pasteurella multocida</em></td>
<td>1</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td><em>Mannhaemia haemolytica</em></td>
<td>2</td>
<td>16</td>
<td>4</td>
</tr>
</tbody>
</table>

Fig. 1. MIC distribution of sulfamethoxazole/trimethoprim for *Pasteurella multocida* (A) and *Mannhaemia haemolytica* (B) isolated from lung or bronchoalveolar lavage of lambs with respiratory symptoms. Epidemiological breakpoint (ECOFF) is indicated by a red line (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
families to monitor more precisely the antimicrobial susceptibility of these ovine pathogens but there is a shortage of veterinary medicinal products for use in lambs with these active ingredients. Thus, information gained would have been of little practical use.

The measurement of growth inhibition is carried out by minimum inhibitory concentration (MIC) determination. The MIC is the lowest antimicrobial concentration that inhibits in vitro the growth of the target bacteria in specific conditions of incubation in vitro (usually after 18 to 24 h in a culture medium at 37 °C and with a standard amount of inoculums) (Mckellar et al., 2004). In this study, the antimicrobial susceptibility was determined using artificial culture media as recommended by international guidelines on antimicrobial susceptibility determination (CLSI, 2018). This methodology does not emulate the natural biophase in which bacteria grow in vivo, such as blood, or interstitial and intracellular fluid. This could lead to a deviation when using the information obtained in vitro to predict the clinical outcomes in vivo, but the results are more reproducible and comparable between laboratories and the prediction of clinical efficacy, taking into account this pharmacodynamic information, is acceptable from a practical point of view (Mckellar et al., 2004).

Our samples were collected from diagnostic specimens and we do not have the whole history of antimicrobial treatments received in those animals before arriving at the finishing farm. This could have biased the results towards a more resistant bacterial population that

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**Fig. 2.** MIC distribution of tetracycline for *Pasteurella multocida* (A) and *Mannhaemia haemolytica* (B) isolated from lung or bronchoalveolar lavage of lambs with respiratory symptoms. Epidemiological breakpoint (ECOFF) is indicated by a red line (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
may not be representative for animals that have never received any antimicrobial therapy. The methods used to test the activity of antimicrobials against pathogens are mainly agar dilution, broth microdilution, E-test and diffusion disk (Kelly et al., 1999). Nowadays, broth microdilution is the standard method for this determination because the diffusion disk technique and E-test were found to be unreliable for some antimicrobials (e.g., colistin) due to poor diffusion properties in agar. Nevertheless, other methods may also be used if correctly validated as in the case of the E-test. This procedure consists of a continuous stable gradient of antimicrobial agent corresponding to 15 two-fold dilutions on a strip. We have carried out studies with the E-test to test its agreement with broth microdilution (BM) for two ovine pathogens and four antimicrobials. Our results indicate that the agreement is good enough to use in place of BM for our research. The E-test technique is less labor-intensive and more cost-effective than BM for this particular case. The good agreement between the E-test and BM was previously demonstrated for cefditoren with Streptococcus pneumoniae (Kelly et al., 1999) and for telithromycin with pneumococci (Davies et al., 2000). Nevertheless, it must be studied on a case-by-case basis as a lack of agreement between the E-test and BM was recently published for colistin and enterobacteriaceae, probably due to its poor diffusion properties in agar (Turlej-Rogacka et al., 2018).

Fig. 3. MIC distribution of enrofloxacin for Pasteurella multocida (A) and Mannhaemia haemolytica (B) isolated from lung or bronchoalveolar lavage of lambs with respiratory symptoms. Epidemiological breakpoint (ECOFF) is indicated by a red line (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Empirical treatment is generally based on knowledge of susceptibility patterns of the different bacterial pathogens to antimicrobial agents used in the particular animal species. However, there is a shortage of information regarding the antimicrobial susceptibility among disease-causing bacteria from lambs. For the current work, clinical veterinary breakpoints are not available for lambs. Thus, isolates were not categorized as susceptible or resistant from a clinical point of view. It is, therefore, important to present the MIC frequency distributions to allow some interpretation from a practical point of view (Schwarz et al., 2001). This is feasible because the susceptible population, referred to as wild type by EUCAST, is also characterized by the absence of acquired resistance mechanisms and/or mutations leading to resistance.

Unfortunately, it is not possible to compare the MIC data in our study with other studies in lambs because there are no recent studies using similar antimicrobial agents and guidelines. The information available is very old, was obtained by obsolete methodology (Diker et al., 1994), comes from a geographical area unrelated to the European context (Marru et al., 2013) and/or was obtained with pathogens isolated from mastitis cases (Lollai et al., 2016; Serrano-Rodríguez et al., 2017). On the other hand, the majority of bovine respiratory cases are associated with Pasteurella multocida (PM) and Mannhaemia haemolytica (MH) infections, which makes comparisons across species feasible. Our study demonstrates that, taken as a whole, antimicrobial

Fig. 4. MIC distribution of doxycycline for Pasteurella multocida (A) and Mannhaemia haemolytica (B) isolated from lung or bronchoalveolar lavage of lambs with respiratory symptoms. Epidemiological breakpoint (ECOFF) is indicated by a red line (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
susceptibility of PM and MH in Spain and Portugal is relatively high for important veterinary pathogens are necessary to provide evidence-based guidance for antimicrobial therapy of bacterial diseases.

Declaration of Competing Interest

Authors do not have any conflicts of interests about this research work.

Acknowledgements

This work was possible due to the technical support provided by Nanta SA, which belong to the Nutreco group.

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